Differential Infiltration of Macrophages and Prostaglandin Production by Different Uterine Leiomyomas

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Abstract

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, 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 (Mϕ) accumulation in different uterine fibroids and the production of chemokine and prostaglandin 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 intramural myoma (IMM) and 18 women with subserosal myoma (SSM). CD68 immunoreactive Mϕ were identified in these tissues by immunohistochemistry. A fraction of corresponding tissues were homogenized and levels of monocyte chemotactic protein-1 (MCP-1) and prostaglandin F2α (PGF2α) were

measured by ELISA. **Results:** Macrophage infiltration in the myoma nodule and corresponding endometrium of women with SMM and IMM was significantly higher than that of SSM or control women. 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 $M\phi$ infiltration and was significantly higher in SMM and IMM than that of SSM. A positive correlation was observed between MCP-1 concentration and accumulated $M\phi$ numbers in the endometrium of women with SMM and IMM but not in SSM. The tissue levels of PGF2α were also significantly higher in the nodule and corresponding endometrium of women with SMM and IMM than that in SSM or control women. **Conclusion:** Higher production of MCP-1 could be responsible for the increased accumulation of $M\phi$ in submucosal and intramural myomas. The augmented inflammatory reaction in endometrium and increased PGF2α levels might be detrimental to reproductive outcome in women with submucosal or intramural myomas.

Key Words: macrophages / uterine myoma / MCP-1 / PGF2α / infertility.

Introduction

Uterine fibroids (leiomyoma, myoma) are the most common tumors found in women in the reproductive age group. Their occurrence increases with age. Various prevalence rates have been quoted in literature ranges from 20%-50% of women over the age of 30 years (Wallach EE, 1992; Verkauf BS, 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% to 10% of cases by a number of mechanisms (The Practice Committee of the ASRM, 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 reproductive (ART) treatment (Rackow BW and Arici A, 2005). It is well accepted that the anatomical location of the fibroid is an important factor, with submucous, intramural and subserosal fibroids being in decreasing order of importance, in causing infertility (Bajekal and Li, 2000; Rackow BW and Arici A, 2005). Submucousal or

intramural myoma 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% to 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 (Darai et al., 1997; Goldenberg M et al., 1995). There are five retrospective cohort studies that examined the impact of fibroids on the results of assisted conception (Bajekal and Li, 2000; Rackow BW and Arici A, 2005). The pregnancy rate per embryo transfer in submucous, intramural and subserosal fibroids were 9, 16 and 37%

respectively, compared with an average of 30% in control subjects. The miscarriage rate in the various types of fibroids were 40% for submucous, 33% for intramural, 33% for subserosal myoma 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 submucous 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. Stoval 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 intramural myomas, 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 containing uterine fibroids.

Therefore, we investigated the accumulation of macrophages (Mϕ) in different uterine myomas and their autologous myometrium or endometrium and examined their relationship with the production of chemokine by these tissues. Since variable production of prostaglandin F2α (PGF2α) by myometrium and their autologous endometrium could be a contributing factor for uterine contraction, we also examined the tissue levels of PGF2α in different myomas, their adjacent myometrium and corresponding endometrium. We analyzed these results with 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 a total of 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 anemia with or without

associated complaint of dysmenorrhea. The uterine fibroids in all these women were diagnosed by ultrasonograghapy and magnetic resonance image 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 submucosal myoma (SMM), intramural myoma (IMM) and subserosal myoma (SSM) were based according to 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 less than 50% extension into the serosal surface; SSM (n=18) as the myoma nodule with more than 50% extension into the serosal surface. In the case of mixed myoma, any nodule distorting the caliber of uterine cavity was categorized as submucosal myoma. In the case of multiple myoma except SMM, nodule with maximum size was selected. Among women with SMM, trans-cervical hysterosocopy 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 study and control women were coexistent with either endometriosis or adenomyosis. Six women with SMM, 10 with IMM and five with SSM had hormonal medication within six months before operation. Five women in SMM group complained of secondary sterility and wished for baby. Two of these five women with SMM were associated with the findings 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. Since 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 endometrum from 20 women without uterine myoma was also evaluated. A total of 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 for formalin-fixed paraffin-embedded tissue blocks for subsequent histopathological and immunohistochemical study.

A fraction of biopsy specimen from myoma nodule, adjacent myometrium and corresponding endometrium of women with different uterine myomas and also endometrium of control women were homogenized in homogenizing buffer using a Polytron homogenizer (Kinematics, Luzern, Switzerland). The respective tissue suspension was centrifuged at 1500 rpm for 5 minutes to obtain the supernatant and stored at -80 ℃ for the subsequent measurement of chemokine (MCP-1) and prostaglandin F2 α (PGF2 α).

Antibodies and reagents used. We performed immunohistochemical studies to investigate the immunoreaction of CD68 for macrophages (M ϕ) in intact tissues. CD68

(KP1), a mouse monoclonal antibody was derived 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 M ϕ, is a glycosylated trans-membrane glycoprotein that is mainly located in lysosomes. It belongs to a family of lysosomal granules (Holness and Simmons, 1993). Non-immune mouse immunoglobulin (Ig) G1 antibody in 1:50 dilution was used as a negative control.

The cell lysis buffer used for tissue homogenization consisted of the following reagents: 5 mM Tris-HCl (pH 7.4), 5 mM NaCl, 1 mM CaCl2, 2 mM ethyleneglycol-*bis*-(β aminoethyl ether)- *N,N,N´ , N´* -tetraacetic acid, 1 mM MgCl2, 2 mM DTT, 25 μ g/mL aprotinin, and 25 μ g/mL leupeptin. This homogenizing buffer was prepared according to the instruction as published previously (Fujimoto et al., 2000).

