Differences in the blood serum levels of soluble HLA-G concentrations between the menstrual cycle phases and menopause in patients with ovarian endometriosis and uterine leiomyoma

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Abstract

BACKGROUND: HLA-G is an antigen whose participation in the regulation of the immune system is well documented. The aim of the present study has therefore been to evaluate the sHLA-G blood serum concentrations levels in both women with ovarian endometriosis and women with uterine leiomyomas.

METHODS: In our study, the soluble HLA-G concentration level was evaluated in the blood serum samples obtained from 98 women who underwent laparotomies or laparoscopies due to either ovarian endometriosis or leiomyomatous uterus. The control group consisted of 42 women, including women on whom a diagnostic laparoscopy identified no lesions, and volunteers—healthy women who returned their blood serum samples during menstrual bleeding.

RESULTS: Patients who underwent surgical treatment because of ovarian endometriosis or uterine leiomyoma, as well as patients from the control group, exhibited no sHLA-G blood serum concentration level fluctuations between the proliferative and secretory menstrual cycle phases. The sHLA-G levels were significantly lower in the patients with ovarian endometriosis and in the patients from the control group during the menstrual cycle phase than in those patients with leiomyoma. A similar relation between the sHLA-G levels of the postmenopausal patients suffering from leiomyoma and the control patients was found. In contrast, the postmenopausal women suffering from endometriosis were typified by levels of sHLA-G blood serum concentration comparable to those of the patients with leiomyoma, and the levels were significantly higher than those observed in the blood sera of the postmenopausal patients from the control group.

CONCLUSION: The soluble HLA-G blood serum level would seem to be a useful marker for evaluating the status of the microenvironment, where the tumor-immune cell and ectopic and eutopic endometrial interactions take place.

ORIGINAL ARTICLE

1. INTRODUCTION

Human leukocyte antigen–G (HLA-G), a nonclassical Class Ib molecule, is observed within immune cells and in different organs, such as the uterus, thymus, and placenta, among others [1–4]. Initially, HLA-G was found at the feto-maternal interface – specifically, on the extravillous cytotrophoblast in the first and third trimester [1]. HLA-G is an antigen whose participation in the regulation of the immune system has been well documented [4]. Moreover, HLA-G expression has been identified on neoplasmatic cells, including melanoma cells [3,5], lung cancer cells [6], neuroblastoma cells [2], choriocarcinoma cells [1], ovarian cancer cells [7–8], and others. This protein also participates in the evasion of immune surveillance by cancer cells [2–3]. As a class I histocompatibility complex (MHC), HLA-G in human beings is expressed in both forms---membrane–bound and soluble [9]. The soluble form of this protein may be responsible for the inhibition and even the apoptosis of NK cells and cytotoxic T lymphocytes [9–12]. The presence of the soluble form of HLA-G has been revealed in the blood sera of women during pregnancy as well as in the blood sera of cancer patients [2,3], patients with immune-mediated disorders (such as multiple sclerosis (MS) and rheumatoid arthritis (RA) [13–14]), and even healthy women [3]. In a study by Rebmann *et al.*, the sHLA-G blood serum concentrations of patients with melanoma, ovarian cancer, and breast cancer were significantly higher than those found in the healthy controls [3]. Soluble HLA-G has moreover been found in the blood sera of heart-transplant patients, and it has been documented that sHLA-G blood serum concentration correlates with better heart-graft tolerance [15]. The presence of sHLA-G has also been revealed in amniotic fluid [2], cerebral fluid [13], and peritoneal fluid [3,8,16]. In women with endometriosis, sHLA-G has been detected in peritoneal fluid [16]. Additionally, the presence of HLA-G positive endometrial cells has been revealed in peritoneal fluid during retrograde menstruation [17]. The membrane-bound form of HLA-G has been identified in ectopic endometrial cells, in ovarian endometrial lesions and, in patients with adenomyosis, in ectopic endometrial cells within the uterine wall [18,19]. Although no HLA-G expression has been observed in eutopic endometrium during the proliferative and secretory cycle phases, the presence of HLA-G positive endometrial cells has been identified during menstrual bleeding [17]. Furthermore, Wang *et al.* has detected HLA-G expression in eutopic endometrium during the different menstrual cycle phases in women with adenomyosis [19].

