

# Suppressed apoptotic susceptibility in human endometrial epithelial cells pretreated with hepatocyte growth factor

T. Tanaka, K. Mizuno, N. Umesaki, S. Ogita

*Department of Obstetrics & Gynecology, Osaka City University Medical School, Osaka (Japan)*

## Summary

Hepatocyte growth factor (HGF) and its receptor are strongly expressed perimenstrually in human endometrial epithelium, but their functions remain unknown. In this study the effects of HGF on Fas-mediated apoptosis in the human endometrial epithelial cell line HHUA were investigated. It was found that HGF inhibited Fas-mediated growth suppression and DNA fragmentation in the cells without any increase in Fas antigen expression on the cells. This results suggests that HGF inhibits Fas-mediated apoptotic signals in the endometrium, and possibly plays a role in reshaping the endometrium during menstruation.

**Key words:** HGF; C-met; Endometrium; Apoptosis; Menstruation.

## Introduction

A recent paper has suggested that human menstruation involves an apoptotic phenomenon in endometrial tissues [1]. Apoptotic cells have been found in normal human endometrial epithelium, and reportedly increase in number especially during the secretory phase [2, 3] and perimenstruation period [1]. Moreover, an apoptotic cytokine receptor Fas (CD95) antigen is constitutively expressed in the endometrial epithelium [3-5]. Expression of the bcl-2 product, which is a major apoptosis suppresser, was found to increase in the proliferative epithelium and decrease dramatically in the secretory epithelium [3, 4, 6, 7]. The underexpression of bcl-2 in the secretory endometrial epithelium may result in increased apoptotic susceptibility in the secretory epithelium and thus might be a trigger for menstruation, although the regulatory mechanism of endometrial apoptotic susceptibility is not yet fully understood.

Hepatocyte growth factor (HGF) is expressed constitutively in normal human endometrial epithelium [8, 9]. Expressions of HGF mRNA in the endometrium and serum HGF levels vary throughout the menstrual cycle and reach their peak during the perimenstrual period [8, 9]. Because HGF stimulates cell proliferation of and causes morphological changes in endometrial epithelial cells, HGF may play an important role in the repair of endometrial tissue after menstrual shedding of endometrium [8].

The normal human endometrial epithelium constitutively expresses HGF receptor (c-met) which is a tyrosine kinase type cytokine receptor and whose expression levels are menstrual cycle dependent [8]. Little is known about the biological functions of the cyclic expression of endometrial c-met, although recent reports have shown that HGF can modulate some aspects of apoptosis in hepatocytic cells [10-13]. These reports also indicated that the

possible modulatory effects of HGF on apoptosis were specific to either the differentiation stage or the apoptotic cell lineage. Shen and Novak [14] have recently reported that a receptor type tyrosine kinase c-met is associated with Fas antigen. According to their findings, the Fas-mediated apoptotic signal activates tyrosine phosphorylation of c-met without any addition of HGF. The report hypothesizes that Fas-signalling and c-met activation are related to and modulated by each other. To examine the modulatory effects of HGF on Fas-mediated endometrial apoptosis, we carried out the present study by using a human endometrial epithelial cell line susceptible to Fas-mediated apoptosis.

## Materials and Methods

### *Cell Line and Cell Culture*

In this study, a highly-differentiated human endometrial adenocarcinoma cell line, HHUA [15] was used. HHUA cells were obtained from Riken Cell Bank (Tsukuba, Japan) and cultured in OPTI-MEM (GIBCO-BRL, Gaithersburg, MD, USA) / 5% fetal calf serum (FCS) (GIBCO-BRL) / Penicillin (PC) (100 U/ml) (GIBCO-BRL) / Streptomycin (100 mg/ml) (SM) (GIBCO-BRL).

