Human Reproduction Vol.18, No.12 pp. 2595–2598, 2003 DOI: 10.1093/humrep/deg491

Women with endometriosis have increased levels of placental growth factor in the peritoneal fluid compared with women with cystadenomas

Nobuhiro Suzumori[1], Mayumi Sugiura-Ogasawara, Kinue Katano and Kaoru Suzumori

Department of Obstetrics and Gynecology, Nagoya City University, Graduate School of Medicine, 1 Kawasumi, Mizuho-ku, Mizuho-cho, Nagoya 467-8601, Japan

[1]To whom correspondence should be addressed. E-mail: og.n.suz@med.nagoya-cu.ac.jp

BACKGROUND: To assess the release of placental growth factor (PlGF) into peritoneal fluid in women with and without endometriosis, we measured its concentration with reference to disease stage, the presence of red endometriotic lesions and the phase of menstrual cycle. METHODS: Surgery was scheduled in the proliferative or secretory phase of the menstrual cycle for 59 women with (n = 35) or without (n = 24) endometriosis. The latter group comprised women undergoing surgery for ovarian cystadenomas. PlGF concentrations in the peritoneal fluid were measured using an enzyme-linked immunosorbent assay. RESULTS: PlGF concentration in the peritoneal fluid was markedly elevated in the endometriosis patients (median 189 pg/ml, interquartile range 84–475 pg/ml) as compared with the controls (88 pg/ml, 41–213 pg/ml; $P < 0.001$), especially in women with red lesions. Significantly greater values during the secretory phase of the menstrual cycle as compared with the proliferative phase were observed in both the control (cystadenoma) group ($P < 0.05$) and the endometriosis group ($P < 0.001$). CONCLUSIONS: Our findings suggest that production of PlGF is sensitive to the cyclic changes in ovarian steroids and may contribute to the pathogenesis of endometriosis, especially that of red lesions, by promoting neovascularization.

Key words: angiogenesis/menstrual cycle/neovascularization/PlGF/red lesions

Introduction

Placental growth factor (PlGF) is a secreted, disulfide-linked dimeric glycoprotein originally cloned from a term placenta cDNA library (Maglione *et al*., 1991). PlGF expression has also been detected in endothelial and epithelial cells, as well as various tumours (Hauser *et al*., 1993; Cao *et al*., 1996). Based on its 53% overall amino acid (aa) residue similarity with vascular endothelial growth factor (VEGF), PlGF has been classified as a member of the VEGF family of growth factors (Maglione *et al*., 1991). It occurs in at least three isoforms, PlGF-1 (PlGF149), PlGF-2 (PlGF170) and PlGF-3 (PlGF221), due to alternative splicing of the primary PlGF transcript (Maglione *et al*., 1993). A highly basic 21-aa insertion in the carboxyterminal region of PlGF-2 results in high heparin-binding affinity, which is lacking with PlGF-1 and PlGF-3 (Oura *et al*., 2003). In cells co-expressing VEGF and PlGF mRNA, a heterodimeric VEGF/PlGF protein has been detected, in addition to the homodimeric VEGF and PlGF forms (DiSalvo *et al*., 1995; Cao *et al*., 1996). The VEGF/PlGF heterodimer has been shown to promote capillary growth *in vivo* (Cao *et al*., 1996).

Endometriosis features progressive growth that is estrogen-dependent (Rock *et al*., 1992), and several lines of evidence suggest that the angiogenic factor, VEGF, is also involved in the maintenance and development of endometriotic lesions (McLaren *et al*., 1996; Li *et al*., 2001). An important condition for endometriotic tissues following adhesion and implantation is the establishment of neovascularization or angiogenesis (Folkman, 1995; Kats *et al*., 2002). Recently, several angiogenic molecules have been shown to demonstrate elevated concentrations in the peritoneal fluid of endometriosis patients, examples being VEGF, interleukin (IL)-8 and ENA-78 (Arici *et al*., 1996; McLaren *et al*., 1996; Mahnke *et al*., 2000; Calhaz-Jorge *et al*., 2003; Mueller *et al*., 2003). Recent data suggest that gene therapy with angiogenic inhibitors might be highly effective for the control of endometriosis, even in a host with preserved estrogen levels (Dabrosin *et al*., 2002). Here we speculated that the concentration of PlGF, an angiogenic factor, might be elevated in the peritoneal fluid of women with endometriosis. To our knowledge, no such report of PlGF levels in endometriosis patients has been published previously.

