Differential infiltration of macrophages and prostaglandin production by different uterine leiomyomas

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BACKGROUND: The association between uterine myoma and infertility is still controversial. The anatomical defect of endometrium by uterine fibroids could be a factor for reducing pregnancy rates and increasing miscarriage rates. However, pregnancy and implantation rates were found to be significantly lower in women with intramural myomas (IMMs), when there was no deformity of uterine cavity. This could be due to other biological factors such as increased accumulation of inflammatory cells within fibroid tissue and corresponding endometrium that might impair fertility. Therefore, we tried to investigate the pattern of macrophage (M\textsubscript{ϕ}) accumulation in different uterine fibroids and the production of chemokine and prostaglandin (PG) by these tissues.

METHODS: The selection criteria of uterine fibroids were based on the classification of European Society of Hysteroscopy. Biopsy specimens were collected from respective nodules and autologous endometrium of 20 women with submucosal myoma (SMM), 29 women with IMM and 18 women with subserosal myoma (SSM). CD68 immunoreactive M\textsubscript{ϕ}s were identified in these tissues by immunohistochemistry. A fraction of corresponding tissues were homogenized, and levels of monocyte chemotactic protein-1 (MCP-1) and PGF\textsubscript{2α} were measured by enzyme-linked immunosorbent assay (ELISA).

RESULTS: M\textsubscript{ϕ} infiltration in the myoma nodule and corresponding endometrium of women with SMM and IMM was significantly higher than that of women with SSM or control women (\(P < 0.01\) and \(P < 0.05\), respectively). This tissue accumulation of inflammatory cells was independent of the sizes of the myoma nodules and phases of menstrual cycle. The tissue concentration of MCP-1 corresponded to increased M\textsubscript{ϕ} infiltration and was significantly higher in women with SMM and IMM than that in women with SSM (\(P < 0.05\) for each). A positive correlation was observed between MCP-1 concentration and accumulated M\textsubscript{ϕ} numbers in the endometrium of women with SMM and IMM but not in women with SSM. The tissue levels of PGF\textsubscript{2α} were also significantly higher in the nodule and corresponding endometrium of women with SMM and IMM than that in SSM or control women (\(P < 0.05\) for each).

CONCLUSIONS: Higher production of MCP-1 could be responsible for the increased accumulation of M\textsubscript{ϕ} in women with SMM and IMM. The augmented inflammatory reaction in endometrium and increased PGF\textsubscript{2α} levels might be detrimental to reproductive outcome in women with SMM or IMM.

Key words: infertility/macrophages/monocyte chemotactic protein-1/prostaglandin F\textsubscript{2α}/uterine myoma

Introduction

Uterine fibroids (leiomyoma and myoma) are the most common tumours found in women in the reproductive age group. Their occurrence increases with age. Various prevalence rates have been quoted in literature ranging from 20 to 50% of women over the age of 30 years (Verkauf, 1992; Wallach, 1992; Eldar-Geva et al., 1998). Therefore, it is not surprising to detect uterine fibroids in women with a history of infertility or reproductive wastage from time to time. The clinical symptoms and severity usually depend on the size, position and number of fibroids present (Eldar-Geva et al., 1998).

The degree to which uterine fibroids contribute to infertility is controversial. It has been estimated that uterine myomas are associated with infertility in 5–10% of cases by a number of mechanisms (The Practice Committee of the American Society for Reproductive Medicine, 2004). The role of fibroids in infertility was evaluated indirectly by fertility performance after myomectomy. The effect of submucosal, intramural and subserosal uterine fibroids was also investigated on the reproductive outcome of assisted reproduction treatments (ART) (Rackow and Arici, 2005). It is well accepted that the anatomical location of the fibroid is an important factor, with submucosal, intramural and subserosal fibroids being in decreasing order of
importance, in causing infertility (Bajekal and Li, 2000; Rackow and Arici, 2005). Submucosal myoma (SMM) or intramural myoma (IMM) may cause dysfunctional uterine contractility that may interfere with sperm migration, ovum transport or nidation (Hunt and Wallach, 1974; Buttram and Reiter, 1981; Vollenhoven et al., 1990). In addition, uterine myoma may be associated with implantation failure or gestation discontinuation due to focal endometrial vascular disturbance as well as endometrial inflammation, secretion of vasoactive substances or an enhanced endometrial androgen environment (Deligdish and Lowenthal, 1970; Buttram and Reiter, 1981).

