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Effects of a Gonadotropin-releasing Hormone Agonist on Rat Ovarian Adenocarcinoma Cell Lines *in vitro* and *in vivo*

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To evaluate the biologic effects of the gonadotropin-releasing hormone (GnRH) agonist buserelin on rat ovarian adenocarcinoma cells *in vivo* and *in vitro*, female Wistar rats with primary ovarian adenocarcinoma induced by 7, 12-dimethylbenz(*a*)anthracene (DMBA) and the DMBA-OC-1 cell line established from a DMBA-induced rat tumor were used in this study. *In vivo*, daily administration of buserelin significantly suppressed the release of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and progesterone as compared with controls. Buserelin did not inhibit the growth of DMBA-induced tumors. However, histopathologically, there was increased central necrosis and a decrease in the number of neoplastic cells, with proliferation of connective tissue, in the group treated with buserelin. *In vitro*, FSH-induced proliferation of DMBA-OC-1 cells was suppressed by buserelin. Thus, this basic experimental study supports the potential use of a GnRH agonist to suppress the growth of ovarian cancer.

Key words: GnRH agonist — Ovarian cancer — Rat — *In vivo* — *In vitro*

Epithelial ovarian cancer is a common cause of death by cancer in Japan as well as in Western countries.[1)] Despite maximum cytoreductive surgery followed by drugs such as platinum and paclitaxel, most ovarian cancers recur, and patients eventually die of disease that is resistant to available cytotoxic agents.[2, 3)] Therefore it is of great clinical importance to find new therapeutic options for advanced and recurrent ovarian cancer.[4)]

Experimental data have demonstrated an influence of human gonadotropins on the growth of ovarian cancer cell lines and gonadotropins have been implicated in ovarian carcinogenesis. Recently, binding sites for gonadotropin hormones and gonadotropin-releasing hormone (GnRH) have been demonstrated on ovarian cancer cells.[5, 6)] The presence of these receptors supports the hypothesis that ovarian cancer is a hormone-sensitive tumor. It has been reported that both follicle-stimulating hormone (FSH) and estrogen stimulate the growth of ovarian cancer.[7)] *In vivo* and *in vitro* evidence suggests that GnRH agonists (GnRHa) may exert direct inhibitory effects on tumor growth, separate from their indirect steroid hormone-mediated effects.[8–11)] GnRHa have been used successfully in the therapy of hormone-sensitive cancers, such as breast cancer and prostate cancer.[12–14)] However, in cases of ovarian cancer, clinical results are limited and controversial.[15–19)] In this study, we evaluated the biologic effects of the GnRHa buserelin on rat ovarian cancer cells *in vivo* and *in vitro*.

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MATERIALS AND METHODS

In vivo Primary ovarian adenocarcinomas were induced by the local application of 7, 12-dimethylbenz(*a*)anthracene (DMBA) to the ovaries of Wistar-strain rats, as previously described,[20)] by serial implantation in the dorsal subcutis of newborn rats of the same strain. Tumors were measured with sliding calipers two times per week by the same observer. Tumor volume was assessed by measuring diameters along two major axes (length and width) and calculating the volume using the formula length (mm)×(width in mm)^2/2. Experiments were initiated when the tumors reached 0.1 cm^3 in volume. To evaluate the effects of GnRHa administration, the 20 tumor-bearing rats in which tumors reached 0.1 cm^3 in volume were selected. These rats were randomized into two treatment groups (group A and group B), and the following treatment was started simultaneously in both groups. The rats in group A received GnRHa buserelin injections ([D-Ser(tBu)6,desGly10-Pro9NEt]-GnRH) (Hoechst Marion Roussel Co., Frankfurt, Germany) (n=10). The buserelin was diluted in oil and administered intramuscularly every day for 8 weeks (10 μg/kg/day). Group B was a control group receiving daily intramuscular injections of oil for the same period (0.05 ml/day for 8 weeks) (n=10). All the rats were killed 1 week after the completion of the buserelin treatment. Tumor growth was analyzed in treated rats versus control rats using Student's t test. Serum samples were obtained for hormonal assays to measure luteinizing hormone (LH), FSH (enzyme immunoassay; E-test, Toso Co., Yamaguchi), estrogen, and progesterone values