Since endometriosis is influenced by the cyclic change in ovarian steroids, we also examined here whether the PlGF concentration varies with the phase of the menstrual cycle. In addition, several findings suggest greater neovascularization within endometriotic implants in red as compared with black or white lesions (Nisolle *et al*., 1993). Therefore, the present study

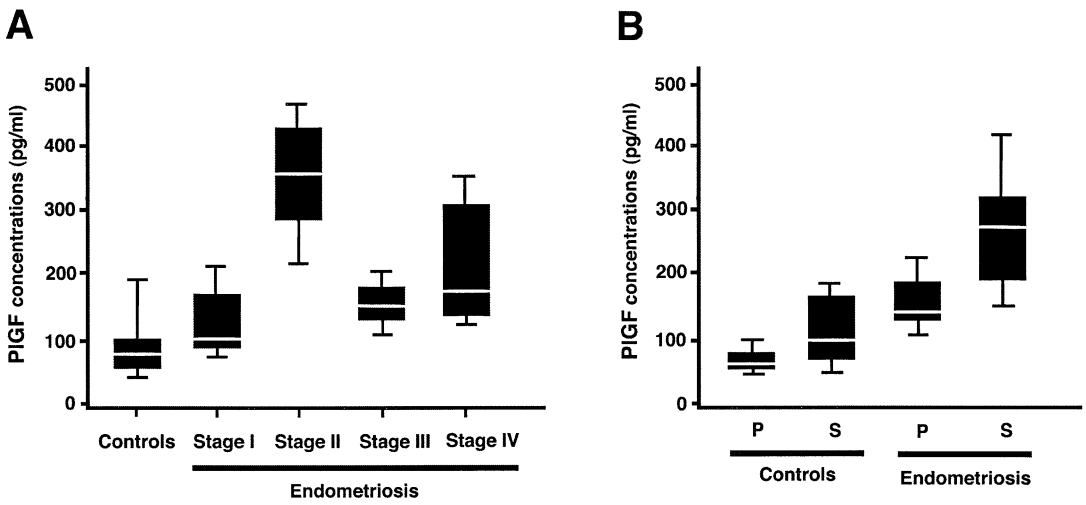

Figure 1. Concentrations of PlGF in peritoneal fluid as determined by ELISA. The boxes delimit values falling between the 25th and the 75th percentiles and the horizontal lines refer to the median scores. (**A**) The difference between the control (cystadenoma) group (n = 24) and the endometriosis group (n = 35) is significant ($P < 0.001$). In the endometriosis patients, elevated PlGF levels ($P < 0.001$) are apparent in the stage II group, as compared with the other stage groups. (**B**) Significant differences in PlGF levels with stage of menstrual cycle are evident in both the control (cystadenoma) group ($P < 0.05$) and the endometriosis group ($P < 0.001$). P = proliferative phase; S = secretory phase.

was designed to assess variation with the presence of red lesions as well as with menstrual cycle and endometriosis stage.

Materials and methods

Subjects

Thirty-five women between 21 and 46 years of age with endometriosis and 24 controls were enrolled in this study. All of the women had regular (25–35 day) menstrual cycles. The basal body temperature (BBT) was used to determine their phase in the menstrual cycle, and the visualization of either a corpus luteum or a dominant follicle was also taken into consideration. The actual presentation of the cases was pain and/or ovarian cysts. None of the patients had been on medication for at least 3 months prior to surgery and none had taken any long-acting drugs. The control group consisted of 24 women with ovarian cystadenomas (diameter 4–11 cm) with no evidence of endometriosis or pelvic adhesions, because data for normal disease-free women were not available. The study protocol was approved by the institutional review board of Nagoya City University, and informed consent was obtained from all of the study subjects.

The patients with endometriosis were scored according to the American Society of Reproductive Medicine classification 1996 (American Society for Reproductive Medicine, 1997), and included individuals with or without red lesions. Samples of peritoneal fluid were obtained during laparoscopic surgery or laparotomy (for cystectomy, adnexectomy or total abdominal hysterectomy). There was no contradiction between BBT and follicle or corpus luteum. We studied eight cases of laparotomy [study group, n = 4 (proliferative phase, n = 2; secretory phase, n = 2), all of them stage IV; control group, n = 4 (proliferative phase, n = 2; secretory phase, n = 2)] and 51 cases of laparoscopy (study group, n = 31; control group, n = 20) for this study.

Collection of peritoneal fluid

Aspiration of the peritoneal fluid during surgery was performed under direct visualization from the posterior cul de sac and anterior vesico-uterine fold. Patients with bleeding into the peritoneal cavity from the abdominal walls were excluded. The volume of peritoneal fluid was then measured. After clarification by centrifugation at 2000 *g* for 10 min, the supernatants were stored at –80°C until assayed.