There are several reports describing similar reproductive outcome with a post-operative pregnancy rate of 54–58.2% after abdominal myomectomy in a group of patients with no other apparent cause for their infertility (Eldar-Geva et al., 1998). Finally, fertility outcome has been shown to increase after either laparoscopic myomectomy or hysteroscopic resection of submucosal fibroids with clinical outcome similar to those seen after myomectomy at laparotomy (Goldenberg et al., 1995; Darai et al., 1997). There are five retrospective cohort studies that examined the impact of fibroids on the results of assisted conception (Bajekal and Li, 2000; Rackow and Arici, 2005). The pregnancy rate per embryo transfer in women with submucosal, intramural and subserosal fibroids was 9, 16 and 37%, respectively, compared with an average of 30% in control subjects. The miscarriage rate in the various types of fibroids was 40% for SMM, 33% for IMM and 33% for subserosal myoma (SSM) compared with a total of 16.4% among all the control subjects in all five series. The results are consistent with the commonly held view that submucosal fibroids have the most detrimental effect, intramural fibroids a modest impact and subserosal fibroids have the least impact on pregnancy rate.

Farhi et al. (1995) demonstrated that the pregnancy rate after ART was impaired only when the fibroids caused deformation of the uterine cavity. Stovall et al. (1998) reported that even after excluding patients with submucosal fibroids, the presence of fibroids reduced the efficacy of ART treatment. In another ART clinical trial, Eldar-Geva et al. (1998) showed that pregnancy and implantation rates were significantly lower in patients with IMM, even when there was no deformation of the uterine cavity. In contrast, reproductive outcome was not influenced by the presence of subserosal fibroids. This indicates that anatomical deformity of uterine cavity is not the only factor that may impair reproductive outcome in women with uterine fibroids. We speculated that this could be due to other biological factors such as infiltration of inflammatory cells within fibroid tissue, adjacent myometrium and corresponding endometrium that might impair fertility or may cause miscarriage in women with uterine fibroids.

Therefore, we investigated the accumulation of macrophages (Mφs) in different uterine myomas and their autologous myometrium or endometrium and examined their relationship with the production of chemokine by these tissues. Because variable production of prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) by myometrium and their autologous endometrium could be a contributing factor for uterine contraction, we also examined the tissue levels of PGF$_{2\alpha}$ in different myomas, their adjacent myometrium and corresponding endometrium. We compared these results with those from the endometrium of age-matched control women without uterine myomas.

**Materials and methods**

**Subjects**

The subjects in this study were women of reproductive age. From February 2004 to April 2005, biopsy specimens were collected from 67 women containing variable sizes of different uterine leiomyomas who underwent hysteroscopy, laparoscopy or laparotomy during this period. All these women were admitted to our hospital with the complaint of abnormal genital bleeding, hypermenorrhoea or anaemia with or without associated complaint of dysmenorrhoea. The uterine fibroids in all these women were diagnosed by ultrasonography and magnetic resonance imaging (MRI) before operation. The control group (n = 20) consisted of fertile women without any evidence of uterine myoma and who were operated for dermoid cysts by laparoscopy.

The criteria for the selection of SMM, IMM and SSM were based on the classification of the European Society of Hysteroscopy (Bajekal and Li, 2000). Accordingly, we classified SMM (n = 20) as the myoma that distorts uterine cavity; IMM (n = 29) as the myoma nodule with no cavity deformity and with <50% extension into the serosal surface; and SSM (n = 18) as the myoma nodule with >50% extension into the serosal surface. In the case of mixed myoma, any nodule distorting the calibre of uterine cavity was categorized as SMM. In the case of multiple myoma except SMM, nodule with maximum size was selected. Among women with SMM, transcervical hysteroscopy was performed in 10 women and hysterectomy in 10 women; among the women with IMM, laparoscopic myomectomy was performed in 13 women and hysterectomy in 16 women. All women with SSM underwent only laparoscopic myomectomy. Therefore, no biopsy specimen from the myometrium could be collected from women with SSM. A fraction of these studies and control women also had either endometriosis or adenomyosis. Six women with SMM, 10 with IMM and 5 with SSM had hormonal medication within 6 months of the operation. Five women in SMM group complained of secondary sterility and wished for a baby, and two of these women showed signs of endometriosis in pelvic cavity.

The phase of the menstrual cycle in women without hormonal therapy was determined by histological dating of eutopic endometrium samples taken simultaneously with myoma nodule. All biopsy specimens were collected in accordance with the guidelines of the Declaration of Helsinki and with the approval of the Nagasaki University Institutional Review Board. An informed consent was obtained from all women.

**Biopsy specimens**

Biopsy specimens from the respective myoma nodule, autologous myometrium or endometrium were collected from these women during operation. Biopsy of each myoma nodule and adjacent myometrium was sampled at random. Because biopsy specimens from the endometrium could not be collected from all study women, there was a heterogeneous distribution in the sample number among myoma nodule, myometrium and endometrium. As a control, eutopic endometrium from 20 women without uterine myoma was also evaluated. Three to four biopsy specimens from different anatomical locations of the eutopic endometrium were also studied for women with myoma who underwent hysterectomy. These anatomical sites of endometria included endometrium adjacent to myoma nodule, endometrium contra-lateral to myoma nodule, endometrium from fundal area and endometrium close to the cervix. All collected biopsy specimens were prepared as formalin-fixed paraffin-embedded tissue blocks for subsequent histopathological and immunohistochemical study.
A fraction of biopsy specimens from myoma nodule, adjacent myometrium and corresponding endometrium of women with different uterine myomas and also endometrium of control women were homogenized using a Polytron homogenizer (Kinematics, Luzern, Switzerland) in a buffer [5 mM Tris-HCl (pH 7.4), 5 mM NaCl, 1 mM CaCl₂, 2 mM EGTA, 1 mM MgCl₂, 2 mM dithiothreitol, 25 μg/mL aprotinin and 25 μg/mL leupeptin] prepared according to Fujimoto et al. (2000). The respective tissue suspension was centrifuged at 400 × g for 5 min to obtain the supernatant and stored at −80°C for the subsequent measurement of chemokine (MCP-1) and PGF₂α levels.