Immunohistochemistry. The details of immunohistochemical staining were described elsewhere (Ishimaru et al., 2004; Khan et al., 2003, 2004). Briefly, five-micrometer thick paraffin-embedded tissues were deparaffinized in xylene and rehydrated in phosphate-buffered saline. After immersion in 0.3% H_2O_2/methanol to block endogenous peroxidase activity, sections were preincubated with 10% normal goat serum to prevent

nonspecific binding and then incubated overnight at 4℃ with anti-CD68 antibody. The slides

were subsequently incubated with biotinylated second antibody for 10 minutes, followed by

incubation with avidin-peroxidase for 10 minutes and visualized with diaminobenzidine.

Finally, the tissue sections were counterstained with Mayer's hematoxylene, dehydrated with

serial alcohols, cleared in xylene, and mounted.

The immunoreactive CD68 spots were counted in five different fields of one

section (x200 magnification) by light microscopy and expressed as the mean Mϕ number

per field in one specimen. We used a combination of a x20 objective and a x10 ocular

(0.785mm2/field). The number of Mϕ per field in each biopsy specimen was recounted and

confirmed by a second observer who did not know the history of these patients.

Measurement of MCP-1 and PGF2α. The tissue concentrations of MCP-1 and

PGF2α in the homogenized supernatant of myoma nodule, <u>myometrium</u> 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 (Quantikine; R&D System, Minneapolis, MN) according to the

manufacturer's instructions and as described recently (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 less than 5.0 pg/mL 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

and the X^2 test for any difference between two groups. Differences in Mϕ number and

MCP-1 or PGF2α concentration between two groups were analyzed by the nonparametric

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 detail clinical profiles of control women and women with different uterine leiomyomas are shown in Table 1. The control women were significantly younger than women with any types of myomas. There was no difference in the mean ages between women with submucosal myomas (SMM) and subserosal myomas (SSM) or between women with intramural myomas (IMM) and SMM. However, the mean ages of women with IMM were significantly higher than that of SSM (41.4 ± 6.4 vs. 36.3 ± 5.3 years, mean $\pm$ SD, $p<0.01$). The mean size of the fibroids in women with IMM and SSM was significantly larger than that in women with SMM ($p<0.001$ and $p<0.05$, respectively). A variable number of other diseases such as endometriosis or adenomyosis were coexistent with either 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 shown in Table 1.

M ϕ infiltration in myoma nodules, autologous myometrium and endometrium. When we distributed the M ϕ infiltration in different myoma nodules and their autologous myometrium or endometrium, we found that M ϕ 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 was 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 or autologous myometrium derived from women with SMM and IMM (Figure 1), 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 subserosal myomas, because there was no cases of hysterectomy in this group of women.

Quantitative analysis of Mϕ infiltration in different leiomyomas. Initially, we tried to analyze the macrophage infiltration separately in the cases with or without coexistent diseases but we did not find any significant difference in the accumulation of these inflammatory cells in either endometrium or in myoma nodule or in autologous myometrium between them. 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 ($\pm$ SEM) per field in the endometria, myoma nodules and

autologous myometrium between women with and without coexistent diseases are shown in

Table 2.

The mean macrophage (Mϕ) number per field in different myoma nodules and

autologous myometrium or endometrium of women with submucosal myoma (SMM),

intramural myoma (IMM) and subserosal myoma (SSM) are 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 vs. SSM; $p<0.001$, IMM vs. SSM, Figure 2A).

However, no significant difference in Mϕ infiltration was observed between the myoma

nodules or surrounding myometrium derived from women with SMM and IMM (Figure 2A).

Again, we found that Mϕ infiltration in the corresponding endometrium of women

with submucosal myoma was significantly higher than that of intramural myoma, subserosal

myoma or control women ($p<0.05$, SMM vs. IMM; $p<0.01$, SMM vs. 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 vs. 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). Since a small size of the study population in each group of the myomas underwent hormonal therapy for a variable period of three to six months, we found a decreased tendency of Mϕ infiltration in the myoma nodule, myometrium and endometria of women who received hormonal therapy (with GnRHa treatment) without displaying any significant difference from the corresponding tissues of women who did not receive any hormonal therapy (without GnRHa treatment).

The mean Mϕ number ($\pm$ SEM) per field in the endometria, myoma nodule and myometrium derived from different leiomoymas of women without GnRHa treatment vs.

with GnRHa treatment and their p values are described in Table 3.

In order to examine any difference in M ϕ infiltration according to the anatomical location of the endometrium, we collected endometrial tissues from different anatomical sites of women who underwent hysterectomy. We found an apparent increase in the accumulation of M ϕ in the endometrium adjacent to SMM nodule or IMM nodule, but there was no significant difference in M ϕ 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 monocyte chemotaxis protein-1 (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 that 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 vs. SSM; $p < 0.05$, IMM vs. SSM (Figure 3A); endometrium, $p < 0.01$, SMM vs. SSM or control; $p < 0.05$, IMM vs. SSM or control (Figure 3B). These findings of MCP-1 in the myoma

nodule and endometrium corresponded to increased accumulation of Mϕ in the similar

tissues derived from women with SMM or IMM as shown in Figure 2A and 2B. 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. Since we

found an increased infiltration of macrophages 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 M

ϕ in the endometrium of women harboring different myomas. We found a significant

positive correlation between MCP-1 concentration and tissue infiltration of Mϕ in the

endometrium of women with SMM (R^2=0.377, p<0.01, Figure 4A) and 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 SSM (R^2=0.158, p=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 M ϕ in the myoma nodule and myometrium of women with SMM and IMM.

Tissue levels of PGF2 α in myoma nodule, myometrium and endometrium.