### *Cell Proliferation Assay*

Cell proliferation was assayed with the Non-RI Colourimetric Assay Kit, XTT (Boehringer-Mannheim, Mannheim, Germany). The stimulatory effect of anti-Fas IgM or HGF on the cell growth of HHUA cells was assayed as follows. On day 1, HHUA cells in the log phase were detached in 0.25% trypsin / 1mM EDTA (GIBCO-BRL) and cultured overnight in 96 well-culture plates (5,000 cells/well) containing OPTI-MEM/5% FCS/PC/SM. On day 2, anti-Fas IgM (clone CH-11) (MBL, Nagoya, Japan) or recombinant human HGF (rhHGF) (R&D Systems, Minneapolis, MN, USA) was added to the cells. On day 4, viable cells were counted. In order to examine the modulatory effect of HGF, HHUA cells were treated first with rhHGF (1 ng/ml) for 2 days. Then anti-Fas IgM was added to the cells

Received September 1, 1998

revised manuscript accepted for publication September 30, 1998

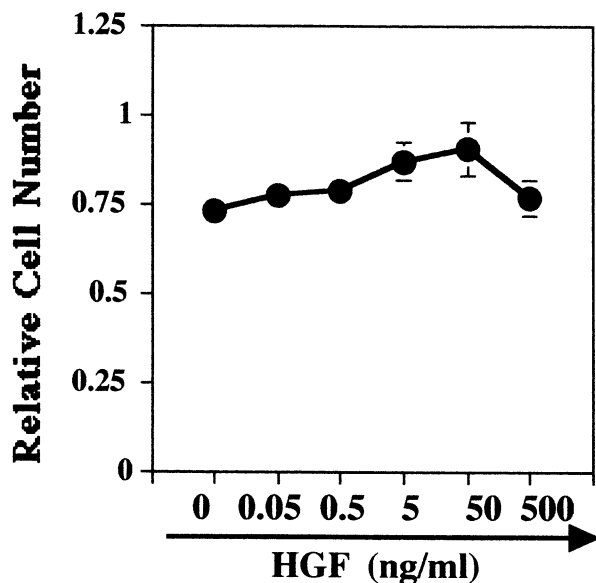


Figure 1. — Cell proliferation effects of HGF on HHUA cells. Relative cell numbers are expressed as absorbance at 450 nm by the cell proliferation assay kit and represent the mean  $\pm$  SEM of 5 experiments (the error bars of some of the points lie within the size of the symbols).

followed by a 2-day culture. On day 5, cell growth was evaluated using the kit. More than three assays were performed for each experiment to verify the results. Statistical analyses were performed by Student's *t*-test or ANOVA depending on the protocol. Significance was set at  $p < 0.05$ . Figures represent means  $\pm$  SEM of 5 experiments.

#### DNA Fragmentation Assay

HHUA cells in the log phase were detached in trypsin/EDTA, cultured overnight in culture dishes ( $3 \times 10^6$  cells/dish) containing OPTI-MEM/5% FCS/PC/SM, and rhHGF (1 ng/ml) was added to the cells on the second day. On day 3, anti-Fas IgM was added, and on the fifth day genomic DNAs were extracted from all the cells, including dead ones, with a DNA extraction kit, SepaGene (Sankyo-Junyaku Co., Ltd., Tokyo, Japan), and treated with 100  $\mu$ g/ml of RNaseA (Sigma, St. Louis, MO, USA) in TE (10 mM Tris, 2 mM EDTA, pH8.0) for 90 minutes at 37°C to remove any RNA contamination. The genomic DNA from approximately  $5 \times 10^5$  cells was then electrophoresed in 1.5% agarose gel at 50 volts for about 2 hours, stained with 5  $\mu$ g/ml of ethidium bromide, and visualized by UV fluorescence.

#### Flowcytometric Assay

HHUA cells stimulated with 1 ng/ml of rhHGF for 2 days were detached from the culture flask by phosphate buffered saline (PBS)/3 mM EDTA, and stained according to the following procedure:  $3 \times 10^5$  cells were incubated with an excess dose of anti-Fas IgG (clone UB2) (MBL, Nagoya, Japan) for 20 minutes at 4°C, and then washed twice with washing buffer (PBS/2% FCS/0.1% NaN<sub>3</sub>). Next, the cells reacted with FITC-conjugated goat anti-mouse IgG (H+L) (GIBCO-BRL) for 20 minutes at 4°C, were washed twice, and finally suspended in 200  $\mu$ l of the washing buffer for analysis with a Coulter-Epics Flowcytometer.