Antibodies used

We performed immunohistochemical studies to investigate the immunoreaction of CD68 for Mφ in intact tissues. A CD68 (KP1) mouse monoclonal antibody was obtained from Dako, Denmark. A 1:50 dilution was used. CD68 antigen (clone KP1), which we used for our current study as a marker of matured and activated Mφ, is a glycosylated transmembrane glycoprotein that is mainly located in lysosomes. It belongs to a family of lysosomal granules (Holness and Simmons, 1993) biotinylated anti-mouse IgG was used as a second antibody. Non-immune mouse immunoglobulin G1 antibody in 1:50 dilution was used as a negative control.

Immunohistochemistry

The details of immunohistochemical staining were described elsewhere (Khan et al., 2003, 2004; Ishimaru et al., 2004). Briefly, 5-μm-thick paraffin-embedded tissues were deparaffinized in xylene and rehydrated in phosphate-buffered saline (PBS). After immersion in 0.3% H₂O₂/methanol to block endogenous peroxidase activity, sections (Khan was used as a negative control. Non-immune mouse immunoglobulin G1 antibody in 1:50 dilution was used. CD68 antigen (clone KP1), which we used for our current study as a marker of matured and activated Mφ, is a glycosylated transmembrane glycoprotein that is mainly located in lysosomes. It belongs to a family of lysosomal granules (Holness and Simmons, 1993) biotinylated anti-mouse IgG was used as a second antibody. Non-immune mouse immunoglobulin G1 antibody in 1:50 dilution was used as a negative control.

Measurement of MCP-1 and PGF₂α

The tissue concentrations of monocyte chemotactic protein-1 (MCP-1) and PGF₂α in the homogenized supernatant of myoma nodule, myometrium and corresponding endometrium of women with uterine myoma and endometrium of control women were measured in duplicate using a commercially available sandwich enzyme-linked immunosorbent assay (ELISA) (Quantikine; R&D System, Minneapolis, MN, USA) according to the manufacturer’s instructions and as described previously (Parent et al., 2003; Khan et al., 2004; Tamura et al., 2004). The protein concentration of samples was measured by the method of Bradford (1976) to standardize MCP-1 and PGF₂α levels.

The antibodies used in MCP-1 and PGF₂α determination do not cross-react with other cytokines. The limit of detection was <5.0 and <6.78 pg/mL for MCP-1 and PGF₂α, respectively. Both the intra-assay and inter-assay coefficients of variation were <10% for both of these assays. The tissue concentrations of MCP-1 and PGF₂α were expressed as pg/μg protein.

Statistical analysis

All results are expressed as either mean ± SEM or mean ± SD. The clinical characteristics of the subjects were compared with one-way analysis of variance (ANOVA) and the χ² test for any difference between two groups. Differences in Mφ number and MCP-1 or PGF₂α concentration between two groups were analysed by the non-parametric Mann–Whitney U-test or Student’s t-test. For comparisons among groups, the Kruskal–Wallis test was used. Pearson’s correlation coefficient was used to evaluate the relationship between two groups. A value of P < 0.05 was considered statistically significant.

Results

The detailed clinical profiles of control women and women with different uterine leiomyomas are summarized in Table I. The control women were significantly younger than those of women with any types of myomas. There was no difference in the mean ages between women with SMM and SSM or between women with IMM and SMM. However, the mean ages of women with IMM were significantly higher than those of women with SSM (41.4 ± 6.4 versus 36.3 ± 5.3 years, P < 0.01). The mean size of the fibroids in women with IMM and SMM was significantly larger than that in women with SSM (P < 0.0001 and P < 0.05, respectively). A number of other diseases such as endometriosis or adenomyosis were coexistent in control women and women with different leiomyomas. The distribution of women with or without coexistent diseases, women with or without hormonal therapy and phases of menstrual cycle are summarized in Table I.