As a result of an alternative splicing of a primary HLA-G transcript, seven isoforms of the protein are generated. The soluble form of HLA-G can be either secreted (e.g., isoform HLA-G5) or proteolytically cleaved from the cell surface (isoform HLA-G1) [2,3,20–22]. The soluble form of this protein can be derived from immune cells, mainly macrophages/monocytes [2,3,23]. Macrophages are predominant in immune cell infiltration into ectopic endometrium lesions [24–26] and into leiomyoma nodules [27], as well as into the menstrual endometrium [28–29]. Although HLA-G expression in eutopic and ectopic endometrium has been well documented, there is no data currently available on the sHLA-G blood serum concentration in women with endometriosis and in women with leiomyomatous uterus. A dysfunction of macrophages has been observed in both endometriosis and uterine leiomyoma, and this is significant as proper immune cell activity regulation is crucial for the participation of the endometrium in reproductive processes [30–40]. The aim of the present study has therefore been to evaluate the sHLA-G blood serum concentration levels in both women with ovarian endometriosis and women with uterine leiomyomas.

2. MATERIALS AND METHODS

2.1. Human subject

In this study, we have analyzed the blood serum samples obtained from 140 patients, including 50 patients with ovarian endometriosis, 48 suffering from leiomyomatosis, and 42 patients from a control group. The samples were classified according to the menstrual cycle phase in which they were collected. None of the patients included in our study received hormonal treatment of any kind. All the patients had undergone surgical treatment in the Gynecology and Oncology Department of the Jagiellonian University, Krakow, Poland between January and September of 2008. The patient's consent was obtained in each case. Prior to the study, the approval of the Jagiellonian University Ethical Committee (KBET/135/B/2007) was also obtained.

Ovarian endometriosis (OE)

The group of patients with ovarian endometriosis consisted of 50 women who had undergone laparoscopic procedures or laparotomies. The existence of endometriosis was confirmed in all cases through histopathological examination. Only the patients with advanced endometriosis were classified to this group (III and IV clinical stage of endometriosis according to the revised classification of the American Fertility Society from 1985). Based on the assessment of each patient's level of progesterone, estradiol, and FSH in blood serum (Table 1), these groups were then divided into the following subgroups: OE-proliferative, OE-secretory, and OE-postmenopause.

Uterine leiomyoma (UL)

The 48 patients selected for this group had undergone myomectomies and hysterectomies due to leiomyomatosis in laparoscopic procedures or in laparotomies. Based on the histopathological verification of endometrial tissue samples and on the assessment of the levels

of progesterone, estradiol, and FSH in blood sera (table 1), these 48 patients were then classified into the following subgroups: UL- proliferative, UL-secretory, and UL-postmenopause.

Control group (C)

The 42 patients in the control group were selected from those who had undergone laparoscopic procedures due to infertility and in whom fallopian tube occlusion had been diagnosed as a cause of infertility. Additionally, healthy female volunteers who returned their blood serum samples during menstrual bleeding were included in this group. The volunteers had normal, regular menstrual periods, and gynecological and vaginal ultrasound examinations before and after their menstrual periods revealed no pathological lesions within the reproductive tract. According to the level of progesterone, estradiol, and FSH in the blood serum (Table 1) of each patient, the group was then divided into the following subgroups: C-proliferative, C-secretory, C-menstruation, and C-postmenopause.

2.2. ELISA

The blood was collected in a serum collection tube prior to surgery in patients who were undergoing laparoscopy or laparotomy, and during menstrual bleeding from the C-menstruation patient group. A clot was allowed to form at room temperature for 30–60 minutes. The tube was then placed on ice for 30 minutes in order to contract the clot. After this, the serum samples were centrifuged at 3000xg for 10 minutes at room temperature. The supernatants 1.0–2.0 ml were collected and stored at –80°C. The soluble human leukocyte antigen-G (sHLA-G) was detected using the sHLA-G sandwich ELISA kit (BioVendor-Exibo, Czech Republic). Briefly stated, the blood plasma samples were diluted twice and incubated for 1 hour in the 96-well microplate precoated with the monoclonal anti-sHLA-G antibodies. Following incubation, the wells were washed and then filled with the monoclonal anti-human beta-2 -microglobulin antibodies labeled with horseradish peroxidase. After an additional 1 hour of incubation, the wells were again washed, and the color reaction was developed using tetramethyl benzidine (TMB) substrate. The absorbance values were measured at 450 nm on a microplate reader followed by calculation of the sHLA-G concentrations. The assay was calibrated using a set of sHLA-G standards provided by the producer of the kit.