Measurement of PlGF concentrations

Amounts of PlGF in the peritoneal fluid were determined with a PlGF enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. Samples from all patients were measured in parallel and in duplicate to control for interassay variance. The optical density of each well was measured at dual wavelengths of 450 and 570 nm. Concentrations of PlGF were calculated by interpolation from a standard curve. The sensitivity of the PlGF ELISA was 7 pg/ml.

Statistical analysis

Calculated values were expressed as medians and interquartile ranges, and comparisons were made with the Mann–Whitney and Kruskal–Wallis non-parametric tests. A P-value <0.05 was regarded as statistically significant.

Results

The study (n = 35, mean age ± SEM 36 ± 1.6 years) and control (n = 24, 37 ± 1.8 years) groups were matched for parity (both groups: mean, interquartile range 0, 0–2). Peritoneal fluid samples from the women with endometriosis contained significantly higher concentrations of PlGF (median 189 pg/ml, interquartile range 84–475 pg/ml) than those of the controls with ovarian cystadenomas (88 pg/ml, 41–213 pg/ml) ($P < 0.001$, Mann–Whitney test).

The endometriosis patients were staged from I to IV depending on the severity of disease based on the Revised American Society for Reproductive Medicine classification (American Society for Reproductive Medicine, 1997). The stage distribution at surgery was as follows: stage I, n = 8 (proliferative phase, n = 4; secretory phase, n = 4; 101 pg/ml), 84–235 pg/ml; stage II, n = 7 (proliferative phase, n = 4;

secretory phase, $n = 3$), 357 pg/ml, 219–475 pg/ml; stage III, $n = 12$ (proliferative phase, $n = 7$; secretory phase, $n = 5$), 173 pg/ml, 105–235 pg/ml; and stage IV, $n = 8$ (proliferative phase, $n = 5$; secretory phase, $n = 3$), 192 pg/ml, 122–346 pg/ml. There was a significant difference in PlGF levels among the endometriosis stage I–IV groups ($P < 0.001$, Kruskal–Wallis test) (Figure 1A). Red lesions were identified in 22 patients, and appeared to be lacking in the other 13. Patients with peritoneal implants classified as red lesions (stage I, $n = 3$; stage II, $n = 6$; stage III, $n = 7$; stage IV, $n = 6$) had significantly higher concentrations of PlGF [$n = 22$ (proliferative phase, $n = 13$; secretory phase, $n = 9$), 181 pg/ml, 133–475 pg/ml) in their peritoneal fluid than in those without [$n = 13$ (proliferative phase, $n = 7$; secretory phase, $n = 6$), 134 pg/ml, 84–307 pg/ml; $P < 0.001$]. There were notable differences in volume of the peritoneal fluid between cases with (mean ± SEM 22 ± 2.7 ml) and without (10 ± 1.8 ml) endometriosis ($P < 0.001$).

Significantly greater values were observed in the secretory phase of the menstrual cycle as compared with the proliferative phase in both the control (cystadenoma) group (proliferative phase, $n = 12$, 66 pg/ml, 41–101 pg/ml; secretory phase, $n = 12$, 114 pg/ml, 45–213 pg/ml; $P < 0.05$) and the endometriosis group (proliferative phase, $n = 20$, 144 pg/ml, 84–346 pg/ml; secretory phase, $n = 15$, 287 pg/ml, 105–475 pg/ml; $P < 0.001$) (Figure 1B).

Discussion

In the present study, the concentration of PlGF in peritoneal fluid was found to be significantly elevated in women with endometriosis compared with the controls with cystadenomas. Patients with endometriosis evaluated in the secretory phase had significantly higher concentrations of PlGF in the peritoneal fluid than in the proliferative phase.

VEGF and its receptors are overexpressed in several inflammatory diseases (Dvorak *et al*., 1995). Recent data indicate that expression of PlGF is up-regulated during inflammation and with inflammatory human skin diseases (Oura *et al*., 2003). Endometriosis causes an inflammatory reaction in the pelvis, changing the characteristics of the peritoneal fluid and its cellular components (Halme, 1991; Ramey *et al*., 1993; Hill *et al*., 1988). Our results suggest that the inflammation associated with endometriosis, through increased levels of peritoneal fluid PlGF, may promote angiogenesis and support the progressive growth and activity of endometriosis.