Since PGF2 α is involved in causing uterine contraction and vasoconstriction of spiral arteries of the endometrium, we therefore measured the tissue levels of PGF2 α in the myoma nodule, myometrium and corresponding endometrium of different myomas as shown in Figure 5. A variable tissue concentration of PGF2 α was observed in the different myoma nodules, myometrium and their corresponding endometrium. The tissue levels of PGF2 α were significantly higher in the myoma nodule and corresponding endometrium derived from women with SMM and IMM than that of similar tissues derived from SSM or control women (Figure 5A and 5B). An apparent increase in PGF2 α levels was observed in the myometrium of SMM and IMM but there was no significant difference in myometrial concentration of PGF2 α between SMM and IMM or in PGF2 α levels between myoma nodule and myometrium of women with SMM and IMM (Figure 5A). The statistical differences of PGF2 α between myoma nodules and endometria of different leiomyomas are as follows: myoma nodule, $p<0.05$, both SMM and IMM vs. SSM (Figure 5A); endometrium,

p<0.01, both SMM and IMM vs. control; p<0.05, both SMM and IMM vs. SSM (Figure 5B).

Since the number of biopsy specimen derived from endometrium was small, we analyzed the tissue levels of PGF2α 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 PGF2α 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 PGF2α when compared with early proliferative phase (Figure 5C).

Discussion

We demonstrated for the first time that the inflammatory reaction and the biological activity of the myoma nodule and corresponding endometrium of women with submucosal and intramural myomas are different from that of women with subserosal myoma and control women. This was evidenced 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 PGF2α in the tissue homogenates of different myoma nodules, myometrium and autologous endometrium that were simultaneously

collected during surgery.

We found that the degree of inflammatory reaction as manifested by the infiltration of M ϕ in the myoma nodules and their corresponding endometrium was significantly stronger in the biopsy specimens derived from women with submucosal and intramural myomas when compared with that of women with subserosal myoma and control women whose uteri were free of myoma nodules. In fact, we demonstrated that the tissue infiltration of M ϕ in the myoma nodule and autologous endometrium was the highest in women containing submucosal myoma, intermediate in intramural myoma and the least in either subserosal myoma or in the endometrium of control women. When we examined the infiltration of M ϕ in myoma nodule alone, we found that the distribution in the accumulation of these inflammatory cells was similar between submucosal and intramural myoma but was significantly higher than that of specimens derived from subserosal myoma nodules. A similar inflammatory reaction was also observed in the surrounding healthy myometrium of women with submucosal and intramural myomas 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 $M\phi$ appeared to be the highest in the corresponding endometrium of myoma nodules originated in the subendometrial myometrium (junctional zone), modest in that of myoma nodule having intramural origin and the least in that of subserosal origin.

Since the origin of submucosal myoma 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 submucosal myoma 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 submucosal myoma with resultant ischemic or hypoxic change might be responsible for producing different angiogenic factors and an augmented endometrial vascular perfusion (Carmeliet P, 2000). Recently, Xavier P et al (2005) demonstrated that

sub-endometrial 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 intramural myoma causing moderate production of MCP-1

and a modest inflammatory reaction in their endometrium.

A number of ART clinical trials in women containing different types of uterine

myomas demonstrated that submucosal myomas are the most detrimental in reducing

pregnancy rate, 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 the anatomical deformity of the uterine cavity caused by submucosal myomas.

However, the parallel ART clinical trials in women containing intramural myoma without

any cavity deformity showed that the pregnancy and implantation rates were also

significantly decreased and almost similar to that of submucosal myomas when they

compared the ART results of women containing either subserosal myoma or women who

were free of any uterine pathology (Eldar-Geva et al., 1998; Oliveira et al., 2004). We

speculate that this adverse effect of intramural myoma on the worse fertility outcome might

be due to a sustained inflammatory reaction in the autologous endometrium of women

containing intramural myomas as we demonstrated in our current study. In fact, we found that

the corresponding endometrium of women containing either submucosal myoma or

intramural myoma harbored abundant infiltration of M ϕ irrespective of the presence of

cavity deformity of the uterus. These increased tissue infiltrations of M ϕ in the endometrium

were significantly higher than that of similar tissues of women containing either subserosal

myoma or control women. These results indicate that endometria of women containing

submucosal myoma or intramural myoma develop a similar degree of in-situ inflammatory

change that might be responsible in creating adverse reproductive outcome.

Our findings of increased tissue infiltration of M ϕ in the myoma nodules and

corresponding endometrium of women with submucosal and intramural myomas 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 nodules are the most detrimental in causing either decreased pregnancy rate or

increasing miscarriage rate. However, our findings revealing a diffuse inflammatory reaction

involving different anatomical location 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 a submucosal myoma or an intramural myoma in their uterus.

Since the vascularity of the submucosal and intramural myomas are different from that of subserosal myomas (Walocha et al., 2003; Brosens J et al., 2003) and the production and secretion of MCP-1, a potent chemo-attractant protein, are mainly from the vascular endothelial cells, macrophages, and smooth muscle cells (Khan et al., 2004; Seli et al., 2002), we tried to examine the relationship between the concentrations of MCP-1 and accumulation of M ϕ 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 submucosal or intramural myomas and these findings corresponded to an increased infiltration of M ϕ in the corresponding tissues of the similar women. We also found a positive correlation between the production of MCP-1 and accumulation of inflammatory cells in the endometria of both groups of women containing either submucosal myoma or intramural myoma. This indicates that a 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 consequent recruitment of inflammatory cells.