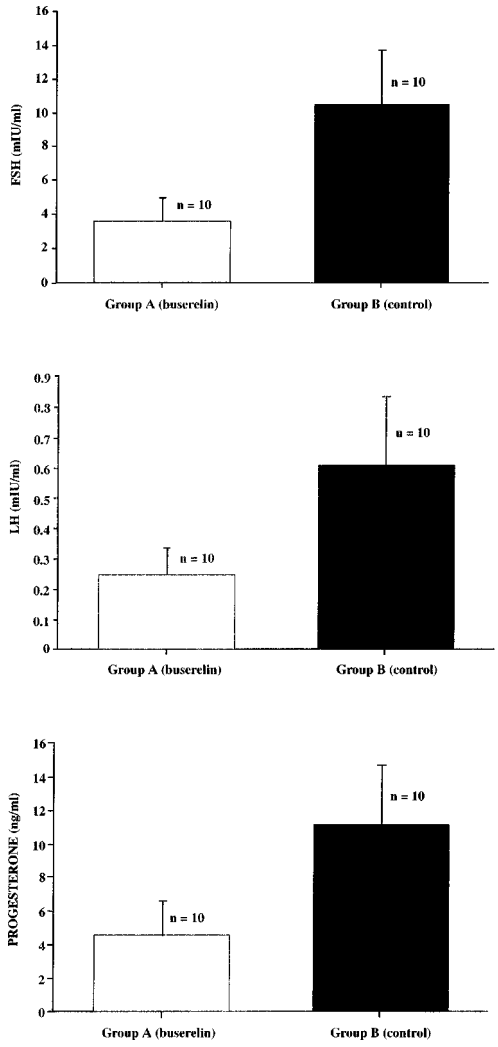

Fig. 1. Serum FSH, LH, and progesterone concentrations in Wistar-strain rats bearing ovarian adenocarcinoma transplants, with or without GnRHa buserelin treatment. Tumors were grown to a volume of 0.1 cm^3. The rats were treated with either buserelin, 10 μg/kg/day (n=10), or normal saline, 0.05 ml/day (n=10) for 8 weeks. Rats were killed 1 week after completion of buserelin treatment.

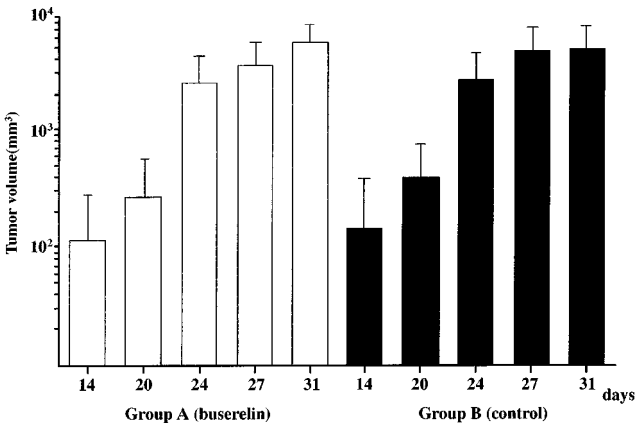

Fig. 2. The *in vivo* effect of buserelin on rat ovarian cancer induced by 7,12-dimethylbenz(*a*)anthracene. No significant difference in tumor growth was observed between buserelin-treated rats and control rats.

(radioimmunoassay; DPC kit, Nippon DPC Co., Chiba) when the animals were killed. Histologic sections were fixed immediately in Bouin's solution and were stained with hematoxylin and eosin.