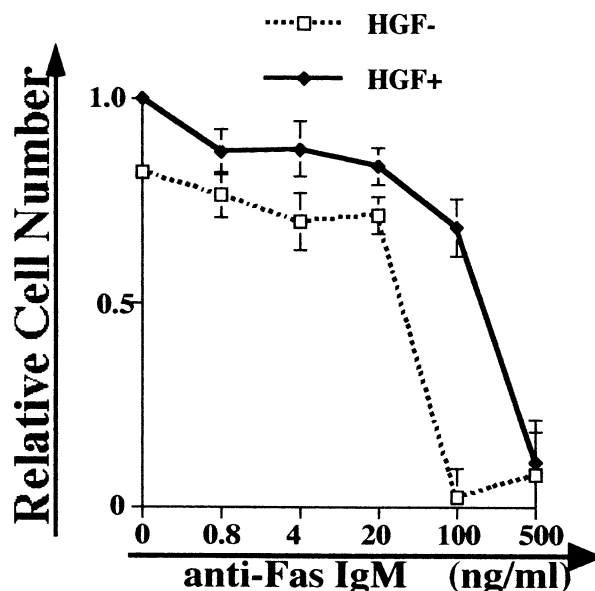


Figure 2. — Inhibition by HGF of Fas-mediated cell growth suppression in HHUA cells. Relative cell numbers are expressed as absorbance at 450 nm by the cell proliferation assay kit and represent the mean  $\pm$  SEM of 5 experiments (the error bars of some of the points lie within the size of the symbols). This experiment was repeated three separate times.

#### Results

HHUA expresses functional estrogen receptors, functional progesterone receptors [15], Fas (CD95) [16] and EGF-receptors (Fig. 4). Anti-Fas IgM treatment of HHUA demonstrated dose-dependent suppression of cell growth (Fig. 2) and induced DNA fragmentation in the cells (Fig. 3) in a dose-dependent fashion. The fragmentation was accompanied by reduction in cell size and nuclear pyknosis [16]. Northern blot analysis revealed a moderate expression of *c-met* mRNA and a very low expression of *bcl-2* mRNA (data not shown). These results indicate that HHUA cells are phenotypically similar to secretory epithelial cells.

HHUA cell proliferation was slightly stimulated by rhHGF alone (Fig. 1). However, pretreatment of the cells with a low concentration of rhHGF (1 ng/ml) strongly inhibited the Fas-mediated growth suppression in HHUA cells in a reproducible fashion (Fig. 2). Next, the effects of rhHGF pretreatment on DNA fragmentation were examined in order to determine whether Fas-mediated growth suppression inhibited by rhHGF pretreatment was caused by inhibition of apoptosis. As shown in Fig. 3, pretreating HHUA cells with rhHGF reduced DNA fragmentation in the anti-Fas IgM-treated cells. This indicates that pretreatment of the cells with rhHGF inhibited Fas-mediated apoptotic signals.

Next, we examined whether inhibition of the Fas-mediated apoptosis by rhHGF was associated with a decrease in Fas expression on the cell surface and for this purpose carried out a flow cytometric analysis of HHUA cells that were stimulated with rhHGF. Expression levels of Fas on the cells were not affected by rhHGF (Fig. 4).

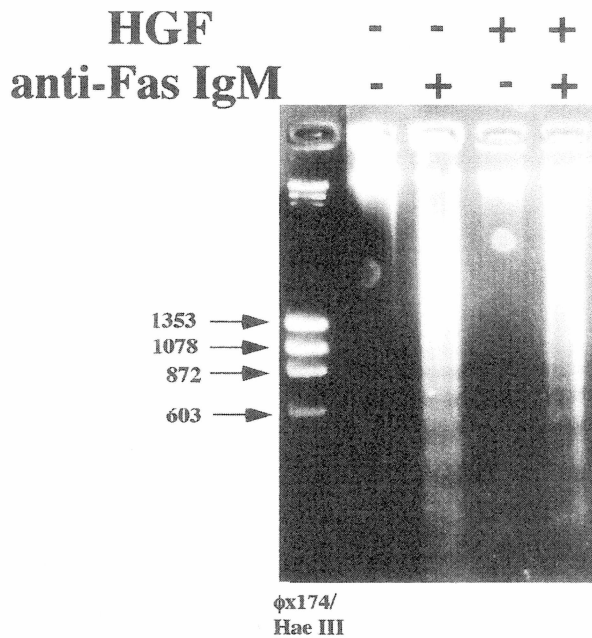


Figure 3. — Inhibition by HGF of Fas-mediated DNA fragmentation in HHUA cells. This assay was repeated three times. A typical electrophoretic pattern is shown.