| Table I. Clinical profiles of women with uterine leiomyomas |
|-----------------|-----------------|-----------------|-----------------|
|                 | Control (n = 20) | SMM (n = 20)    | IMM (n = 29)    | SSM (n = 18)    |
| Age (years)     | 29.3 ± 3.2*,**  | 37.0 ± 8.2**,   | 41.4 ± 6.4*,    | 36.3 ± 5.3*,**  |
| Range in age (years) | 21–36          | 17–50           | 28–51           | 28–44           |
| Size (cm)       | 2.9 ± 0.8***   | 5.1 ± 1.8**    | 4.9 ± 2.8*     |                |
| Range in size (cm) | 1.8–4.0       | 3.5–12         | 2.5–10         |                |
| With coexistent diseases: endometriosis/adenomyosis | 4/0            | 7/1            | 2/11           | 6/2            |
| Without coexistent diseases | 16              | 12             | 16             | 10             |
| GnRHα therapy (<+) | 14/6          | 19/10          | 13/5           |                |
| Menstrual cycle: P/S/M | 10/10/0       | 4/10/0         | 4/10/5         | 3/9/1          |

GnRHα, GnRH agonist; IMM, intramural myoma; M, menstrual phase; P, proliferative phase; S, secretory phase; SMM, submucosal myoma; SSM, subserosal myoma. The results are expressed as mean ± SD. Age, *P < 0.01, IMM versus SMM and control; **P < 0.05, SMM or SSM versus control; size, †P < 0.05, SSM versus SMM; ‡P < 0.001, IMM versus SMM.
Macrophage infiltration in different leiomyomas of women with and without coexistent diseases. The mean Mϕ number of different myomas that were associated with or without combined data of Mϕ or autologous myometrium. Therefore, we presented our significant differences between them in the accumulation of the cases with or without coexistent diseases, but we found no marked difference in Table II.

The endometria, myoma nodules and autologous myometrium derived from women with SMM and IMM (IMM) (d, e and f) and subserosal myoma (SSM) (g and h) is shown. All these tissue sections were derived from the similar proliferative phases of the menstrual cycle. Final magnification was ×200 using a light microscope.

### Mϕ infiltration in myoma nodules, autologous myometrium and endometrium

When we analysed the Mϕ infiltration in different myoma nodules and their autologous myometrium or endometrium, we found that Mϕ infiltration, as shown by CD68-positive brown spots, appeared to be higher in the nodules and endometria of women with SMM and IMM than that of corresponding tissues derived from women with SSM (Figure 1). This increased accumulation of inflammatory cells in endometria of women with SMM and IMM also appeared to be higher than that of control women (data not shown). Although there was no apparent difference in Mϕ infiltration between myoma nodules and autologous myometrium derived from women with SMM and IMM (Figure 1), the accumulation of these inflammatory cells appeared to be higher in the endometrium of women with SMM than that in the endometrium of IMM or SSM (Figure 1).

We could not study the Mϕ infiltration in the autologous myometrium of women with SSMs, because there were no cases of hysterectomy in this group of women.

### Quantitative analysis of Mϕ infiltration in different leiomyomas

Initially, we tried to analyse the Mϕ infiltration separately in the cases with or without coexistent diseases, but we found no significant differences between them in the accumulation of these inflammatory cells in either endometrium or myoma nodule or autologous myometrium. Therefore, we presented our combined data of Mϕ infiltration among the corresponding tissues of different myomas that were associated with or without coexistent diseases. The mean Mϕ number (±SEM) per field in the endometria, myoma nodules and autologous myometrium between women with and without coexistent diseases is summarized in Table II.

### Table II. Macrophage infiltration in different leiomyomas of women with and without coexistent diseases

<table>
<thead>
<tr>
<th>Type of tissues</th>
<th>With coexistent diseases</th>
<th>Without coexistent diseases</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endometrium</td>
<td>32.0 ± 1.6</td>
<td>29.9 ± 1.5</td>
<td>Ns</td>
</tr>
<tr>
<td>Submucosal myoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endometrium</td>
<td>61.6 ± 5.4</td>
<td>56.4 ± 5.1</td>
<td>Ns</td>
</tr>
<tr>
<td>Myoma nodule</td>
<td>46.1 ± 4.6</td>
<td>42.4 ± 7.2</td>
<td>Ns</td>
</tr>
<tr>
<td>Myometrium</td>
<td>43.9 ± 5.1</td>
<td>45.0 ± 7.8</td>
<td>Ns</td>
</tr>
<tr>
<td>Intramural myoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endometrium</td>
<td>39.3 ± 3.7</td>
<td>40.6 ± 4.5</td>
<td>Ns</td>
</tr>
<tr>
<td>Myoma nodule</td>
<td>48.2 ± 3.4</td>
<td>58.8 ± 5.6</td>
<td>Ns</td>
</tr>
<tr>
<td>Myometrium</td>
<td>43.6 ± 2.5</td>
<td>46.2 ± 5.2</td>
<td>Ns</td>
</tr>
<tr>
<td>Subserosal myoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endometrium</td>
<td>31.2 ± 2.9</td>
<td>34.5 ± 11.9</td>
<td>Ns</td>
</tr>
<tr>
<td>Myoma nodule</td>
<td>29.7 ± 3.5</td>
<td>32.8 ± 6.5</td>
<td>Ns</td>
</tr>
</tbody>
</table>

Ns, not significant.
The results are expressed as mean ± SEM. Tissue infiltration of macrophages (Mϕs) was quantitated as mean Mϕ number per field.