In vitro *Cell proliferation experiments*. The DMBA-OC-1 rat ovarian cell line was previously established by the authors.[21] This cell line does not have receptors for estrogen or progesterone. Cells were grown in DM-170 (Kyokuto Co., Tokyo), supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 1.25 U/ml nystatin. Buserelin (1.0 ng/ml), FSH (5.0 mIU/ml), and human chorionic gonadotropin (hCG) (10 mIU/ml) were added to the cell cultures as sterile concentrates freshly prepared in a culture medium. Both buserelin and FSH were added simultaneously. In the control, hormonal agents were not present in the cell culture. One week before the experiments, the cells were transferred to a medium containing 10% charcoal-treated FCS. Phenol red was not used because of its potential estrogenic effects.[22] Cells seeded into 35-mm Falcon culture dishes (10^5 cells per dish) were incubated for 10 days. Cell density was quantified every 24 h by the amino black 10B staining method described by Vilcek *et al*.[23]

FSH-binding assay. For the FSH-binding assay, DMBA-OC-1 cells were cultured under the following conditions: (1) FSH 5 mIU/ml, (2) FSH 25 mIU/ml, (3) buserelin 5.0 ng/ml, (4) FSH 5 mIU/ml and buserelin 5.0 ng/ml, (5) control (no addition of hormonal agents). In separate experiments, cells were seeded in 5 dishes and incubated for 48 h. FSH concentration in the conditioned media was quantified by an FSH Elmotech kit (Mochida Co., Tokyo) before and after incubation. After 48 h of incubation, the FSH level in the culture supernatant was determined and the obtained value was subtracted from FSH added to the culture before incubation, which was considered to represent the cell-bound FSH. The value was divided by the number of cells after 48 h of incubation and then converted into the number per 10^6 cells. The FSH binding assay was performed using this calculated value. Cell counts were carried out in quadruplicate. Each experiment was repeated three times. Statistical evaluation was performed by analysis of variance, followed by the

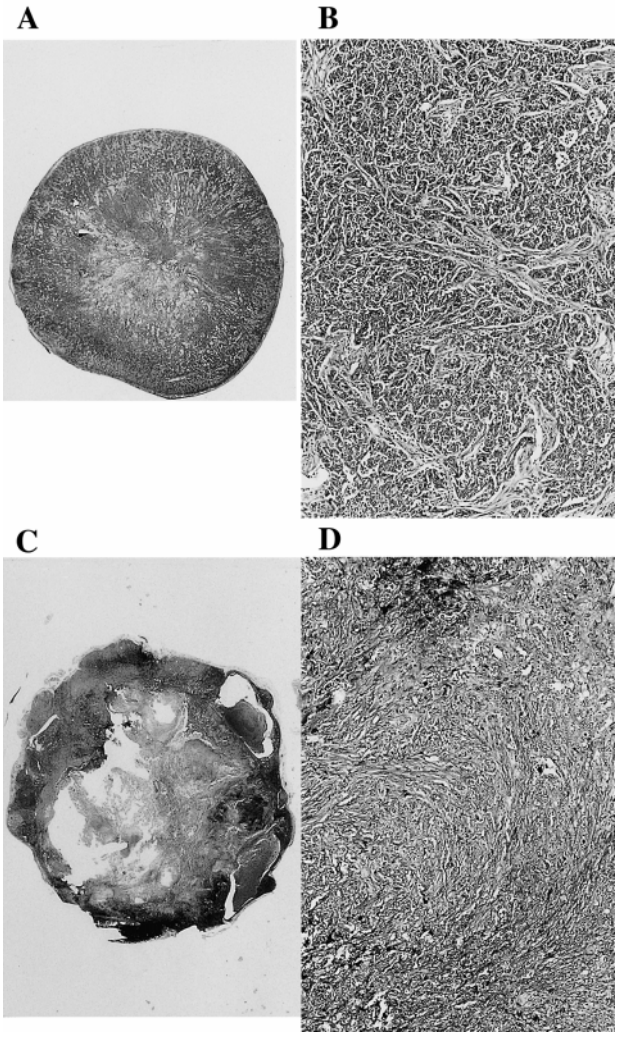

Fig. 3. Morphology of transplanted tumors. A: Low magnification of a section from a transplant showing a solid tumor with slight central necrosis. B: Higher magnification of the margin of a tumor showing undifferentiated adenocarcinoma surrounded by a scant, delicate fibrovascular stroma. C: Low magnification of a buserelin-treated tumor showing irregular enlargement of the central necrosis. D: Higher magnification of a buserelin-treated tumor showing proliferation of loose connective tissue with microvascular development and nests of degenerated neoplastic cells.

Student-Newman-Keuls test. A value of $P<0.05$ was considered to be statistically significant.