PlGF and VEGF have been shown to be equipotent in stimulating tissue factor production and chemotaxis in monocytes (DiSalvo *et al*., 1995). A recent report suggested that PlGF stimulated angiogenesis and collateral growth in ischaemic heart and limb with efficiency at least comparable to VEGF (Luttun *et al*., 2002). Peritoneal VEGF concentrations in women with endometriosis are reported to be significantly higher in the proliferative phase than in the secretory phase (McLaren *et al*., 1996). However, in our study both the endometriosis group and controls with cystadenomas demonstrated significantly higher PlGF levels in the secretory phase. A recent report suggested that expression of PlGF mRNA occurs in uterine natural killer cells, with highest levels found in the midsecretory phase of the cycle (Li *et al*., 2001), coincident with increase of lymphocytes in the human endometrium (King *et al*., 1998). Our data are in line with PlGF production by lymphocytes in the peritoneal cavity and regulation by ovarian steroids. PlGF is thought to play a role in regulating trophoblast function and the early process of placentation (Tjoa *et al*., 2001), so that our findings may point to a potential contribution of PlGF to implantation and invasion of endometriotic tissues.

Several reports suggest that red lesions represent active endometriosis because they feature more pronounced angiogenesis than their black or white counterparts (Wiegerinck *et al*., 1993; Iwabe *et al*., 1998). Early red lesions have been reported to be characterized by invasion of the extracellular matrix (Spuijbroek *et al*., 1992). Our findings thus suggest that PlGF in the peritoneal fluid may be involved in the neovascularization of endometriotic tissues.

We conclude that PlGF is an important factor that may contribute to the pathogenesis of endometriosis, especially with red lesions, possibly promoting neovascularization. Further studies are now needed to define the actual role of PlGF in regulation of angiogenesis in endometriosis and how its production may be regulated by ovarian steroids.

References

American Society for Reproductive Medicine (1997) Revised American Society for Reproductive Medicine classification of endometriosis: 1996. *Fertil. Steril.*, **67**, 817–821.

Arici, A., Tazuke, S.I., Attar, E., Kliman, H.J. and Olive, D.L. (1996) Interleukin-8 concentration in peritoneal fluid of patients with endometriosis and modulation of interleukin-8 expression in human mesothelial cells. *Mol. Hum. Reprod.*, **2**, 40–45.

Calhaz-Jorge, C., Costa, A.P., Santos, M.C. and Palma-Carlos, M.L. (2003) Peritoneal fluid concentrations of interleukin-8 in patients with endometriosis depend on the severity of the disorder and are higher in the luteal phase. *Hum. Reprod.*, **18**, 593–597.

Cao, Y., Chen, H., Zhou, L., Chiang, M.K., Anand-Apte, B., Weatherbee, J.A., Wang, Y., Fang, F., Flanagan, J.G. and Tsang, M.L. (1996) Heterodimers of placenta growth factor/vascular endothelial growth factor. Endothelial activity, tumor cell expression, and high affinity binding to Flk-1/KDR. *J. Biol. Chem.*, **271**, 3154–3162.

Dabrosin, C., Gyorffy, S., Margetts, P., Ross, C. and Gauldie, J. (2002) Therapeutic effect of angiostatin gene transfer in a murine model of endometriosis. *Am. J. Pathol.*, **161**, 909–918.

DiSalvo, J., Bayne, M.L., Conn, G., Kwok, P.W., Trivedi, P.G., Soderman, D.D., Palisi, T.M., Sullivan, K.A. and Thomas, K.A. (1995) Purification and characterization of a naturally occurring vascular endothelial growth factor/placenta growth factor heterodimer. *J. Biol. Chem.*, **270**, 7717–7723.

Dvorak, H.F., Detmar, M., Claffey, K.P., Nagy, J.A., van de Water, L. and Senger, D.R. (1995) Vascular permeability factor/vascular endothelial growth factor: an important mediator of angiogenesis in malignancy and inflammation. *Int. Arch. Allergy Immunol.*, **107**, 233–235.

Folkman, J. (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med.*, **1**, 27–31.

Halme, J. (1991) Role of peritoneal inflammation in endometriosis-associated infertility. *Ann. N. Y. Acad. Sci.*, **622**, 266–274.

Hauser, S. and Weich, H.A. (1993) A heparin-binding form of placenta growth factor (PlGF-2) is expressed in human umbilical vein endothelial cells and in placenta. *Growth Factors*, **9**, 259–268.

Hill, J.A., Faris, H.M., Schiff, I. and Anderson, D.J. (1988) Characterization of leukocyte subpopulations in the peritoneal fluid of women with endometriosis. *Fertil. Steril.*, **50**, 216–222.