The mean Mϕ number per field in different myoma nodules and autologous myometrium or endometrium of women with SMM, IMM and SSM is shown in Figure 2. We found that the tissue infiltration of Mϕ in the myoma nodule of SMM and IMM was significantly higher than that of SSM (P < 0.01, SMM versus SSM; P < 0.001, IMM versus SSM, Figure 2A). However, no significant difference in Mϕ infiltration was observed between the myoma nodules and surrounding myometrium derived from women with SMM and IMM (Figure 2A).

Again, we found that Mϕ infiltration in the corresponding endometrium of women with SMM was significantly higher than that of women with IMM, SSM or control women (P < 0.05, SMM versus IMM; P < 0.01, SMM versus SSM or control women, Figure 2B). The Mϕ infiltration in the endometrium of women with IMM was also significantly higher than that of women with SSM or control women (P < 0.05, IMM versus SSM or control women, Figure 2B). No significant difference was observed in the accumulation of these inflammatory cells between endometria derived from women with SSM and control women. The Kruskal–Wallis test as performed among these four groups of women indicated that tissue infiltration of Mϕ was the highest in the endometrium of women with SMM, intermediate in IMM and the least in SSM or control women.

When we performed quantitative analysis of Mϕ infiltration according to the median size of the respective myoma nodules, we found that Mϕ infiltration in the different myoma nodules, myometrium and their corresponding endometria of SMM, IMM or SSM was not dependent on their sizes (data not shown). Again, we found that the Mϕ infiltration in the endometria of different myomas was independent of the phases of menstrual cycle (data not shown). With only a small study population in each group of the women with myomas who underwent hormonal therapy for a variable period of 3–6 months, although we found a decreased tendency for Mϕ infiltration in the myoma nodule, myometrium and endometrium of women who received GnRH agonist (GnRHa) hormonal therapy, there was no significant difference from the corresponding tissues of women who did not receive GnRHa treatment.
Macrophage infiltration in uterine myomas

To examine any difference in Mϕ infiltration according to the anatomical location within the endometrium, we collected endometrial tissues from different sites in women who underwent hysterectomy. We found an apparent increase in the accumulation of Mϕ in the endometrium adjacent to SMM nodule or IMM nodule, but there was no significant difference in Mϕ infiltration when compared with that in contra-lateral endometrium, fundal endometrium or in endometrium close to the cervix (data not shown).

**MCP-1 concentration in myoma nodule, myometrium and endometrium**

As a chemotactic protein, we tried to measure the tissue concentrations of MCP-1 in the myoma nodule, myometrium and corresponding endometrium of women with different leiomyomas (Figure 3). We found that tissue concentrations of MCP-1 in the myoma nodule and corresponding endometrium of women with SMM and IMM were significantly higher than those in the similar tissues of women with SSM or control women. The statistical differences between them are as follows: myoma nodule, \( P < 0.05 \), SMM versus SSM; \( P < 0.05 \), IMM versus SSM (Figure 3A); endometrium, \( P < 0.01 \), SMM versus SSM or control.

### Table III. Macrophage infiltration in different leiomyomas of women with and without GnRHa treatment

<table>
<thead>
<tr>
<th>Type of tissues</th>
<th>Without GnRHa treatment</th>
<th>With GnRHa treatment</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Submucosal myoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endometrium</td>
<td>59.1 ± 7.0</td>
<td>44.6 ± 8.1</td>
<td>0.396</td>
</tr>
<tr>
<td>Myoma nodule</td>
<td>50.9 ± 10.2</td>
<td>42.6 ± 5.4</td>
<td>0.510</td>
</tr>
<tr>
<td>Myometrium</td>
<td>46.6 ± 7.6</td>
<td>43.8 ± 8.3</td>
<td>0.769</td>
</tr>
<tr>
<td>Intramural myoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endometrium</td>
<td>39.3 ± 3.6</td>
<td>29.4 ± 1.0</td>
<td>0.154</td>
</tr>
<tr>
<td>Myoma nodule</td>
<td>54.5 ± 8.4</td>
<td>44.8 ± 2.6</td>
<td>0.765</td>
</tr>
<tr>
<td>Myometrium</td>
<td>44.4 ± 3.0</td>
<td>41.2 ± 4.7</td>
<td>0.627</td>
</tr>
<tr>
<td>Subserosal myoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endometrium</td>
<td>34.1 ± 3.5</td>
<td>23.3 ± 3.9</td>
<td>0.170</td>
</tr>
<tr>
<td>Myoma nodule</td>
<td>30.6 ± 4.6</td>
<td>27.2 ± 4.7</td>
<td>0.921</td>
</tr>
</tbody>
</table>

GnRHa, GnRH agonist.