RESULTS

***In vivo* experiments** The mean serum FSH concentration of the A group declined significantly (3.7 mIU/ml) compared with that of the B group (10.8 mIU/ml). The serum

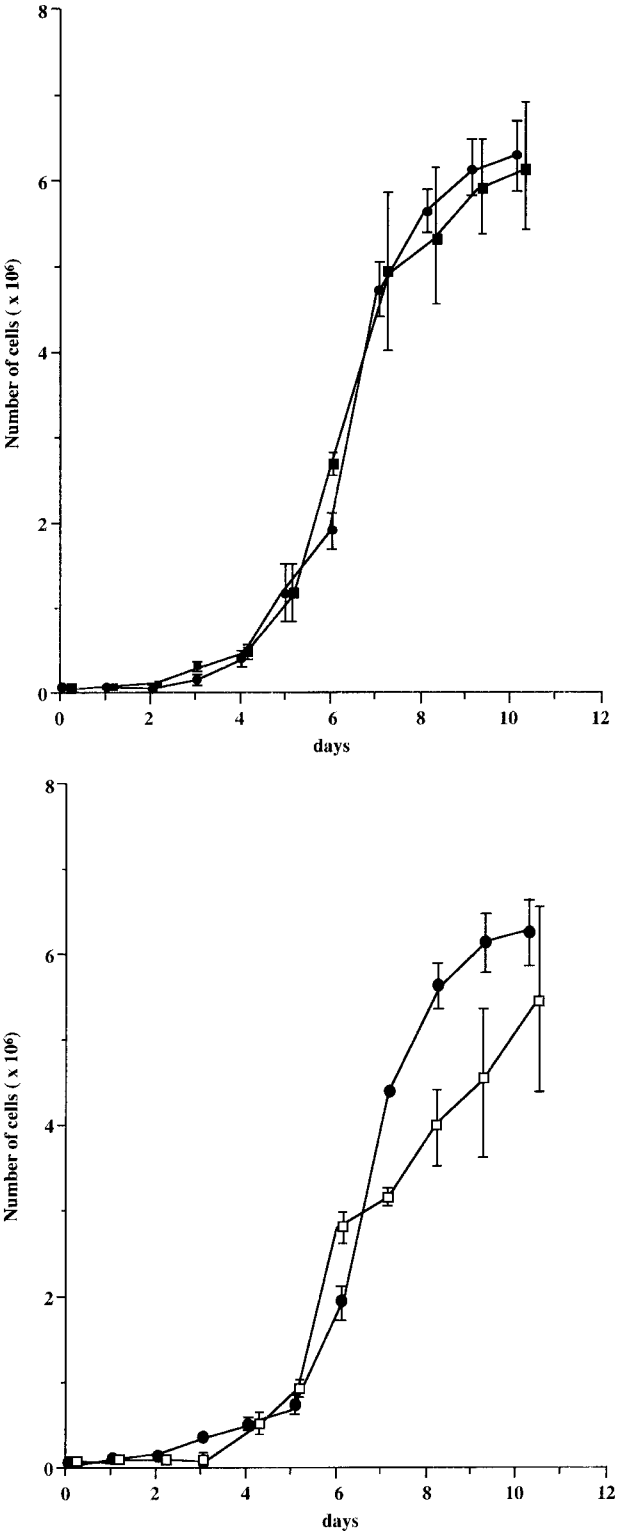

Fig. 4. The *in vitro* effect of hCG and buserelin therapy on the cell number of DMBA-OC-1 rat ovarian cell line. No significant inhibition of growth was observed. ● control, ■ hCG, □ GnRHa.