### Discussion

It has remained unclear what inhibits or enhances apoptosis in endometrial epithelial cells. Although HGF by itself is reported to stimulate endometrial epithelial cell growth [8], a finding supported by our results, pretreatment of HHUA cells with HGF strongly inhibited Fas-mediated growth suppression without any decrease in Fas expression on the cell surface. We therefore con-

cluded that the inhibition of Fas-mediated apoptosis by rhHGF was caused by the inhibition of postreceptor apoptotic signals and the enhancement of cell proliferation, but not by a decrease in receptor expression. This means that HGF inhibition of Fas-mediated apoptosis in HHUA cells was caused partly by reduced susceptibility to anti-Fas IgM without any decrease in Fas expression. As some signals can share common signalling pathways irrespective of which apoptotic stimuli cause the signals [8], it is possible that HGF may inhibit not only Fas-mediated apoptotic signals but also other endometrial apoptotic signals.

Inhibition of endometrial apoptotic susceptibility by HGF probably takes place *in vivo* during the perimenstrual period when serum HGF concentrations increase [8, 9]. Expression of the HGF receptor is found in the endometrial epithelium, and overexpression in the perimenstrual period [8]. The finding that endometrial HGF causes epithelial cell proliferation and morphological changes [8] suggests that HGF plays a crucial role in the regulation of epithelial apoptosis and regeneration of endometrial tissues.

HGF has been reported to regulate apoptosis in hepatic cell lineages: it was found to suppress interferon- $\alpha$ -induced cytotoxicity in mouse hepatocytes [12] and apoptosis in rat hepatic ductal cells treated with 2-acetylaminofluorene [13], and to prevent apoptosis of serum-free C2.8 mouse embryo hepatocytic cells [10, 11]. It can thus be concluded that HGF does have an inhibitory effect on apoptosis in hepatocytic cells. Our study is thought to be the first to show, however, that endometrial Fas-mediated apoptosis can also be inhibited by HGF.

HGF receptor (c-met) is a tyrosine kinase type cytokine receptor [18]. Tyrosine phosphorylation has been reported to be an early event required for Fas signaling in T

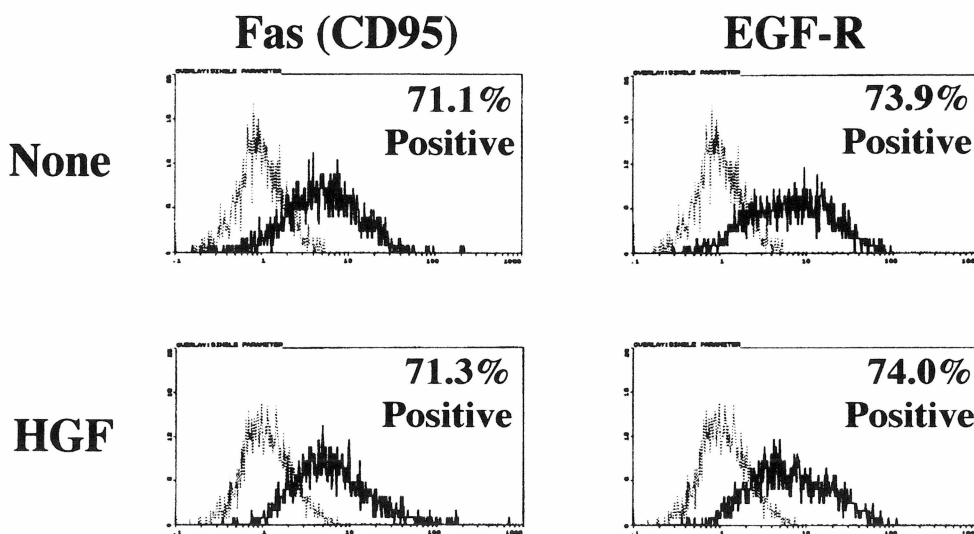


Figure 4. — Flowcytometric analysis of Fas expression on HGF-treated HHUA cells. The thick lines show expression of Fas antigen (CD95) or EGF receptor (EGF-R) on the cell surface, while the thin lines show negative controls (secondary antibody alone). HHUA cells were cultured with 1 ng/ml of rhHGF for 2 days before the analysis. Positive Fas antigen expression was seen in 71.1% of the untreated and 71.3% of the HGF-treated HHUA cells. There was thus no significant difference in Fas expression and EGF-R expression between treated and untreated cells.