The results are expressed as mean ± SEM. Tissue infiltration of macrophages (Mϕs) was quantitated as mean Mϕ number per field.
versus SSM or control; $P < 0.05$, IMM versus SSM or control (Figure 3B). These findings of MCP-1 in the myoma nodule and endometrium corresponded to an increased accumulation of Mϕ in the similar tissues derived from women with SMM or IMM as shown in Figure 2A and B. We also found an increased production of MCP-1 by the autologous myometrium and at a tissue concentration similar to that of myoma nodule in women with SMM and IMM. There was no difference in MCP-1 concentration between myoma nodule and myometrium of women with SMM and IMM (Figure 3A).

**Correlation between MCP-1 concentration and Mϕ infiltration**

Because we found an increased infiltration of Mϕs in SMM and IMM nodule and their corresponding endometrium and a parallel increased production of MCP-1 by these tissues, we tried to examine the relationship between MCP-1 concentration and accumulation of Mϕ in the endometrium of women harbouring different myomas. We found a significant positive correlation between MCP-1 concentration and tissue infiltration of Mϕ in the endometrium of women with submucosal myoma (SMM) ($R^2 = 0.377$, $P < 0.01$, Figure 4A) and intramural myoma (IMM) ($R^2 = 0.480$, $P < 0.01$, Figure 4B). However, we did not find any correlation between them in the endometria of women with subserosal myoma (SSM) ($R^2 = 0.158$, $P = \text{not significant}$, Figure 4C) or in the similar tissues of control women (data not shown). Although data not shown, we also found a significant correlation between MCP-1 concentration and tissue infiltration of Mϕ in the myoma nodule and myometrium of women with SMM and IMM.

**Tissue levels of PGF$_{2\alpha}$ in myoma nodule, myometrium and endometrium**

Because PGF$_{2\alpha}$ is involved in causing uterine contraction and vasoconstriction of spiral arteries of the endometrium, we therefore measured the tissue levels of PGF$_{2\alpha}$ in the myoma nodule, myometrium and corresponding endometrium of different myomas as shown in Figure 5. The tissue levels of PGF$_{2\alpha}$ were significantly higher in the myoma nodule and corresponding endometrium derived from women with SMM and IMM than that of similar tissues derived from women with SSM or control women (Figure 5A and B). There was no significant difference in the myometrial concentration of PGF$_{2\alpha}$ between SMM and IMM or in PGF$_{2\alpha}$ levels between myoma nodule and myometrium of women with SMM and IMM (Figure 5A). The statistical differences of PGF$_{2\alpha}$ between myoma nodules and endometria of different leiomyomas are as follows: myoma nodule, $P < 0.05$, both SMM and IMM versus SSM (Figure 5A); endometrium, $P < 0.01$, both SMM and IMM versus control; $P < 0.05$, both SMM and IMM versus SSM (Figure 5B).

Because the number of biopsy specimen derived from endometrium was small, we analysed the tissue levels of PGF$_{2\alpha}$ in the endometria of all women with SMM, IMM and SSM and according to the phases of menstrual cycle. We found an increasing and significantly higher tissue content of PGF$_{2\alpha}$ as observed from the late proliferative phase to early secretory phase when compared with gradually declining pattern towards late secretory phase (Figure 5C). No significant difference was observed in the tissue levels of PGF$_{2\alpha}$ when compared with early proliferative phase (Figure 5C).

![Figure 4](image)

**Figure 4.** The correlation between the tissue concentration of monocyte chemotactic protein-1 (MCP-1) and the infiltrated macrophage (Mϕ) number per field in the endometria derived from women with submucosal myoma (SMM) (A), intramural myoma (IMM) (B) and subserosal myoma (SSM) (C) is shown. No significant correlation was observed in the endometria derived from control women (data not shown).
We found that the degree of inflammatory reaction as manifested by the infiltration of Mϕ in the myoma nodules and their corresponding endometrium was significantly stronger in the biopsy specimens derived from women with SSM and IMM when compared with that of women with SSM and control women whose uteri were free of myoma nodules. In fact, we demonstrated that the tissue infiltration of Mϕ in the myoma nodule and autologous endometrium was the highest in women containing SMM, intermediate in IMM and the least in either SSM or in the endometrium of control women. When we examined the infiltration of Mϕ in myoma nodule alone, we found that the distribution in the accumulation of these inflammatory cells was similar between SMM and IMM but was significantly higher than that of specimens derived from SSM nodules. A similar inflammatory reaction was also observed in the surrounding healthy myometrium of women with SMMs and IMMs who were recruited for hysterectomy in our study.

It was interesting to observe that this variation in the inflammatory reaction was not confined only to myoma nodules but also equally involved the corresponding endometria of women containing different myoma nodules. Again, the degree of this inflammatory reaction as documented by the tissue infiltration of Mϕ appeared to be the highest in the corresponding endometrium of myoma nodules which originated in the sub-endometrial myometrium (junctional zone), modest in that of myoma nodule of intramural origin and the least in that of subserosal origin.