2.3. Statistical analysis

The distribution of variables in the study groups of women checked with the use of the Shapiro-Wilk test showed that each of them was different from normal. The statistical significance between the groups was determined by the Kruskal-Wallis analysis of variance (ANOVA) test. The Mann-Whitney U test was then used as applicable.

Table 1. Clinical characteristics of patients classified into subgroups in regarding to menstrual cycle phases and after menopause. The data is presented as a mean and standard deviation (±SD).

		Proliferative cycle phase	Secretory cycle phase	Postmenopausal women
Uterine leiomyoma (UL) (n=48)	Age (years)	37.5 (±8.6)	40.8 (±6.2)	55.2 (±8.4)
	FSH (mIU/ml)	5.93 (±1.73)	5.3 (±3.9)	61.7 (±22.3)
	Estradiol (pg/ml)	181.5 (±119.02)	110.6 (±33.8)	17.6 (±9.08)
	Progesteron (ng/ml)	0.49 (±0.22)	8.26 (± 3.87)	0.32 (±0.3)
Ovarian endometriosis (OE) (n=50)	Age (years)	38.4 (±8.6)	35.2 (±6.1)	58.1 (±8.8)
	FSH (mIU/ml)	7.14 (±5.26)	5.17 (±2.14)	65.2 (±19.4)
	Estradiol (pg/ml)	116.3 (±122.9)	114.6 (± 62.6)	32.7 (±36.03)
	Progesteron (ng/ml)	0.95 (±0.79)	7.4 (±6.4)	0.49 (±0.23)
Control group (C) (n=42)	Age (years)	31.3 (±2.12)	30.5 (±3.2)	60.0 (±6.6)
	FSH (mIU/ml)	5.45 (±.76)	5.97 (±4.43)	99.1 (±46.1)
	Estradiol (pg/ml)	278.13 (±220.3)	118.8 (±42.11)	18.6 (±16.5)
	Progesteron (ng/ml)	1.03 (±0.49)	7.32 (±3.62)	0.33 (±0.2)

3. RESULTS

The sHLA-G blood serum concentration levels were assessed in the blood sera obtained from women operated on due to either uterine leiomyoma or ovarian endometriosis during the proliferative and secretory cycle phases. In the control group consisting of healthy women, the blood serum concentration levels were determined in the proliferative and secretory cycle phases as well as during menstrual bleeding. Additionally, the sHLA-G blood serum concentration levels were determined for the postmenopausal patients in each of the examined groups.

The highest levels of blood serum HLA-G concentration were found in the patients suffering from leiomyoma during the secretory and proliferative cycle phases, while the lowest sHLA-G levels were observed in the healthy women at the time of menstruation. Patients who underwent surgical treatment because of ovarian endometriosis and uterine leiomyoma, as well as the patients from the control group, exhibited no sHLA-G blood serum concentration level fluctuation between the proliferative and secretory cycle phases (Figures 1÷3).