Iwabe, T., Harada, T., Tsudo, T., Tanikawa, M., Onohara, Y. and Terakawa, N. (1998) Pathogenetic significance of increased levels of interleukin-8 in

the peritoneal fluid of patients with endometriosis. *Fertil. Steril.*, **69**, 924–930.

Kats, R., Metz, C.N. and Akoum, A. (2002) Macrophage migration inhibitory factor is markedly expressed in active and early-stage endometriotic lesions. *J. Clin. Endocrinol. Metab.*, **87**, 883–889.

King, A., Burrows, T., Verma, S., Hiby, S. and Loke, Y.W. (1998) Human uterine lymphocytes. *Hum. Reprod. Update*, **4**, 480–485.

Li, X.F., Charnock-Jones, D.S., Zhang, E., Hiby, S., Malik, S., Day, K., Licence, D., Bowen, J.M., Gardner, L., King, A. *et al*. (2001) Angiogenic growth factor messenger ribonucleic acids in uterine natural killer cells. *J. Clin. Endocrinol. Metab.*, **86**, 1823–1834.

Luttun, A., Tjwa, M., Moons, L., Wu, Y., Angelillo-Scherrer, A., Liao, F., Nagy, J.A., Hooper, A., Priller, J., De Klerck, B. *et al*. (2002) Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. *Nat. Med.*, **8**, 831–840.

Maglione, D., Guerriero, V., Viglietto, G., Delli-Bovi, P. and Persico, M.G. (1991) Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. *Proc. Natl Acad. Sci. USA*, **88**, 9267–9271.

Maglione, D., Guerriero, V., Viglietto, G., Ferraro, M.G., Aprelikova, O., Alitalo, K., Del Vecchio, S., Lei, K.J., Chou, J.Y. and Persico, M.G. (1993) Two alternative mRNAs coding for the angiogenic factor, placenta growth factor (PlGF), are transcribed from a single gene of chromosome 14. *Oncogene*, **8**, 925–931.

Mahnke, J.L., Dawood, M.Y. and Huang, J.-C. (2000) Vascular endothelial growth factor and interleukin-6 in peritoneal fluid of women with endometriosis. *Fertil. Steril.*, **73**, 166–170.

McLaren, J., Prentice, A., Charnock-Jones, D.S. and Smith, S.K. (1996) Vascular endothelial growth factor (VEGF) concentrations are elevated in peritoneal fluid of women with endometriosis. *Hum. Reprod.*, **11**, 220–223.

Mueller, M.D., Mazzucchelli, L., Buri, C., Lebovic, D.I., Dreher, E. and Taylor, R.N. (2003) Epithelial neutrophil-activating peptide 78 concentrations are elevated in the peritoneal fluid of women with endometriosis. *Fertil. Steril.*, **79**, 815–820.

Nisolle, M., Casanas-Roux, F., Anaf, V., Mine, J.M. and Donnez, J. (1993) Morphometric study of the stromal vascularization in peritoneal endometriosis. *Fertil. Steril.*, **59**, 681–684.

Oura, H., Bertoncini, J., Velasco, P., Brown, L.F., Carmeliet, P. and Detmar, M. (2003) A critical role of placental growth factor in the induction of inflammation and edema formation. *Blood*, **101**, 560–567.

Ramey, J.W. and Archer, D.F. (1993) Peritoneal fluid: its relevance to the development of endometriosis. *Fertil. Steril.*, **60**, 1–14.

Rock, J.A. and Markham, S.M. (1992) Pathogenesis of endometriosis. *Lancet*, **340**, 1264–1271.

Spuijbroek, M.D., Dunselman, G.A., Menheere, P.P. and Evers, J.L. (1992) Early endometriosis invades the extracellular matrix. *Fertil. Steril.*, **58**, 929–933.

Tjoa, M.L., van Vugt, J.M.G., Mulders, M.A.M., Schutgens, R.B.H., Oudejans, C.B.M. and van Wijk, I.J. (2001) Plasma placenta growth factor levels in midtrimester pregnancies. *Obstet. Gynecol.*, **98**, 600–607.

Wiegerinck, M.A., Van Dop, P.A. and Brosens, I.A. (1993) The staging of peritoneal endometriosis by the type of active lesion in addition to the revised American Fertility Society classification. *Fertil. Steril.*, **60**, 461–464.

Submitted on May 13, 2003; resubmitted on June 30, 2003; accepted on August 14, 2003