The dysfunctional uterine contraction as caused by the increased production of PGF2α 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 PGF2α. We measured PGF2α levels in the tissue homogenates of these tissues and found that the tissue contents of PGF2α were significantly higher in the myoma nodules and corresponding endometrium of women with either submucosal myoma or intramural myoma when compared with that in the similar tissues derived from subserosal myoma or control women. Since PGF2α 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 PGF2α 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 are important for successful implantation or progressive sperm transport. Our findings of enhanced tissue levels of $PGF2\alpha$ during the periovulatory period of menstrual cycle may have some clinical implications to determine fertility outcome in women containing either submucosal myoma or intramural myoma.

Leiomyomas do not contract and their prostaglandin receptors are down regulated relative to the myometrium as indicated by cDNA arrays study of Tsibris et al., (2002). We found an apparent increase of $PGF2\alpha$ in the myometrium comparing 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 $PGF2\alpha$ levels in myoma nodules and adjacent myometrium of women with submucosal and intramural myomas may support a possible cause of augmented uterine contraction by the local transport of $PGF2\alpha$ to the junctional zone or endometrium by vascular channels.

Recently Nishino M et al. (2005) reported that uterine peristaltic movements were partly interrupted by submucosal myomas. Their findings of lower uterine contractility was focal, adjacent to myomas, detected by cine magnetic resonance image (MRI) and was observed in only one third of their studied cases. The uterine contractility was well preserved in the remaining part of the sub-endometrial 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 PGF2α in women with submucosal and intramural myoma may biologically explain their cine MRI findings. In addition to uterine contractility, PGF2α-induced vasoconstriction with resulting ischemic or hypoxic change in and around the site of implanting nidus could an additional factor in producing adverse fertility outcome in women with submucosal or intramural myoma.

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 Mϕ 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; Ishimaru et al., 1994; Khan et al., 2005a, 2005b). The decreased fertility outcome in women containing uterine fibroids as described in the previous reports (Eldar-Geva et al., 1998; Bajekal and Li, 2000) can be explained by a range of biological activities of infiltrated $M\phi$ in the endometrium and surface endometrial damage as caused by the ischemic or hypoxic changes of the spiral arteries secondary to PGF2α-induced vasoconstriction. The combined inflammatory 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 of women harboring submucosal myoma or intramural myoma in their uterus.

Our results have some biological and clinical implications. (1) Besides cavity deformity, submucosal myoma nodules may also cause a strong and diffuse inflammatory reaction in the autologous endometrium. (2) Even when there is no cavity deformity, presence of intramural myoma nodule may also create an inflamed endometrium. (3) Endometria of control women and women with subserosal myoma display a minimal inflammatory change and may not impair fertility outcome. (4) Surgical or medical treatments should be considered in infertile women who have submucosal and/or intramural

fibroids before resorting to ART treatment.

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

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Figure Legends

Figure 1. Shows the immunohistochemical localization of M ϕ infiltration as indicated by the CD68 immunoreactive spots in the biopsy specimens derived from the endometrium (*a, d, g*), myoma nodules (*b, e, h*), and myometrium (*c, f*) of women with submucosal myoma (*a, b, c*), intramural myoma (*d, e, f*), and subserosal myoma (*g, h*). All these tissue sections were derived from the similar proliferative phases of the menstrual cycle. The infiltration of M ϕ appeared to be higher in the sections derived from the myoma nodules (*b and e*) and autologous endometria (*a and d*) of women with submucosal myoma and intramural myomas when compared with that of similar sections (*h and g*) derived from women with subserosal myoma. Final magnification was adjusted at x200 using a light microscope.

Figure 2. Shows the mean M ϕ number per field in the myoma nodules or myometrium (**A**) and autologous endometria (**B**) of women with submucosal myoma (SMM), intramural myoma (IMM), subserosal myoma (SSM) and control women. The results are expressed as mean $\pm$ SEM. **A**. myoma nodules, *p<0.01, SMM vs. SSM; ‡p<0.001, IMM vs. SSM. **B**. endometrium, ‡p<0.01, SMM vs. SSM or control; *p<0.05, SMM vs. IMM;

#p<0.05, IMM vs. SSM or control.

Figure 3. Shows the concentrations of MCP-1 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. The results are expressed as mean ± SEM. The higher tissue concentrations of MCP-1 in the myoma nodules and autologous endometria corresponded to the increased infiltration of M ϕ in the parallel tissues derived from women with SMM and IMM as shown in Figure 2. **A**. myoma nodules, *p<0.05, SMM vs. SSM; †p<0.05, IMM vs. SSM. **B**. endometrium, *p<0.01, SMM vs. SSM or control; ‡p<0.05, IMM vs. SSM or control.

Figure 4. Shows the correlation between the tissue concentration of MCP-1 and the infiltrated M ϕ number per field in the endometria derived from women with submucosal myoma (**A**), intramural myoma (**B**) and subserosal myoma (C). There was a significant positive correlation between MCP-1 concentration and accumulation of M ϕ in the endometria derived from women with submucosal myoma (R^2=0.377, p<0.01, **A**) and intramural myoma (R^2=0.480, p<0.01, **B**). In contrast, no correlation was observed between them in the endometria derived from women with subserosal myoma (R^2=0.158, p=NS, not

significant) or in the similar tissues obtained from control women (data not shown).

Figure 5. Shows the levels of PGF2 α 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. The collective tissue content PGF2 α in the endometria derived from all women with SMM, IMM and SSM are also shown according to the phases of the menstrual cycle (**C**). The results are expressed as mean ± SEM. The tissue levels of PGF2 α were significantly higher in the myoma nodules and autologous endometria of women with SMM and IMM when compared with that in the similar tissues derived from women with SSM or control women (**A and B**). The tissue levels of PGF2 α also appeared to be higher in the periovulatory phase of the menstrual cycle (**C**). **A**. myoma nodules, ‡p<0.05, SMM or IMM vs. SSM. An apparent increase in myometrial PGF2 α content was found in SMM and IMM without any significant difference from corresponding myoma nodules. **B**. endometrium, *p<0.05, SMM vs. SSM or control; †p<0.05, IMM vs. SSM or control. **C**. endometrium, *p<0.05, late proliferative phase vs. late secretory phase;†p<0.05, early secretory phase vs. late secretory phase of the menstrual cycle.

