Induction of Apoptosis in Ovarian Carcinoma Cell Line by Gonadotropin–releasing Hormone Agonist

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Summary: Gonadotropin releasing hormone (GnRH) agonist suppresses the growth of the cancer cells in vitro. To evaluate the effect of a GnRH agonist (GnRHA) in ovarian carcinomas, we investigated the interactions of GnRHA with the KOC-2s human ovarian cancer cells. The addition of GnRHA (10^{-8}-10^{-6}M) produced an increase 20-30% in the number of cells (p<0.05). GnRHA (10^{-5} M) produced a slight, statistically insignificant decrease of <10% in the cell count. No DNA fragmentation was produced by GnRHA (10^{-5}-10^{-4}M). However, GnRHA (10^{-5}M) produced internucleosomal cleavage of DNA into fragments with multiples of 180 to 200 bp. This DNA “ladder” pattern is characteristic of apoptosis. The amount of Fas antigen was reduced by each concentration of GnRHA. The addition of GnRHA (10^{-6}M and 10^{-5}M) significantly increased the secretion of TNF-α (p<0.001). The time- and dose-dependent effects of GnRHA might be confined to the KOC-2s cells as demonstrated by growth inhibitions and characteristic internucleosomal DNA fragmentation. However, the effects of GnRHA on secretion of TNF-α and the expression of Fas antigen differed. The present results provide a basis for future studies on the mechanism of apoptotic effect of GnRHA.

Key words ovarian cancer, apoptosis, GnRH agonist, glucocorticoids, DNA-ladder

INTRODUCTION

Effective therapy for patients with epithelial ovarian cancer is elusive, with long-term survival rates persistently below 10% [1]. A poor understanding of the precursor lesions and warning signs continue to hamper the approach to early diagnosis and screening for this disease [1,2]. Increased information on the genetic alterations responsible for the malignant ovarian phenotype would significantly influence the diagnosis and therapy of ovarian carcinoma. The failure of traditional chemotherapeutic regimens to significantly increase survival rates prompted us to question whether ovarian carcinoma cells might be responsive to hormone administration [1,2]. However, despite reports that antiestrogens and progestins may be useful in treating ovarian cancer, results have been disappointing, so that such therapy is not routinely used [3-5]. Gonadotropin releasing hormone (GnRH) may modulate activity in the brain and peripheral organs [6-9]. GnRH agonist (GnRHA) has been used in treating endocrine-dependent cancers, including breast, prostate, pancreas, endometrium, and ovarian cancer [1,10-12]. GnRHA reportedly suppressed the growth of cancer cells in vitro [3,10]. Specific binding sites for GnRH have been demonstrated in certain tumors that are responsive to GnRHA [1,3,8,10,11]. Findings suggest direct inhibitory effects of GnRHAs on tumor growth [5,15]. We evaluated the apoptotic effects of a GnRHA in the KOC-2s ovarian carcinoma cell line.

MATERIALS AND METHODS

Cell line and cell culture conditions

The KOC-2s human ovarian cancer cell line was
previously established by the authors [15]. It represents a clone of a serous adenocarcinoma of the ovary from a 51-year-old patient [15]. This cell line has GnRH receptors with a Kd=10^7M order but lacks receptors for estrogen or progesterone. Cells were grown in Dulbecco’s minimum essential medium (DMEM, Nissui Co., Tokyo) supplemented with 1% heat-incubated fetal calf serum, penicillin 100 units/ml, streptomycin 0.1 mg/ml, and nystatin 1.25 units/ml. GnRHA (10^-8-10^-6M) ([des-Gly10, D-Phe6, Pro9-N-ethylamide]) (Hoechst Japan Co., Tokyo) was added to the cell culture as sterile concentrate freshly prepared in culture medium. One week before the experiments, the cells were transferred to a medium containing 10% charcoal-treated fetal calf serum (FCS). Phenol red was not used to avoid its reported estrogenic effects.