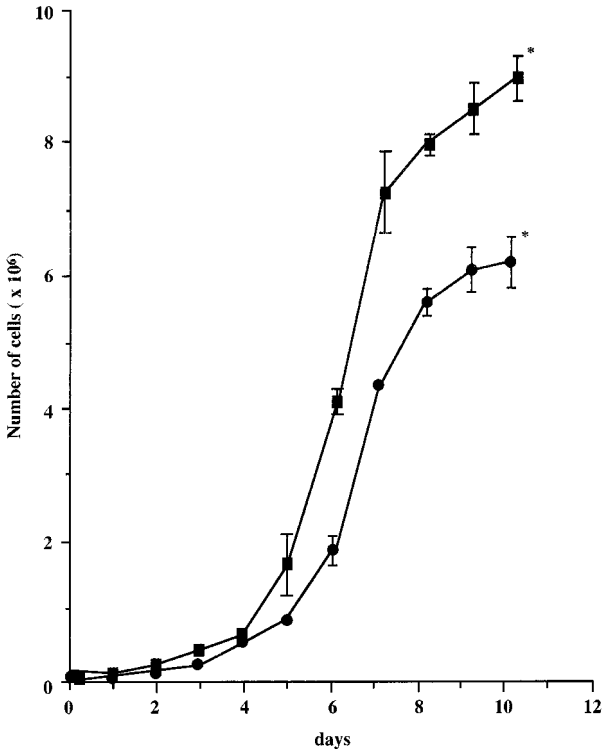

Fig. 5. The *in vitro* effect of FSH on the cell number of DMBA-OC-1 cells. The growth rate as well as cell number were significantly higher in the FSH-treated group (■) compared to the control group (●) (P<0.05).

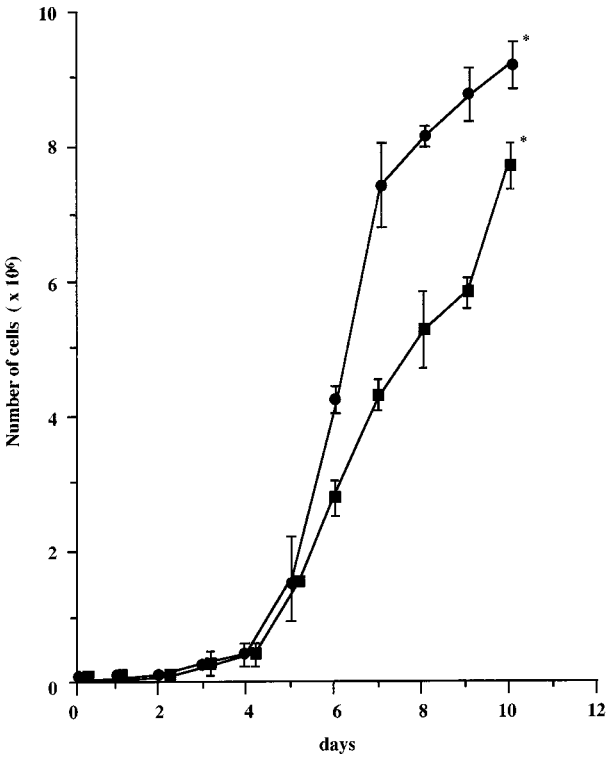

Fig. 6. The *in vitro* effect of the co-administration of FSH and buserelin on the cell number of DMBA-OC-1 cells. When GnRHa was added concomitantly with FSH (■), growth stimulation was significantly suppressed compared with FSH treatment alone (●) (P<0.05).

LH concentration was also significantly lower in the A group (0.26 mIU/ml) than in the B group (0.62 mIU/ml). In addition, serum progesterone concentrations significantly decreased in the A group (4.8 ng/ml) as compared to those of the B group (11.2 ng/ml) (Fig. 1). Estrogen levels were below the detection limit both before and after treatment in both the A and B groups. No significant difference in tumor growth was observed between the A and B groups (Fig. 2). In the B group, the tumors grew as solid tumors and histologic examination revealed undifferentiated adenocarcinomas with a small area of central necrosis. The A group findings included an increase in central necrosis as well as a decrease in the number of tumor cells, with proliferation of connective tissue at the margins of the tumors (Fig.3).