lymphocytes [19-21], although some contradictory reports have stated that tyrosine phosphorylation is not needed for Fas-mediated apoptosis in T cells [22-24]. Recently it was reported that Fas antigen could be co-immunoprecipitated with src-like tyrosine kinases such as Fyn [25] and Lyn [21] and with receptor tyrosine kinases such as c-erbB2 and HGF receptor [14]. The involvement of Fyn or Lyn in Fas signaling in T cells has been thoroughly studied. A decrease in Fas-mediated thymocytic death was demonstrated in Fyn-knockout mice [19] while Fas-mediated apoptosis in lck-deficient T cells was not affected [26-27]. However, the effects of receptor tyrosine kinase activation on Fas signaling remain unknown. Our study represents one of the first reports to show 1) that receptor tyrosine kinase activation can inhibit Fas-mediated apoptotic signals in endometrial cells, and 2) that tyrosine phosphorylation is possibly involved in endometrial Fas signaling. Seen in conjunction with previous reported results, our findings show that certain tyrosine phosphorylated molecules may have an inhibiting effect on apoptosis in the endometrial epithelium similar to that on apoptosis in hepatocytes.

### Acknowledgments

This work was supported in part by a Grant-in-Aid for scientific research (10470347) from the Ministry of Education, Science, Sports and Culture of Japan, and a Grant-in-Aid from The Osaka Medical Research Foundation For Incurable Diseases.