Figure 1

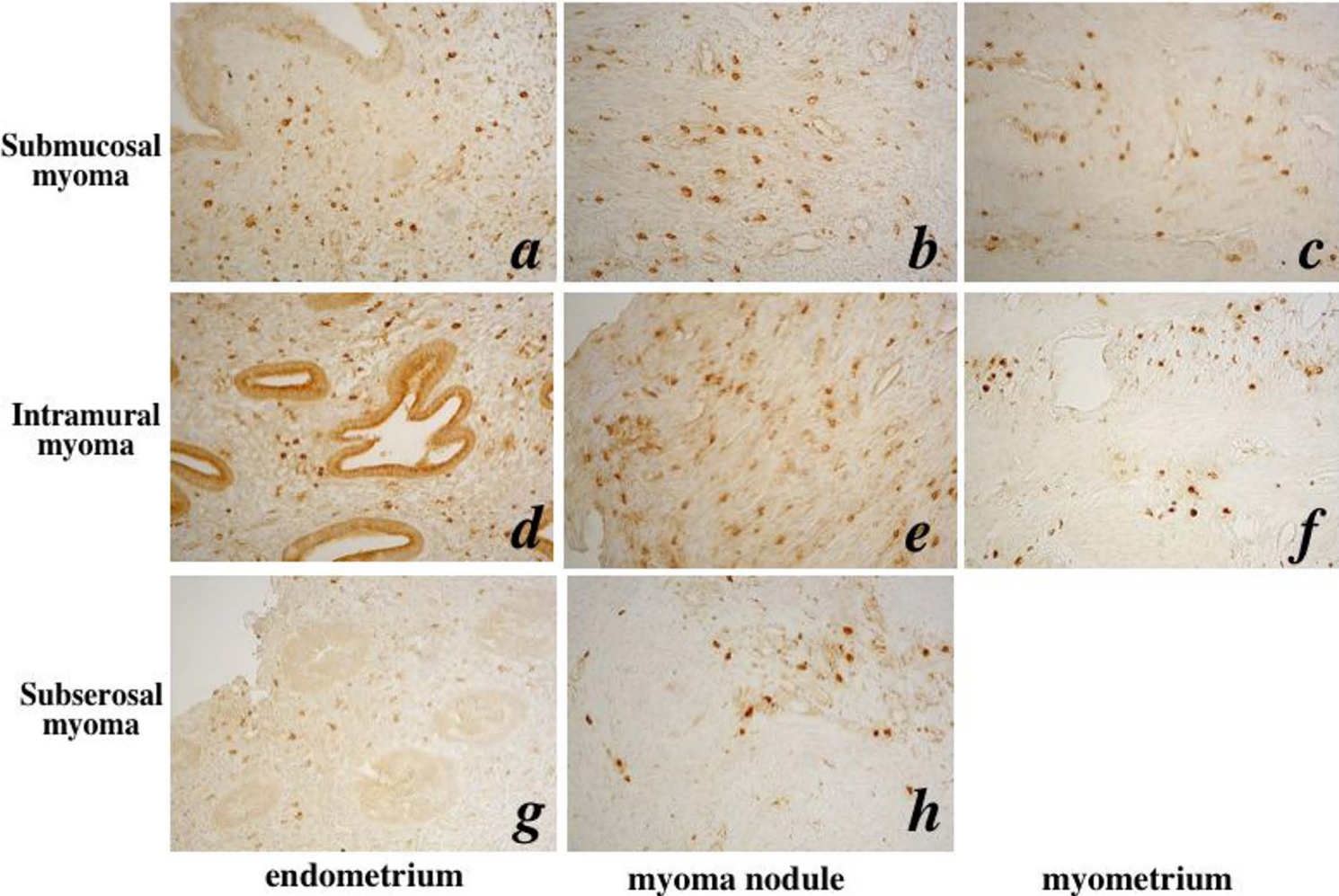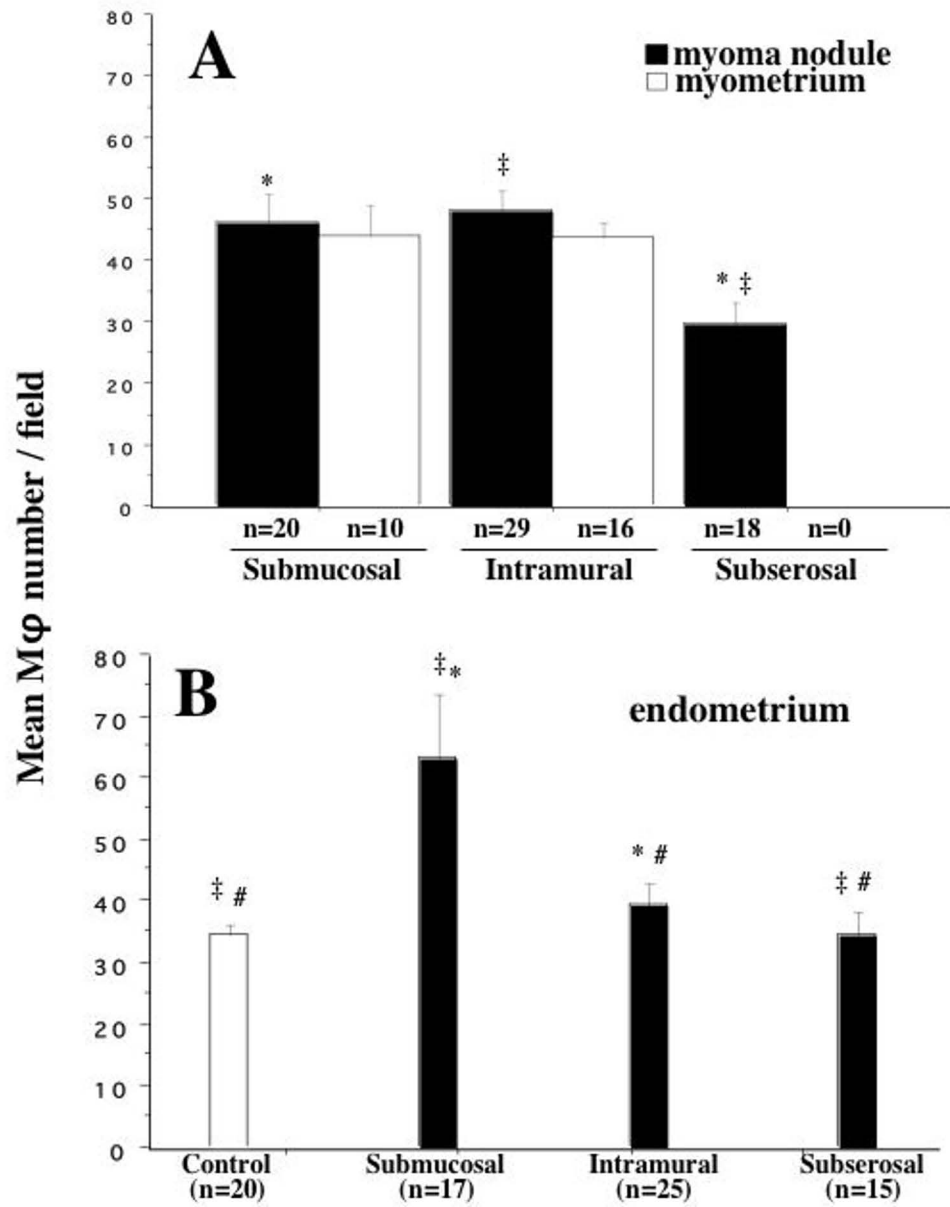