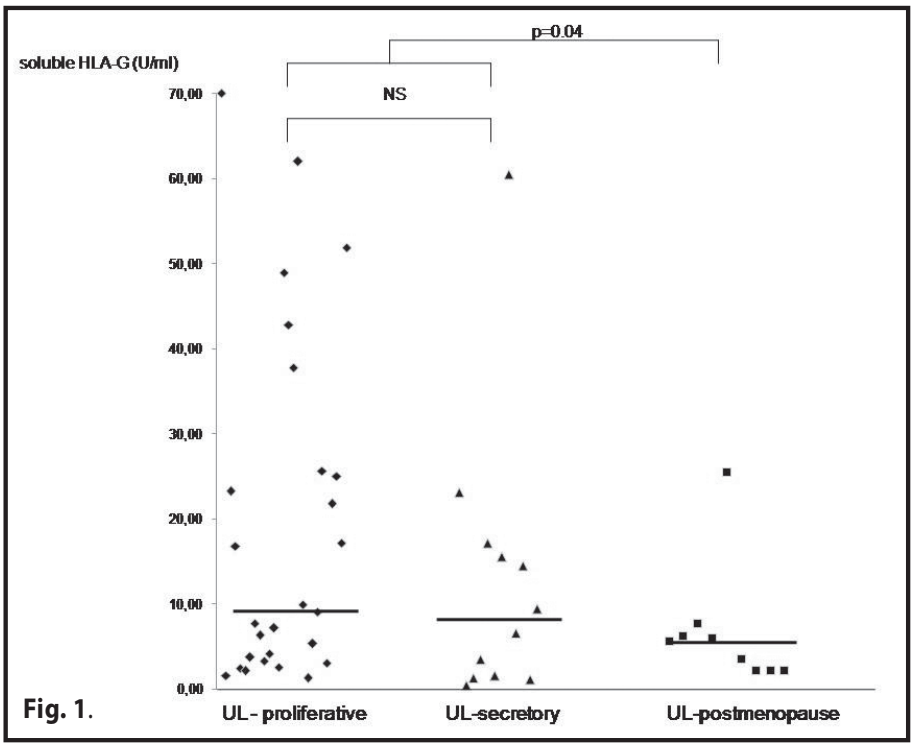

Figure 1. sHLA-G blood serum concentration levels during the menstrual cycle phases in patients operated on due to uterine leiomyoma (UL). Based on the histopathological verification of endometrial tissue samples and on the assessment of the levels of progesterone, estradiol, and FSH in blood sera, these patients were then classified into the following subgroups: UL- proliferative, UL-secretory, and UL-postmenopause.

Fig. 1.

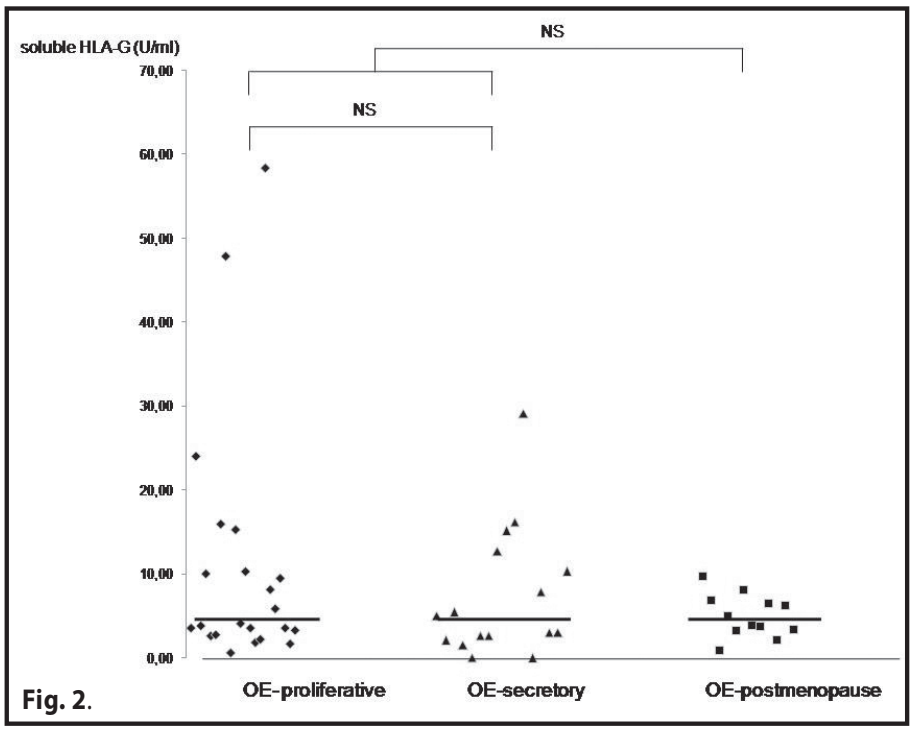

Figure 2. sHLA-G blood serum concentration levels during the menstrual cycle phases in patients operated on due to ovarian endometriosis (OE). Based on the assessment of each patient's level of progesterone, estradiol, and FSH in blood serum, these groups were then divided into the f ollowing subgroups: OE-proliferative, OE-secretory, and OE-postmenopause.

Fig. 2.

