A novel molecular mechanism for anticancer drug-induced ovarian failure: Irinotecan HCl, an anticancer topoisomerase I inhibitor, induces specific FasL expression in granulosa cells of large ovarian follicles to enhance follicular apoptosis

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Abstract. Clinical use of CPT-11 combination chemotherapy frequently induces ovarian dysfunction in premenopausal and perimenopausal cancer patients, but its mechanism remains unclear. Mouse experiments were performed to clarify the molecular mechanism of CPT-11-induced ovarian dysfunction. Clinically therapeutic doses of CPT-11 were injected intraperitoneally into 8-week-old female MCH mice, and their ovaries were examined by the TUNEL assay to detect dead cells. Immunohistochemical examinations were simultaneously performed to detect the expression of activated caspase 3, Fas antigen and Fas ligand (FasL). Furthermore, normal murine ovarian tissue fragments were incubated with recombinant soluble FasL in organ cultures and stained by the TUNEL assay to detect apoptotic cells. Intraperitoneal CPT-11 injections induced specific TUNEL-positive cells and cell death with cleaved caspase 3 expression among large ovarian follicular granulosa cells. Apoptotic follicles (follicles containing ≥10 TUNEL-positive cells per ovarian section) were only found among large follicles. The final apoptotic follicle ratios were ~30% of the total follicles independent of the CPT-11 dose, while CPT-11 dose-dependently enhanced apoptotic processes in murine ovarian follicles. Fas antigen was expressed in most ovarian cells, with extremely high expression levels detected in luteal cells. CPT-11 injections did not significantly increase the Fas expression levels in ovarian cells. Although no FasL expression was detected in normal ovarian tissues, CPT-11 injections significantly induced specific FasL expression in granulosa cells. Incubation of organ-cultured normal murine ovarian tissue fragments with recombinant mouse soluble FasL significantly increased the numbers of TUNEL-positive granulosa and luteal cells. In conclusion, CPT-11 dose-dependently induced specific FasL expression in granulosa cells of developing ovarian follicles. The induced FasL reacted with the Fas antigen constitutively expressed on granulosa cells, such that apoptosis can only be enhanced and induced in granulosa cells in an autocrine and/or paracrine manner. This cell lineage-specific and differentiation stage-specific apoptosis in granulosa cells is thought to be the main molecular mechanism of the ovarian dysfunction induced by CPT-11 combination chemotherapy.

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Introduction

Anticancer chemotherapies sometimes cause severe ovarian damage and lead to premature ovarian failure or severe sterility in patients. Since ovarian blood flow increases in accordance with postpubertal follicular growth, systemic administration of anticancer drugs can initially cause damage to granulosa cells and oocytes, which accelerates ovarian follicular loss. Even when a young female receives irreversible disruption to most of her ovarian follicles, she may undergo premature menopause. Since follicular atresia is controlled by apoptosis during normal follicular development (1-3), normal ovarian follicles are specific organs that are sensitive to apoptotic stimuli. Anticancer drugs may enhance the apoptotic susceptibility of normal ovarian follicles to rapidly reduce the limited numbers of ovarian follicles or create certain damage to oocytes, and finally induce severe ovarian dysfunction or sterility (4-8). However, the molecular mechanism of the ovarian follicular loss induced by anticancer chemotherapy has not yet been clarified.

Due to recent progress, anticancer chemotherapy can often induce complete remission, even in advanced cancer patients. In today's developed countries, the ages of women at marriage and childbirth are increasing. On the other hand, the divorce and remarriage rates are also currently increasing, as well as the incidence of pregnancy in women in their late thirties to forties. Therefore, the numbers of patients in whom advanced cancer is diagnosed and requires chemotherapy before pregnancy are increasing. Recent progress in assisted

reproductive technology (ART) has made it possible to cryopreserve unfertilized oocytes before anticancer chemotherapy in order to perform *in vitro* fertilization of thawed oocytes and embryo transfer after completion of the chemotherapy. Cryopreservation of fresh ovarian tissues and tissue transplantation after thawing is an alternative therapy. However, these ART methods can only obtain small numbers of oocytes, and successful pregnancy rates using these methods are low. If the mechanism of chemotherapy-induced gonadal dysfunction can be clarified, it will be clinically useful for developing certain preventive therapies or treatments for chemotherapy-induced ovarian dysfunction in cancer patients hoping for future pregnancy. However, no definite preventive therapies have been established to date, since most of the molecular mechanisms of ovarian damage induced by various anticancer drugs remain to be elucidated.