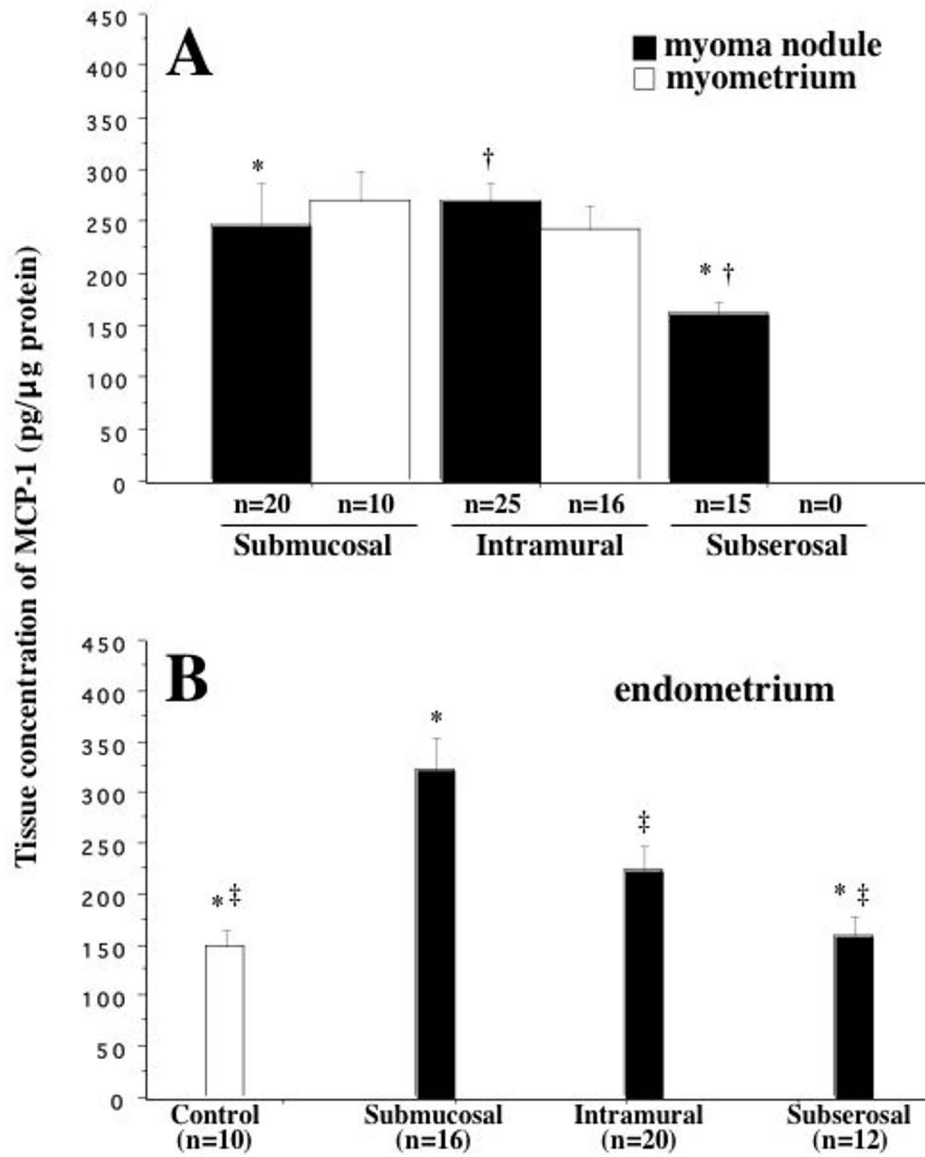# Figure 2