Since the blood serum concentration levels of sHLA-G in women with either leiomyoma or endometriosis as well as in the control group did not appear to fluctuate between the proliferative and secretory cycle phases, in further analyses we put together individuals from the proliferative and secretory subgroups into new subgroups named according to the particular menstrual cycle phase as follows: patients with endometriosis (OEMenstrualCycle – OEMC), patients with leiomyoma (ULMenstrualCycle – ULMC), and patients from the control group in adequate menstrual cycle phase (CMenstrualCycle – CMC).

Significant differences in sHLA-G blood serum concentration levels were observed between the patients in the different menstrual cycle phases and the postmenopausal patients suffering from leiomyoma as well as those derived from the control group. No such differences, however, were observed in the patients suffering from ovarian endometriosis (Table 2).

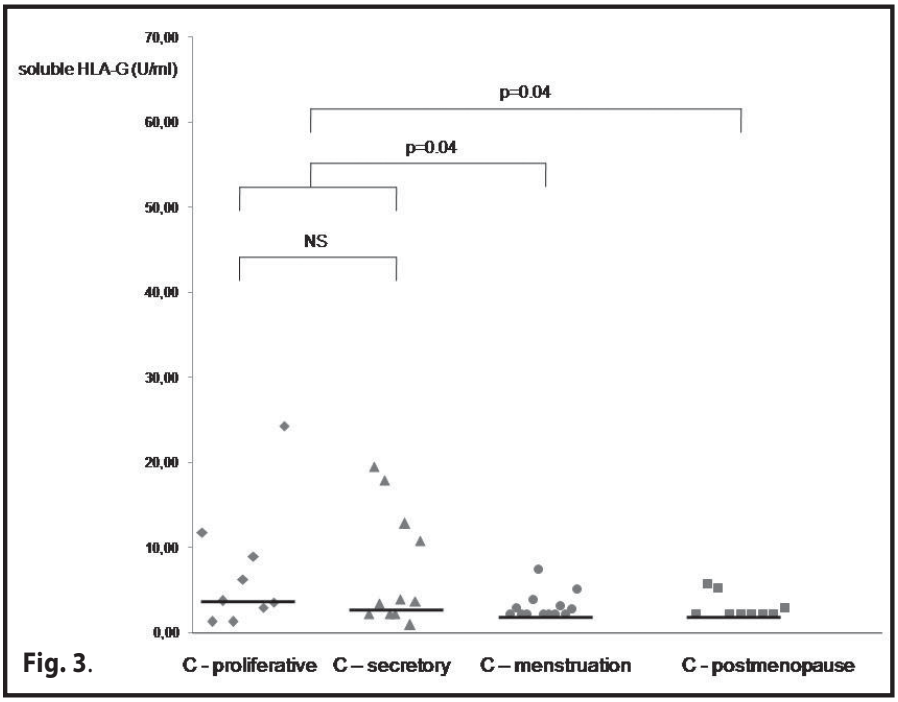

Fig. 3.

Figure 3. sHLA-G blood serum concentration levels during the menstrual cycle phases in patients from the control group (C). According to the level of progesterone, estradiol, and FSH in the blood serum of each patient, the group was then divided into the following subgroups: C-proliferative, C-secretory, C-menstruation, and C-postmenopause.

Table 2. Analysis of sHLA-G serum concentration level (U/ml) in accordance with hormonal status in patients with uterine leiomyoma (UL) and ovarian endometriosis (OE) as well as in patients from the control group (C).

	sHLA-G blood serum concentration (U/ml) (median, IQR)		
	Patients in menstrual cycle phase	Postmenopausal patients	p-value
Uterine leiomyoma (UL) (n=48)	9.01 (20.6)	4.73 (4.01)	0.04
Ovarian endometriosis (OE) (n=50)	3.98 (7.6)	3.58 (3.4)	0.2
Control group (C) (n=42)	3.31 (9.6)	1.28 (0.71)	0.04

IQR – intraquartile range

The sHLA-G levels were significantly lower in the patients with ovarian endometriosis and in the patients from the control group in comparison to the levels found in the blood sera of the patients with leiomyoma (respectively ULMC vs OEMC, p= 0.057; ULMC vs CMC, p= 0.01;). No such differences between sHLA-G levels were observed, however, in the blood sera of the patients with endometriosis when compared with the control patients in the menstrual cycle phase (OEMC vs CMC, p= 0.4). A similar relation in the sHLA-G levels was found to exist between the postmenopausal patients suffering from leiomyoma and the control patients (ULM vs CM, p= 0.09). In contrast, the postmenopausal women suffering from endometriosis were typified by levels of sHLA-G blood serum concentration that were comparable to those of the patients with leiomyoma (OEM vs ULM, p= 0.8). The levels of sHLA-G found in the patients with endometriosis were statistically significantly higher than those observed in the blood sera of the postmenopausal patients from the control group (OEM vs CM, p= 0.04)