Cell proliferation experiments

Cells were seeded into 35 mm Falcon culture dishes (10^5 cells per dish) in a medium that contained 10% charcoal-treated FCS and were incubated for periods of 12, 24, 48, and 72 hs. Cytolytic activity was observed in monolayer cultures by means of phase-contrast microscopy. Cell density was quantified by using the amino black 10B staining method described by Vilcek et al. [13].

Assay for fragmented DNA

After 24 hs, 10^5 cells cultured in a 75 cm² flask were collected, and centrifuged for 4 min at 400 g. The resulting pellet was resuspended in a lysis buffer (10 mM Tris HCl, pH 8.0, 10 mM EDTA, 100 μg/ml Proteinase K (PK) (Sigma Chemical Co., St Louis, MO), 1% SDS), and was incubated at 37 °C, until the mixture became clear. A volume of 100 μg/ml PK was added for a few hours. DNA was extracted by the phenol/chloroform method 1:1 (chloroform-chloroform/isoamyl alcohol 24:1), then precipitated overnight in −20 °C ethanol that contained a 0.3 M final concentration sodium acetate. It was then centrifuged at 12,000 g for 30 min at 4 °C. The pellet was resuspended in TE buffer (0.1 M Tris-HCl, pH 8.0, 10 mM EDTA). DNA was next exposed to RNAase (100 μg/ml) at 37 °C. It was then reextracted with phenol, phenol/chloroform, and chloroform, precipitated with ethanol, and resuspended as described above. Samples of DNA, approximately ~0.2 μg each, were electrophoretically separated on a 1.8% agarose gel containing ethidium bromide, 0.4 μg/ml. DNA was visualized by a UV (302 nm) transilluminator. Gels were photographed with a Polaroid camera.

Analysis of Fas antigen

Cell surface Fas antigen was quantified as follows. The cells (5×10^5 per each period of incubation) were washed with PBS, then reacted on ice for 1 h with 0.1 ml PBS containing 1% FCS, 0.02% NaN₃, and 20 μg/ml anti-Fas IgM. They were reacted for 1 h with PBS containing 0.02% NaN₃, 100 μg/ml phycoerythrin-avidin D (Vector Laboratories, Inc., Burlingame, CA), and 10 μg/ml affinity-purified FITC goat anti-mouse IgM (Cappel Laboratories, Malvern, PA). After being washed in PBS at 4 °C, cell surface phycoerythrin-avidin and FITC anti-mouse IgM were quantified simultaneously on a flow cytometer (Becton & Deckinson, Sanday, UT).

Quantitation of TNF-α in medium

The presence of TNF-α was determined following incubation for 0, 24, 48, and 72 hs by use of the TNF-α ELISA kit (BIOTRA TM, Amersham, London).

Data analysis

Cell counts were carried out in quadruplicate. Each experiment was repeated three times. Statistical evaluation was performed by analysis of variance, followed by the Student-Newman-Keuls test. A value of p<0.05 was considered to be statistically significant.

RESULTS

Time-course studies

In initial experiments, we attempted to identify the conditions under which the growth of the ovarian cancer cells would be most responsive to hormones (Fig. 1). Under these conditions, the number of cells increased by approximately 95% over a 72-hour period (p<0.001 vs. the initial number of cells). The addition of GnRHA (10^-8-10^-6M) produced a significant increase by 20-30% in the number of cells after 72 hs (p<0.05). GnRHA (10^-6M) produced a slight, statistically insignificant decrease of <10% in the cell count.

Dose-dependency of tumor cell inhibition

The drug effect shown in Fig. 1 is a decrease in the time rate of change of cell number after 72 hs; in control cell increases by nearly 100%. Low drug concentrations reduce the increase to 20-30%.
Fig. 1. Effects of GnRHA on the time course of growth of KOC-2s cells. Cells were cultured in media containing 1% FCS and treated with $10^{-8}$M (○), $10^{-7}$M (×), $10^{-6}$M (△), and $10^{-5}$M (□) of GnRHA. The SE bars are not apparent because they are covered by the symbols.