Because the origin of SMM is from the junctional zone or inner myometrium, we suggest that involvement of junctional zone can be more important by producing a stronger inflammatory reaction in contrast to other myoma nodules when there is no involvement of junctional zone. A possible explanation is that, compared with healthy controls or other myoma nodules, patients with SMM might be characterized by endometrium with increased angiogenesis, higher endometrial vascular activity and consequent increased recruitment of inflammatory cells. A direct pressure effect on the endometrium by SMM with resultant ischemic or hypoxic change might be responsible for producing different angiogenic factors and an augmented endometrial vascular perfusion (Carmeliet, 2000). Recently, Xavier et al. (2005) demonstrated that subendometrial and intra-endometrial blood flow was significantly higher in women with endometriosis when compared with healthy controls. A similar endometrial vascular change may also occur in women with IMM causing a moderate production of MCP-1 and a modest inflammatory reaction in their endometrium.

A number of ART clinical trials in women with different types of uterine myomas demonstrated that SMMs are the most detrimental in reducing pregnancy rate and implantation rate and in increasing miscarriage rate (Eldar-Geva et al., 1998; Ribeiro et al., 1999; Bajekal and Li, 2000). These results are reasonably explained by the existence of an anatomical deformity of the uterine cavity caused by SMMs. However, the parallel ART clinical trials in women containing IMM without any cavity deformity showed that the pregnancy and implantation rates were also significantly decreased and almost similar to that of SMMs when they compared the ART results of women containing either SSM or women who were free of any surgery.

**Figure 5.** The levels of prostaglandin F$_{2α}$ (PGF$_{2α}$) in the tissue homogenates derived from the myoma nodules or myometrium (A) and the autologous endometria (B) of women with submucosal myoma (SMM), intramural myoma (IMM), subserosal myoma (SSM) and control women are shown. The collective tissue content of PGF$_{2α}$ in the endometrium derived from all women with SMM, IMM and SSM is also shown according to the phases of the menstrual cycle (C). The results are expressed as mean ± SEM. A, Myoma nodules, *$P$ $<$ 0.05, SMM or IMM versus SSM. B, Endometrium. *$P$ $<$ 0.05, SMM versus IMM or SSM or control; **$P$ $<$ 0.05, IMM versus SSM or control. C, Endometrium. *$P$ $<$ 0.05, late proliferative phase versus late secretory phase; **$P$ $<$ 0.05, early secretory phase versus late secretory phase of the menstrual cycle.

**Discussion**

We demonstrated for the first time that the inflammatory reaction and the biological activity of the myoma nodule and the corresponding endometrium of women with SMMs and IMMs are different from that of women with SSM and control women. This was shown by the findings of a variable tissue infiltration of Mϕ as a marker of inflammatory reaction and increased concentration of MCP-1, an inflammatory-related factor, and PGF$_{2α}$ in the tissue homogenates of different myoma nodules, myometrium and autologous endometrium that were simultaneously collected during surgery.
uterine pathology (Eldar-Geva et al., 1998; Oliveira et al., 2004). We speculate that this adverse effect of IMM on the worse fertility outcome might be due to a sustained inflammatory reaction in the autologous endometrium of women containing IMMs as we demonstrated in our current study. In fact, we found that the corresponding endometrium of women containing either SMM or IMM harboured abundant infiltration of Mϕ irrespective of the presence of cavity deformity of the uterus. These increased tissue infiltrations of Mϕ in the endometrium were significantly higher than that of similar tissues from women with SSM or control women. These results indicate that endometria of women with SMM or IMM develop a similar degree of in situ inflammatory change that might be responsible for creating adverse reproductive outcome.

Our findings of increased tissue infiltration of Mϕ in the myoma nodules and the corresponding endometrium of women with SMMs and IMMs were independent of the size of the nodule, the phases of menstrual cycle and the anatomical location of endometrium. Some previous studies (Eldar-Geva et al., 1998; Bajekal and Li, 2000) suggested that implantation of the blastocyst on the endometrium adjacent to the myoma nodule is the most detrimental in causing either decreased pregnancy rate or increasing miscarriage rate. However, our findings revealing a diffuse inflammatory reaction involving different anatomical locations of the endometrium suggest that the impaired fertility outcome might happen for an implanting blastocyst at any anatomical site of the endometrium of women who contain either an SMM or an IMM in their uterus.

Because the vascularity of the SMMs and IMMs is different from that of SSMs (Brosens et al., 2003; Walocha et al., 2003) and the production and secretion of MCP-1, a potent chemotactic protein, are mainly from the vascular endothelial cells, Mϕs and smooth muscle cells (Seli et al., 2002; Khan et al., 2004), we tried to examine the relationship between the concentrations of MCP-1 and the accumulation of Mϕ in the intact tissues of myoma nodules and corresponding endometrium. We found an increased production of MCP-1 by the tissue homogenates as collected from the myoma nodule, myometrium and corresponding endometrium of women containing SMMs or IMMs, and these findings corresponded to an increased infiltration of Mϕ in the corresponding tissues of the similar women. We also found a positive correlation between the production of MCP-1 and the accumulation of inflammatory cells in the endometria of both groups of women containing either SMM or IMM. This indicates that variable degrees of inflammatory reaction in the endometria of these two groups of women are a consequence of an increased biological activity of the myoma nodules, myometrium or endometrium as documented by an increased production of MCP-1 and a consequent recruitment of inflammatory cells.