4. DISCUSSION

In our study, the sHLA-G levels were significantly lower in patients with ovarian endometriosis and in the patients from the control group in the menstrual cycle phase than in the patients with leiomyoma. A similar relation between the sHLA-G levels of the postmenopausal patients suffering from leiomyoma and the control patients was found. In contrast, the postmenopausal women suffering from endometriosis were typified by levels of sHLA-G blood serum concentration comparable to those of the patients with leiomyoma, and the levels were significantly higher than those observed in the blood sera of the postmenopausal patients from the control group.

To our knowledge, this is the first investigation to focus on the sHLA-G blood serum levels in both women with endometriosis and women with leiomyoma and to be carried out not only in accordance with the menstrual cycle phases, but also with regard to menopause. The presence of sHLA-G in the PF of women with

endometriosis has recently been discovered, but no differences in the sHLA-G concentration levels in the PF between the women with endometriosis and the healthy women were observed [16]. Rebmann *et al.* has found sHLA-G in the blood sera of healthy women, and the level was comparable to the level of sHLA-G in the blood serum identified in our study [3].

The membrane-bound form of HLA-G has been detected in the eutopic endometrial cells of women with endometriosis or leiomyoma only during menstrual bleeding [17]. Furthermore, no HLA-G expression has been identified in the eutopic endometrium of these patients during the proliferative and secretory cycle phases [17,18]. It should be noted, however, that in this particular study, the authors did not examine postmenopausal patients. Meanwhile, Barrier *et al.* has demonstrated that HLA-G protein is usually expressed in the glandular epithelium of ectopic endometrial lesions and is rarely expressed in stromal cells [18]. Additionally, he demonstrated the presence of HLA-G in endometrial cells in the PF in women both with and without endometriosis [18]. Kawashima *et al.* has detected the HLA-G protein in endometrial cells in the PF during retrograde menstruation [17], while, by contrast, Hornung *et al.* observed no HLA-G expression in an endometrial cell culture derived from the PF of women with endometriosis [41]. The latter result is most likely linked to the use in this study of Western blot methodology to detect sHLA-G expression without the concomitant ability to distinguish stroma from glandular epithelium [18]. More recently, Wang *et al.* observed a significantly higher level of HLA-G expression in ectopic endometrium compared to those levels found in eutopic endometrium during the proliferative and secretory cycle phases in women with adenomyosis [19]. Moreover, no such expression was observed in women with leiomyoma [19]. The authors have consequently suggested that endometrial HLA-G expression may result from the presence of inflammation within the endometrium. Such inflammation is a response to immune cell activity and the secretion of various cytokines into the microenvironment of the endometrium [19]. Urosevic *et al.* has identified a correlation between IL-10 and HLA-G expression in the lung cancer nest [6], while Moreau *et al.* has demonstrated that IL-10 may up-regulate HLA-G expression in peripheral blood monocytes [42]. Additionally, the latter study found that IL-10 up-regulates the expression of HLA-G in trophoblast cells [42]. Certainly at the maternal-fetal interface the soluble form of HLA-G may derive from macrophages in response to INF-gama stimulation [23,43]. Although the soluble form of HLA-G does chiefly derive from macrophages [3,23], lymphocyte T CD4+ may also be a source of sHLA-G [15]. After all, HLA-G is an antigen whose participation in the regulation of the immune system has been well documented [2–4], and soluble HLA-G is responsible for the inhibition and apoptosis of CTLs and NK cells [9–12]. Lindaman *et al.* has shown both that sHLA-G in dose-dependent manner inhibits the cytolitic activity of NK cells and that the increase in sHLA-G concentration seen in *in vitro* culture is associated in a concentration-dependent manner with an increase in the apoptosis of NK cells. Finally, Lindaman has posited that sHLA-G abrogates NK cells [9].