***In vitro* experiments** No significant differences in growth rate or the number of cells were observed with either GnRHa or hCG treatment compared with controls (Fig. 4). In contrast, the growth rate and the number of cells were significantly higher in the FSH-treated group than in the controls (Fig. 5). However, when GnRHa was

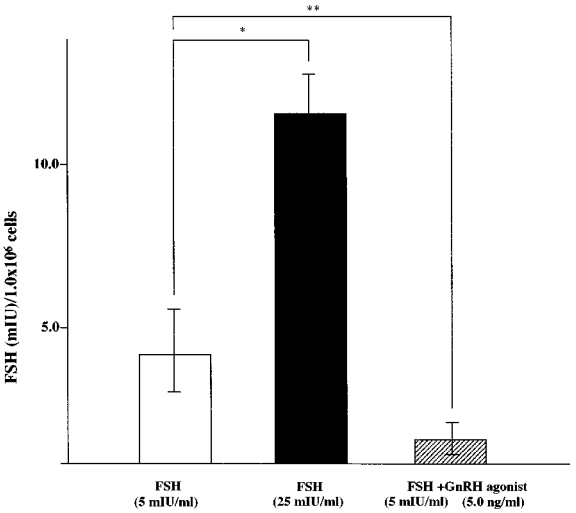

Fig. 7. FSH binding was significantly enhanced in the 25 mIU/ml group compared to the 5 mIU/ml group (P<0.05). When FSH and GnRHa were added concomitantly, FSH binding was significantly suppressed compared with FSH treatment alone (P<0.005).

added concomitantly with FSH, growth stimulation was suppressed (Fig. 6).

In the FSH binding assay, no significant difference was seen in cell proliferation between both groups treated with FSH and the group treated with GnRHa after 48 h of incubation. Between the two groups treated with FSH, i.e., the 5 mIU/ml group and the 25 mIU/ml group, FSH binding was significantly enhanced in the 25 mIU/ml group treated with FSH compared with that in the 5 mIU/ml group. When 5 mIU/ml FSH and 5.0 ng/ml GnRHa were added concomitantly, FSH binding was suppressed (Fig. 7). When the value was corrected to the amount of FSH bound to 10^6 cells, the same results were obtained.

DISCUSSION

Although the half-life of natural GnRH in plasma is about 2 min, the half-lives of GnRH analogues are tens to hundreds of times as long.[24] The increased affinity of these analogues to albumin and to the GnRH receptors results in the prolongation of the half-life of these analogues.[24] This potent and persistent stimulation leads to desensitization of the receptors, which in turn results in a decrease in the responsiveness of the pituitary gland to GnRH.[24] In this study, the synthesis and secretion of gonadotropin in rats were suppressed by buserelin, regardless of species differences between rats and humans. Steroidogenesis in the ovary was also suppressed, as previously reported by Maynard and Nicholson.[25] Gonadal steroid receptors have been detected in ovarian cancer,[5] and a promotive effect of estrogen on ovarian cancer growth has been reported.[5] In addition to decreasing gonadotropin and estrogen by a down-regulation of GnRH receptors in the pituitary gland, GnRH analogues are also postulated to suppress aromatase activity in the interstitial tissue of the tumor and to block estrogen-mediated signaling in the tumor cells, resulting in inhibition of the proliferation of cancer cells.[26–28] In this study, however, changes in tumor volume were not observed after buserelin treatment. This may be due to the lack of estrogen receptors (ER) in the tumor cells used in this experimental model. The rats used in this experiment were immature, and therefore the lack of observed changes in tumor volume might have been due to low serum concentrations of estrogen before buserelin treatment was initiated. *In vitro*, we have shown previously that estrogen can not act as a growth factor for either ER(−) or progesterone receptor (PR)(−) ovarian cancer cell lines.[29] However, the decrease in the number of tumor cells and the proliferation of connective tissue found in this experiment indicate that some local GnRH-responsive factors may have affected the ovarian tumors. As specific GnRH receptors have been detected in ovarian cancer, a direct effect of buserelin on ovarian cancer is possible, although this action may require a higher local concentration of the drug.[6, 8–11, 30, 31]