Figure 3

Figure 4

Submucosal myoma (n=16)
$R^2 = 0.377$, p<0.01

A

Intramural myoma (n=20)
$R^2 = 0.480$, p<0.01

B

Subserosal myoma (n=12)
$R^2 = 0.158$, p=NS

C

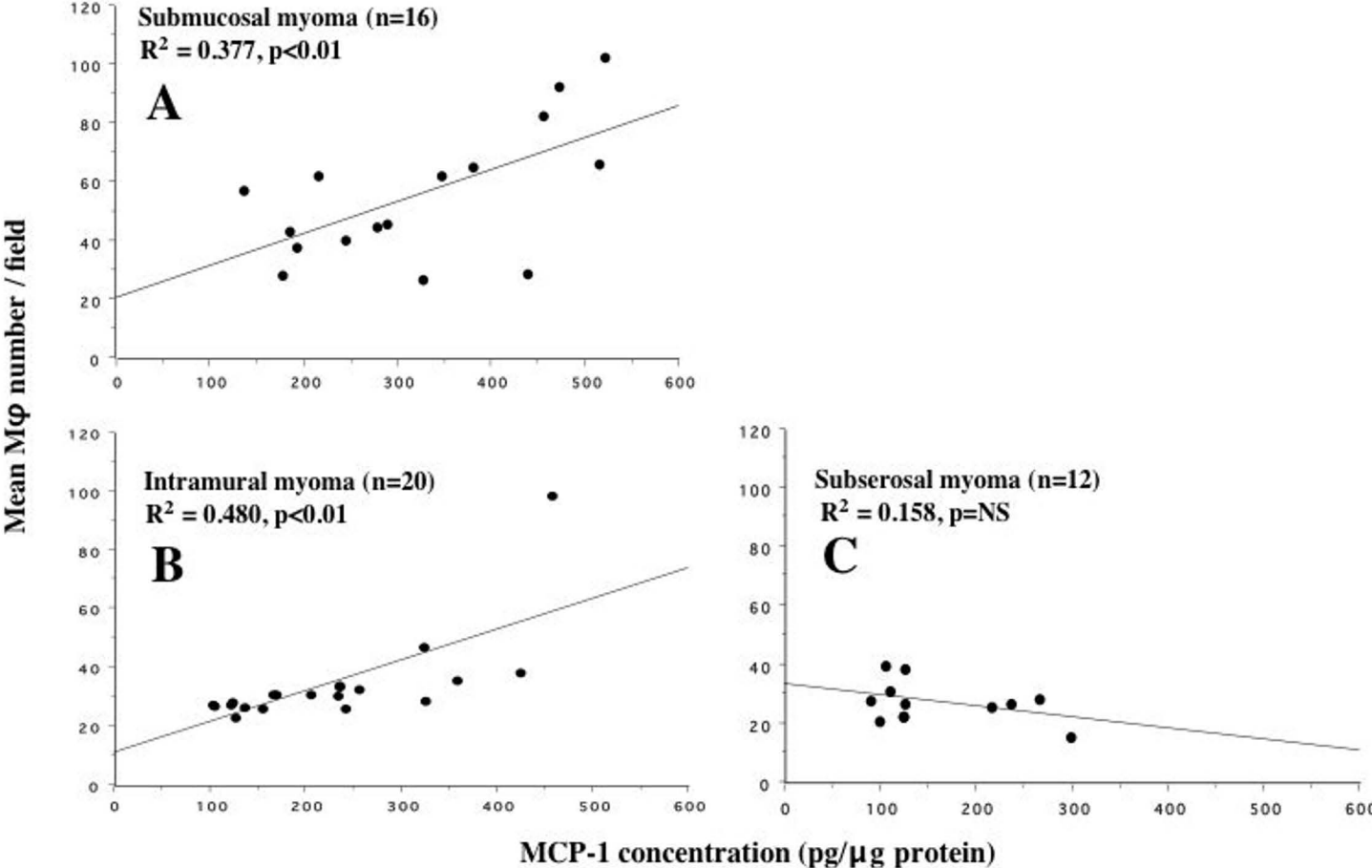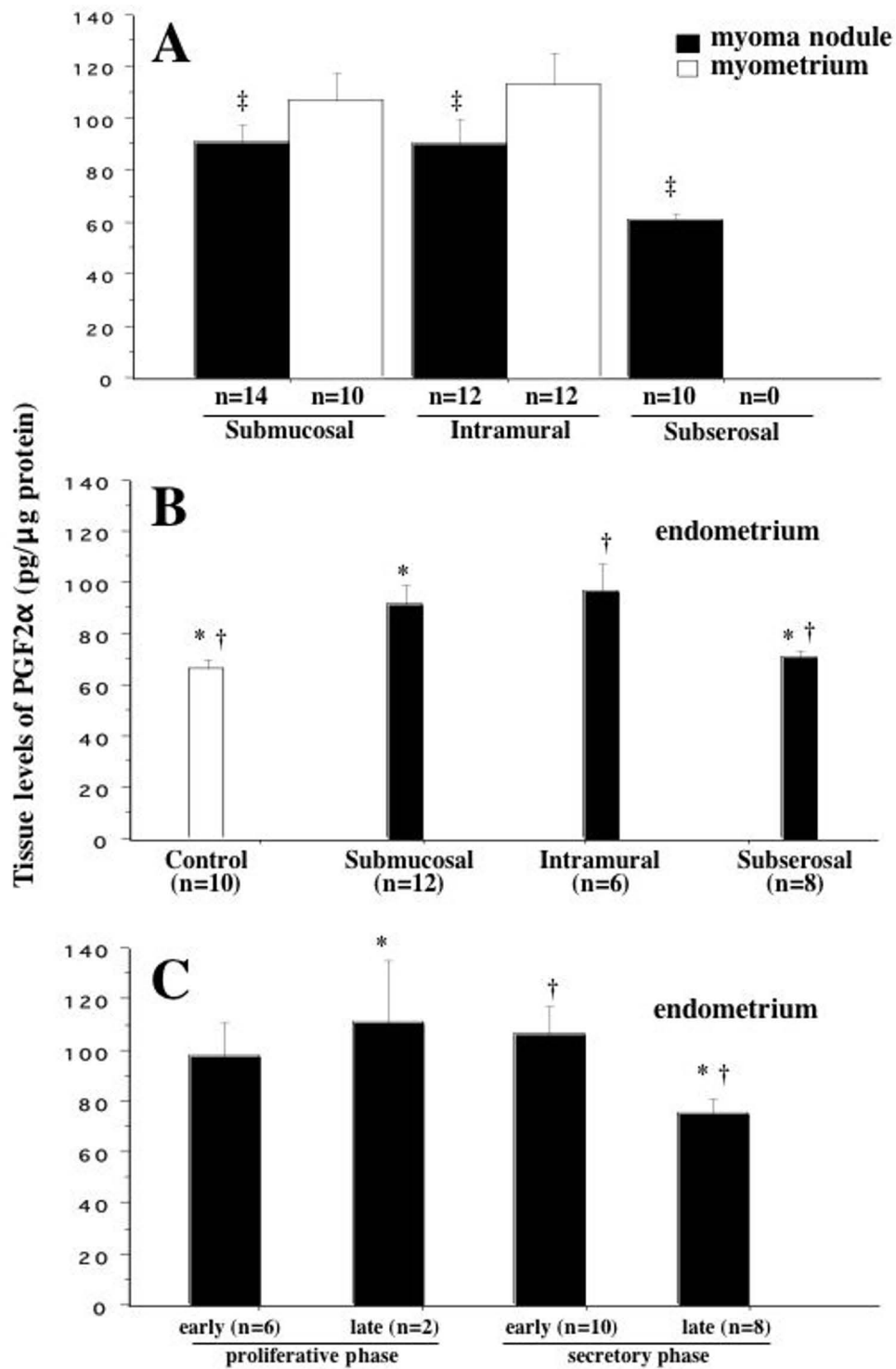Mean Mφ number / field