### References

- [1] Kokawa K., Shikone T., Nakano R.: "Apoptosis in the human uterine endometrium during the menstrual cycle". *J. Clin. Endocrinol. Metab.*, 1996, 81, 4144.
- [2] Tabibzadeh S.: "Signals and molecular pathways involved in apoptosis, with special emphasis on human endometrium". *Hum. Reprod. Update*, 1995, 1, 303.
- [3] Watanabe H., Kanzaki H., Narukawa S., Inoue T., Katsuragawa H., Kaneko Y., Mori T.: "Bcl-2 and Fas expression in eutopic and ectopic human endometrium during the menstrual cycle in relation to endometrial cell apoptosis". *Am. J. Obstet. Gynecol.*, 1997, 176, 360.
- [4] Tabibzadeh S., Babaknia A., Liu R., Zupi E., Marconi D., Romanini C.: "Site and menstrual cycle-dependent expression of proteins of the TNF receptor family, and BCL-2 oncoprotein and phase specific production of TNF- $\alpha$  in human endometrium". *Human Reprod.*, 1994, 10, 277.
- [5] Yamashita H., Kumagai K., Inoki C., Ueki M., Sugimoto O., Otsuki Y.: "The expression of Fas antigen in human endometrium". *Acta Obstet. Gynecol. Jpn.*, 1995, 47, 277.
- [6] Gompel A., Sabourin J. C., Martin A., Yaneva H., Audouin J., Decroix Y., Poitout P.: "Bcl-2 expression in normal endometrium during the menstrual cycle". *Am. J. Pathol.*, 1994, 144, 1195.
- [7] Otsuki Y., Misaki O., Sugimoto O., Ito Y., Tsujimoto Y., Akao Y.: "Cyclic bcl-2 gene expression in human uterine endometrium during menstrual cycle". *Lancet*, 1994, 344, 28.
- [8] Negami A. I., Sasaki H., Kawakami Y., Kamitani N., Kotsuji F., Tominaga T., Nakamura T.: "Serum human hepatocyte growth factor in human menstrual cycle and pregnancy: a novel serum marker of regeneration and reconstruction of human endometrium". *Horm. Res.*, 1995, 44 Suppl. 2, 42.
- [9] Tominaga T.: "Studies on the mechanism of embryo implantation". *Acta Obstet. Gynecol. Jpn.*, 1996, 48.
- [10] Revoltella R. P., Borney F., Dal-Canto B., D'Urso C. M.: "Apoptosis of serum-free C2.8 mouse embryo hepatocytic cells caused by hepatocyte growth factor deprivation". *Cytotechnology*, 1993, 13, 13.
- [11] Revoltella R. P., Dal Canto B., Caracciolo L., D'Urso C. M.: "L-carnitine and some of its analogs delay the onset of apoptotic cell death initiated in murine C2.8 hepatocytic cells after hepatocyte growth factor deprivation". *Biochem. Biophys. Acta.*, 1994, 1224, 333.
- [12] Morita M., Watanabe Y., Akaike T.: "Protective effect of hepatocyte growth factor on interferon-gamma-induced cytotoxicity in mouse hepatocytes". *Hepatology*, 1995, 21, 1585.
- [13] Nagy P., Bisgaard H. C., Santoni-Rugiu E., Thorgerisson S. S.: "In vivo infusion of growth factors enhances the mitogenic response of rat hepatic ductal (oval) cells after administration of 2-acetylaminofluorene". *Hepatology*, 1996, 23, 71.
- [14] Shen K., Noval R. F.: "Fas-signaling and effects on receptor tyrosine kinase signal transduction in human breast epithelial cells". *Biochem. Biophys. Res. Commun.*, 1997, 230, 89.
- [15] Ishiwata I., Ishiwata C., Soma M., Arai J., Ishikawa H.: "Establishment of human endometrial adenocarcinoma cell line containing estradiol-17 $\beta$  and progesterone receptors". *Gynecol. Oncol.*, 1984, 17, 281.
- [16] Tanaka T., Umesaki N., Mizuno K., Chang L., Miyama M., Ohtaki S., Ogita S.: "Enhancement of apoptotic susceptibility in human endometrial epithelial cell line HHUA by transforming growth factor- $\beta$ 1". *Horm. Metab. Res.*, 1998, 30, 61.
- [17] Nagata S.: "Apoptosis by death receptor". *Cell*, 1997, 88, 355.
- [18] Park M., Dean M., Cooper C. S., Schmidt M., O'Brien S. J., Blair D. G., Woude G. F. V.: "Mechanism of met oncogene activation". *Cell*, 1986, 45, 895.
- [19] Eischen C. M., Dick C. J., Leibson P. J.: "Tyrosine kinase activation provides an early and requisite signal for Fas-induced apoptosis". *J. Immunol.*, 1994, 153, 1947.
- [20] Szabo I., Gulbins E., Apfel H., Zhang X., Barth P., Busch A. E. et al.: "Tyrosine phosphorylation-dependent suppression of a voltage-gated K<sup>+</sup> channel in T lymphocytes upon Fas stimulation". *J. Biol. Chem.*, 1996, 271, 20465.
- [21] Schlottmann K. E., Gulbins E., Lau S. M., Coggeshall K. M.: "Activation of Src-family tyrosine kinases during Fas-induced apoptosis". *J. Leuko. Biol.*, 1996, 60, 546.
- [22] Abreu-Martin M. T., Vidrich A., Lynch D. H., Targan S. R.: "Divergent induction of apoptosis and IL-8 secretion in HT-29 cells in response to TNF-alpha and ligation of Fas antigen". *J. Immunol.*, 1995, 155, 4147.
- [23] Mollereau B., Deckert M., Deas O., Rieux-Laucat F., Hirsch F., Bernard A. et al.: "CD2-induced apoptosis in activated human peripheral T cells: a Fas-independent pathway that requires early protein tyrosine phosphorylation". *J. Immunol.*, 1996, 156, 3184.
- [24] Janssen O., Leng-Janssen B., Oberg H. H., Robertson M. J., Kabelitz D.: "Induction of cell death via Fas (CD95, Apo-1) may be associated with but is not dependent on Fas-induced tyrosine phosphorylation". *Immunol. Lett.*, 1996, 49, 63.
- [25] Atkinson E. A., Ostergaard H., Kane K., Pinkoski M. J., Caputo A., Olszowy M. W., Bleackley R. C.: "A physical interaction between the cell death protein Fas and the tyrosine kinase p59fyn". *J. Biol. Chem.*, 1996, 271, 5968.
- [26] Schraven B., Peter M. E.: "APO-1(CD95)-mediated apoptosis in Jurkat cells does not involve src kinases or CD45". *FEBS Lett.*, 1995, 368, 491.
- [27] Latinis K. M., Koretzky G. A.: "Fas ligation induces apoptosis and Jun kinase activation independently of CD45 and Lck in human T cells". *Blood*, 1996, 87, 871.

Address reprint requests to:  
TETSUJI TANAKA, M.D., Ph.D.  
Department of Obstetrics & Gynecology  
Osaka City University Medical School  
1-5-7 Asahi-machi, Abeno-ku  
Osaka 545-8585 (Japan)