The microenvironment of the endometrium is where the interaction of endometrial and immune cells takes place during reproductive processes. The proper activity of immune cells (such as dNK cells, macrophages, and others infiltrating endometrium) regulated by endometrial cells is necessary not only for successful implantation, but also for the cessation of the molecular changes that take place during menstruation, beginning with decidualization. These processes are more prominent during menstrual bleeding than during the different cycle phases. During menstruation the endometrium is infiltrated not so much by dNK cells (such as has been observed in the secretory cycle phase [44]) as by a subsequent increase in the number of neutrophils and macrophages [28–29]. The increasing number of macrophages associated with HLA-G expression in endometrial cells and with the presence of inflammation in the endometrial microenvironment during menstruation results in a low level of sHLA-G in the blood sera, most likely from the emergence of menstrual lochia and the start of menstrual bleeding, as the menstrual lochia contain HLA-G positive cells [17] and probably soluble isoforms of this protein. In our study, the blood serum sHLA-G concentration level was lowest during menstrual bleeding and, moreover, this value was significantly lower than that found in any of the different menstrual cycle phases. Retrograde menstruation enables the dissemination of endometrial cells within the peritoneal cavity and – if the ectopic endometrial cells can escape immune system surveillance – the subsequent development of endometriosis (The escape of ectopic endometrial cells from immune system surveillance is currently under study). As in the cancer microenvironment, macrophages seem to play a dual role in this phenomenon [45–49]. On the one hand, macrophages infiltrating an endometriosis lesion may participate in the rejection of tumor cells, but on the other hand, may actually assist in tumor-immune system counterattack. Pistoia *et al.* has demonstrated that monocytes in the neuroblastoma microenvironment may secrete sHLA-G in response to activation by cytokines (IL-10 among others) deriving from tumor cells [2]. An increased infiltration of endometriosis lesions by macrophages has been identified in the peritoneal lesions of early, active endometriosis [25]. Moreover, women with endometriosis are typified by an increase in monocyte activity in the peripheral blood, and the ability of these cells to increase the secretion of IL-10 has been documented [24]. Furthermore, sHLA-G inhibits NK cells and cytotoxic lymphocytes, which in turn protect tumors from the immune system

[2]. The increasing concentration of sHLA-G in the blood sera of patients with various neoplasms reduces immune surveillance, thus favoring the progression of cancer [3]. This provides a plausible explanation for the higher concentration levels of sHLA-G in the postmenopausal patients with endometriosis in comparison to the control group. As in cases of patients with leiomyoma, the higher concentration levels may be linked to macrophage dysfunction. Miura *et al.* has demonstrated that myoma nodules, myometrium, and endometrium are all widely infiltrated by macrophages [27]. Moreover, both the increase in macrophage activity in the peritoneal fluid [46], and the increase in cytokine concentration linked with inflammatory processes within the endometrium have been observed in women with uterine leiomyoma [27]. The degree of macrophage infiltration within the endometrium, myometrium, and myoma nodules did not differ in the subsequent menstrual cycle phases and, furthermore, was independent of the presence of associated endometriosis lesions [27]. In our study, no differences were found between the sHLA-G levels in the secretory and proliferative cycle phases of patients with endometriosis or of patients with leiomyoma. Additionally, Mira *et al.* has shown that GnRH analogue therapy in women with leiomyoma results in a decrease in macrophage infiltration into the endometrium, myometrium, and myoma nodules [27]. GnRH analogue therapy brings about a hormonal status in the patient comparable to that observed in postmenopausal patients. In our study, we have seen significantly lower sHLA-G levels in postmenopausal patients in comparison to the levels found in women during the various menstrual cycle phases.

In sum, sHLA-G molecules participate in the regulation of the immune response in the endometrium microenvironment during pregnancy [1–4,9–12]. The blood serum concentration level of sHLA-G increases in pathological conditions – such as tumors and immune-mediated disorders – in which the dysfunction of immune cells has been observed, as it has in the blood sera of women with endometriosis and leiomyoma in our study. The sHLA-G concentration level would thus appear to provide important information about disturbances in the immune system associated with this pathological condition.

5. CONCLUSIONS

Overall, the soluble HLA-G blood serum level would seem to be a useful marker for evaluating the status of the microenvironment, where the tumor-immune cell and ectopic and eutopic endometrial interactions take place.

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