The dysfunctional uterine contraction as caused by the increased production of PGF$_{2\alpha}$ is reported to be involved in the abnormal sperm migration, defective transport of fertilized egg and impaired nidation (Bajekal and Li, 2000). We speculated that in addition to inflammatory reaction in the myoma nodules, myometrium and endometrium, these corresponding tissues derived from women with different myoma nodules might produce different tissue levels of PGF$_{2\alpha}$. We measured PGF$_{2\alpha}$ levels in homogenates of these tissues and found that the contents of PGF$_{2\alpha}$ were significantly higher in the myoma nodules and corresponding endometrium of women with either SMM or IMM when compared with that in the similar tissues derived from women with SSM or control women. Because PGF$_{2\alpha}$ is predominantly produced by Mϕ or mesenchymal cells in endometrium or myometrium, our findings of augmented inflammatory reaction in the myoma nodule, myometrium and endometrium corresponded to the increased levels of PGF$_{2\alpha}$ as produced by these tissues.

Lyons et al. (1991) reported that the frequency, amplitude and direction of inner myometrial contraction waves are dependent on the phase of the menstrual cycle, and the degree of cervicofundal contraction from the late proliferative phase to luteal phase is important for successful implantation or progressive sperm transport. Our findings of enhanced tissue levels of PGF$_{2\alpha}$ during the periovulatory period of menstrual cycle may have some clinical implications, by determining fertility outcome in women with either SMM or IMM.

Leiomyomas do not contract, and their PG receptors are down-regulated relative to the myometrium as indicated by complementary DNA arrays study of Tsibris et al. (2002). We found an apparent increase of PGF$_{2\alpha}$ in the myometrium compared to myoma nodules. It has already been demonstrated that uterine contraction and peristaltic movement are mainly exerted by the subendometrial myometrium (junctional zone) and endometrium (Kido et al., 2005). As a result, our findings of increased PGF$_{2\alpha}$ levels in myoma nodules and adjacent myometrium of women with SMMs and IMMs may support a possible cause of augmented uterine contraction by the local transport of PGF$_{2\alpha}$ to the junctional zone or endometrium by vascular channels.

Recently Nishino et al. (2005) reported that uterine peristaltic movements were partly interrupted by SMMs. Their findings of lower uterine contractility were focal, adjacent to myomas, detected by cine MRI and were observed in only one-third of their studied cases. The uterine contractility was well preserved in the remaining part of the subendometrial myometrium. The authors concluded that loss of peristalsis and focal myometrial movements may represent dysfunctional uterine contractility and may be related with pregnancy loss. Our findings of higher tissue concentration of PGF$_{2\alpha}$ in women with SMM and IMM may biologically explain their cine MRI findings. In addition to uterine contractility, PGF$_{2\alpha}$-induced vasoconstriction with resulting ischemic or hypoxic change in and around the site of implanting nidus could be an additional factor in producing adverse fertility outcome in women with SMM or IMM.

The relationship between increased inflammatory reaction in the endometrium and infertility is unclear and still remains controversial. Several in vitro studies from our laboratory and others demonstrated that Mϕs retain potential phagocytic activities, and they have the ability to produce different pro-apoptotic cytokines and reactive oxygen species and to secrete different Th2-type cytokines for the production of auto-antibodies (Muscato et al., 1982; Ishimura et al., 1994; Khan et al., 2005a,b). The decreased fertility outcome in women containing uterine fibroids as described in the previous reports
reaction of the endometrium and increased PGF2α production as demonstrated in our current study could be a possible mechanism in causing either infertility or miscarriage in women harbouring SMM or IMM in their uterus.

Our results have some biological and clinical implications. (i) Besides cavity deformity, SMM nodules may also cause a strong and diffuse inflammatory reaction in the autologous endometrium. (ii) Even when there is no cavity deformity, the presence of IMM nodule may also create an inflamed endometrium. (iii) Endometria of control women and women with SSM display a minimal inflammatory change and may not have impaired fertility outcome. (iv) Surgical or medical treatments should be considered in infertile women who have submucosal and/or intramural fibroids before resorting to ART.

The main limitation of our current study is that we do not have any evidence to support the association between the existence of an inflamed endometrium and the consequent achievement of pregnancy after removal of the culprit myoma nodules. In fact, five women with SMM in our study who complained of secondary infertility are currently being followed up after removal of their nodules. Further multi-centre prospective studies are necessary to strengthen the implications of our current findings as a possible cause of infertility.

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