Fig. 2. Effect of GnRHA on KOC-2s cells as a function of time and dose. Each point represents the mean of triplicate cultures. All data are plotted as % of control, taken as 100. SD of each point was <11% of the mean.
Higher drug concentrations cause a decrease in cell number. The effect of $10^{-5}$M is highly significant ($p<0.005$). Studies of the dose dependency of tumor cell inhibition are conducted by counting the cells after 24, 48 and 72 hs (Fig. 2). Fig. 2 shows that at every concentration in the range $10^{-8}$-$10^{-5}$M the drug treated cultures have a lower number of cells than the control cultures. This effect is greater in the longer incubation with the drug. There is a slight dose-dependence of the effect; a concentration of $10^{-3}$M produced a significant decrease of 40% in the cell count ($p<0.01$).

**Fragmentation of DNA**

No DNA fragmentation was produced by GnRHA ($10^{-5}$-$10^{-6}$M). However, GnRHA ($10^{-5}$M) produced internucleosomal cleavage of DNA into fragments with multiples of 180 to 200 bp (Fig. 3). This DNA "ladder" pattern is characteristic of apoptosis [19,20].

**Flow cytometric analysis**

The amount of Fas antigen was reduced by each tested concentration of GnRHA (Fig. 4).

**Production of TNF-α**

The production of TNF-α in control media did not increase over time (Fig. 5). The secretion of TNF-α following the addition of GnRHA ($10^{-6}$-$10^{-7}$M) resembled the control findings at all time intervals. However, the addition of high concentrations of GnRHA ($10^{-4}$M and $10^{-5}$M) significantly increased...
Fig. 5. Secretion of TNF-α from KOC-2s cells in control condition and in presence of various concentrations of GnRHA. KOC-2s cells were plated in 25-cm² culture flasks for 3 days. Spent growth media were then stored at -80 °C for the analysis of TNF-α concentration by ELISA, as well as for counting the number of cells harvested. The SE bars are not apparent because they are covered by the symbols.

the secretion of TNF-α. The maximum increase occurred after 72 hs (p<0.001).

DISCUSSION

We previously demonstrated that KOC-2s cells express HER-2/neu, but the mRNA was not increased by exposure to GnRHA [10]. High concentrations of GnRHA also induced apoptosis in those cells.

Concerning the mechanisms of the apoptosis produced by GnRHA on ovarian cancer cells, Billig et al. [11] suggested that it was mediated by specific GnRH receptors based on the ability of a GnRH antagonist to block the effect of GnRH. A GnRHA-induced increase in apoptotic DNA fragmentation that was dose dependent was seen in the ovary and in the granulosa cells [11]. In contrast, our present study showed that the in vitro proliferation of KOC-2s cells was inhibited by GnRHA in a dose-dependent manner. As this effect occurred at a high concentration (10⁻⁸M) of the agonist, it is likely that the low affinity, high capacity binding site described by Emons et al. is involved in its mediation [8].

The addition of GnRHA to the culture medium of KOC-2s cells induced up-regulation of secretion of TNF-α. By contrast, the expression of Fas in our present study was down-regulated by GnRHA. Fas and the TNF-receptors cause homologous cytoplasmic death transduced by one or more of these surface receptors that would have similar characteristics [14,15,18]. The Fas and TNF systems seem to be mainly restricted to immune system [14,21,22]. However, the mechanism of reduction of Fas expression or of the promoter elements of the Fas gene have not been studied in detail [16-18]. Thus, our results showed different mechanism on the induced apoptosis between Fas and TNF-α. Furthermore, Fas-induced cell death might be quicker than that induced by TNF-receptor. Our results suggest there exist other death-inducing molecules that are functionally related to Fas, and that may help to remove unwanted cells such as ovarian cancer cells.

In summary, the present study demonstrated that the GnRHA directly induced apoptotic DNA fragmentation of the KOC-2s human ovarian carcinoma cells. The time- and dose-dependent effects of this agent might be confined to the KOC-
2s cells as demonstrated by growth inhibitions and characteristic internucleosomal DNA fragmentation. However, the effects of the agent on secretion of TNF-α and the expression of Fas antigen differed. The present results provide a basis for future studies on the mechanism of the apoptotic effect of GnRHA.

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