During the past two decades, considerable experimental evidence has been collected indicating that epithelial ovarian cancer is gonadotropin-dependent.[5, 7] LH and FSH receptors have been described in some of these tumors. The proliferation of ovarian cancer cells can be stimulated *in vitro* by gonadotropins.[5, 6, 11] Suppression of endogenous LH and FSH secretion by GnRHa treatment inhibits the growth of experimental or heterotransplanted ovarian cancers in various animal models.[8–11] Clinically, positive responses to GnRH agonist therapy have been demonstrated in patients with epithelial ovarian cancer who are postmenopausal at diagnosis or postmenopausal following oophorectomy as part of primary cytoreductive surgery.[15–19] Jager *et al*. reported stable disease for up to 20 months, and a slower rise in the tumor marker CA125 in patients treated with D-Trp^6-LH-RH decapeptyl.[15] Parmar *et al*. also reported the encouraging results with decapeptyl that 6 of 41 patients achieved a partial response (15%) for a mean duration of 10 months, and an additional 5 patients had stable disease for 6 months to 12 months. No correlation was found between response and histologic subtype or grade.[16] Kavanagh *et al*. found that 4 of 18 patients (17%) showed clinically objective responses, with a median duration of response of 52 weeks.[17] Interestingly, responders to leuprolide were for the most part patients with well-differentiated tumors. Scambia *et al*. reported that no response was observed among 14 patients with mostly poorly differentiated tumors refractory to cisplatin-based regimens.[18] However, 1 of the 2 patients with well-differentiated tumors in that study showed stable disease for 8 months. In addition, Falkson *et al*. reported the results of cisplatin versus cisplatin plus D-Trp^6-LH-RH in the treatment of ovarian cancer. No differences in objective response and toxicity profile between the two groups were obtained.[19]

The mechanism behind these responses is still not clear, but several theories have been formulated. One suggestion is that LH and/or FSH may act as tumor growth factors, and suppression of their secretion from the anterior pituitary by GnRHa could lead to tumor regression.[7] Alternatively, GnRHa could have an inherent, direct anti-tumor effect at the cell level, independent of or via a receptor-dependent pathway.[8–11] Miller *et al*. have reported significant growth inhibition and tumoricidal effects on the MCF-7 breast cancer cell line with GnRHa *in vitro*, supporting a direct anti-tumor action of GnRHa.[32] Neri *et al*. found no inhibition of growth by GnRHa in the absence of estradiol-17β, suggesting modulation of estradiol and/or its receptor molecule by GnRHa.[33] Slotman *et al*. and Connor *et al*. have demonstrated significant reductions in growth with physiologically high concentrations of buserelin in several ovarian cancer cell lines.[30, 31]

In our *in vitro* study, we were not able to demonstrate inhibition of growth by buserelin on DMBA-OC-1 cells. The proliferation of DMBA-OC-1 cells induced by the administration of FSH was, however, suppressed by co-administration of buserelin. In addition, treatment with GnRHa significantly suppressed the binding of FSH to DMBA-OC-1 cells, suggesting the existence of FSH receptors on DMBA-OC-1 cells and an antagonism between FSH and GnRHa at this receptor level. Ohtani *et al*. have evaluated the effect of FSH on the proliferation of a human ovarian cell line (the HRA cell line) *in vitro* and *in vivo*.[34] In HRA cells, which have both FSH receptors and GnRH receptors, the number of FSH receptors is significantly decreased by treatment with buserelin, and the growth-promoting effect of FSH is suppressed by treatment with buserelin. These results suggest that GnRHa suppresses the effect of FSH through the down-regulation of FSH receptors. Ranta *et al*. have reported a similar phenomenon in normal granulosa cells *in vivo* through an unknown mechanism, suggesting that GnRHa may down-regulate FSH receptors in cells derived from the ovary.[35] According to a report by Ohtani *et al*., cAMP does not decrease after treatment with FSH, suggesting that FSH may activate the protein kinase C pathway in ovarian tumors, though it does not activate this pathway in normal ovarian tissues.[34] Therefore, the down-regulation of FSH receptors by treatment with GnRHa may inactivate PKC activity, possibly resulting in an anti-proliferative effect of GnRHa on cancer cells. This theory is consistent with the phenomenon of GnRHa triggering apoptosis, as evidenced by DNA fragmentation, as a direct effect on ovarian cancer cells.[36–38]

In conclusion, the anti-proliferative effect of GnRHa on ovarian cancer cells was histologically observed in the present study, although no growth inhibition of the ER(−) and PR(−) tumors was obtained in our *in vivo* study. GnRHa may suppress the proliferative effect of FSH through down-regulation of FSH receptors *in vitro*. GnRHa might be a useful option for the therapeutic treatment of ovarian cancer, but further experimental studies are needed.

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