MCP-1 concentration (pg/μg protein)

Figure 5

Table 1. Clinical Profiles of Women with Uterine Leiomyomas

	Control (n=20)	Submucosal (n=20)	Intramural (n=29)	Subserosal (n=18)
Age (yrs) (mean ± SD):	29.3 ± 3.2*†	37.0 ± 8.2†	41.4 ± 6.4*	36.3 ± 5.3*†
Range in age (yrs):	21-36	17-50	28-51	28-44
Size (cm) (mean ± SD):		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
GnRHa therapy: (-)/(+)		14 / 6	19 / 10	13 / 5
Menstrual cycle: P/S/M	10 / 10 / 0	4 / 10 / 0	4 / 10 / 5	3 / 9 / 1

The results are expressed as mean ± SD. Age, *p<0.01, IMM vs. SSM and control; †p<0.05, SMM or SSM vs. control; Size, *p<0.05, SSM vs. SMM; †p<0.0001, IMM vs. SMM. SMM, submucosal myoma; IMM, intramural myoma; SSM, subserosal myoma. P, proliferative phase; S, secretory phase; M, menstrual phase.

Table 2. Macrophage infiltration in different leiomyomas of women with and without coexistent diseases.

Type of tissues	With coexistent diseases	Without coexistent diseases	p value
Control: endometrium	32.0 ± 1.6	29.9 ± 1.5	ns
SMM: endometrium	61.6 ± 5.4	56.4 ± 5.1	ns
myoma nodule	46.1 ± 4.6	42.4 ± 7.2	ns
myometrium	43.9 ± 5.1	45.0 ± 7.8	ns
IMM: endometrium	39.3 ± 3.7	40.6 ± 4.5	ns
myoma nodule	48.2 ± 3.4	58.8 ± 5.6	ns
myometrium	43.6 ± 2.5	46.2 ± 5.2	ns
SSM: endometrium	31.2 ± 2.9	34.5 ± 11.9	ns
myoma nodule	29.7 ± 3.5	32.8 ± 6.5	ns

The results are expressed as mean ± SEM. Tissue infiltration of macrophages (Mφ) was quantitated as mean Mφ number per field. SMM, submucosal myoma; IMM, intramural myoma; SSM, subserosal myoma; ns, not significant.

Table 3. Macrophage infiltration in different leiomyomas of women with and without GnRHa treatment.

Type of tissues	Without GnRHa treatment	With GnRHa treatment	p value
SMM: endometrium	59.1 ± 7.0	44.6 ± 8.1	0.396
myoma nodule	50.9 ± 10.2	42.6 ± 5.4	0.510
myometrium	46.6 ± 7.6	43.8 ± 8.3	0.769
IMM: endometrium	39.3 ± 3.6	29.4 ± 1.0	0.154
myoma nodule	54.5 ± 8.4	44.8 ± 2.6	0.765
myometrium	44.4 ± 3.0	41.2 ± 4.7	0.627
SSM: endometrium	34.1 ± 3.5	23.3 ± 3.9	0.170
myoma nodule	30.6 ± 4.6	27.2 ± 4.7	0.921

The results are expressed as mean ± SEM. Tissue infiltration of macrophages (Mφ) was quantitated as mean Mφ number per field. GnRHa, gonadotropin releasing hormone agonist; SMM, submucosal myoma; IMM, intramural myoma; SSM, subserosal myoma.