Irinotecan HCl (CPT-11), an anticancer prodrug, is converted into its main active metabolite, SN38, by carboxyl esterase in the body. SN38 is the most powerful inhibitor of topoisomerase I and shows a strong antitumor effect by antagonizing DNA synthesis (9). CPT-11 has been used clinically in various cancer chemotherapies for uterine cancer (10-12), ovarian cancer (13,14), lung cancer (15), colorectal cancer (16,17), gastric cancer (18) and malignant lymphoma (19), and high response rates of these therapies have been reported. Although bone marrow suppression such as neutropenia and thrombocytopia, digestive symptoms such as nausea, vomiting and diarrhea and interstitial pneumonia are well known as frequent severe adverse effects of CPT-11, no previous studies have investigated its gonadal toxicity. To the best of our knowledge, there is one report regarding CPT-11-induced gonadal dysfunction indicating that male rats injected with CPT-11 at 6 μg/g body weight exhibited reduced prostate and epididymis volumes (20), but CPT-11-induced ovarian toxicity was not examined in that study.

Recently, we found that premenopausal and perimenopausal cancer patients aged ~50 years and treated with CPT-11 combination chemotherapy frequently complained of menopausal malaise-like symptoms (MMLS), such as hot flushes, episodic sweating, peripheral chillness, irritability and sleeplessness (21,22). In all of these patients, the MMLS were completely relieved by estrogen therapy, indicating that they were induced by estrogen deficiency. Subsequently, we performed endocrinological examinations of pituitary-ovarian functions in CPT-11-treated patients and histopathological examinations of surgically resected normal ovarian tissues from cervical cancer patients after preoperative CPT-11 combination chemotherapy (22). We found that premenopausal patients who were treated with CPT-11 and complained of secondary amenorrhea or ovarian defect symptoms were associated with elevated serum follicle-stimulating hormone (FSH) and lutenizing hormone (LH) levels, and decreased serum estradiol levels. In addition, we found that normal ovaries surgically resected from cervical cancer patients who were preoperatively treated with CPT-11 did not contain any growing follicles. These results suggested that the MMLS and secondary amenorrhea experienced by CPT-11-treated cancer patients could be induced by direct injury to granulosa cells, which represent the main source of estradiol. Since the examined human ovarian tissues were surgically resected ~1 month after the last administration of CPT-11, the pathological results of these surgical specimens could not perfectly explain the direct effects of CPT-11 on normal ovaries. The present study was carried out to investigate the mechanisms of CPT-11-induced ovarian dysfunction using an established mouse animal experimental model of CPT-11-induced ovarian injury in order to identify preventive methods and therapies for anticancer drug-induced ovarian dysfunction. The relationship between the Fas/Fas ligand (FasL) system and CPT-11-induced ovarian dysfunction was also analyzed.

Materials and methods

TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining of ovaries from CPT-11-injected mice. All animal experiments were carried out using 8-week-old female MCH mice (Nihon Clea Co. Ltd., Osaka, Japan). Various doses (20, 100 or 500 μg) of CPT-11 (Yakult Co. Ltd., Tokyo, Japan) in saline were intraperitoneally injected once into the mice at 48 h after an intraperitoneal injection of 5 U of pregnant mare serum gonadotropin (PMSG; Teikoku Zouki Co., Tokyo, Japan) to create similar estrogen conditions among the mice. Mice injected intraperitoneally with saline rather than CPT-11 were used as controls. Under ether anesthesia, the abdomen was opened and the ovaries were taken out at 24, 48, 72 or 96 h after CPT-11 administration, respectively. Next, the ovaries were laparotomically removed, fixed with 0.2% paraformaldehyde on ice overnight and embedded in paraffin. Sections of the embedded tissues were deparaffinized, treated with proteinase K (20 μg/ml; Roche Diagnostic, Mannheim, Germany) for 10 min at room temperature (RT) and washed four times with distilled water for 5 min each at RT. Next, the endogenous peroxidase was blocked by incubating the sections with 3% hydrogen peroxide in phosphate-buffered saline (PBS) for 5 min at RT. Finally, the sections were subjected to TUNEL staining using an ApopTag peroxidase *in situ* apoptosis detection kit (Serologicals Corp., Billerica, MA, USA). Murine ovarian follicles were classified into three types, namely small, medium, and large follicles according to the Pedersen and Peters classification (23). Briefly, large follicles were defined as follicles with at least 4 granulosa cell layers, medium follicles as those with 2-3 granulosa cell layers and small follicles as those with a single granulosa cell monolayer. TUNEL-positive follicles were determined as follicles containing at least 10 TUNEL-positive cells within an ovarian section. All experiments were performed at least three times to verify the results. Graphical data are shown as the mean ± SD and data comparisons were analyzed by t-tests (n=6) and/or one-way ANOVA. Values of $p<0.05$ were determined to indicate a statistically significant difference.

Immunohistochemical studies of ovaries from CPT-11-injected mice. In order to investigate the molecular mechanisms of the apoptosis in ovaries of CPT-11-injected mice, the ovarian tissues used in the experiment described above were immunohistochemically analyzed to detect the intraovarian localizations of apoptosis-related molecules. Deparaffinized ovarian sections were treated with 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity and

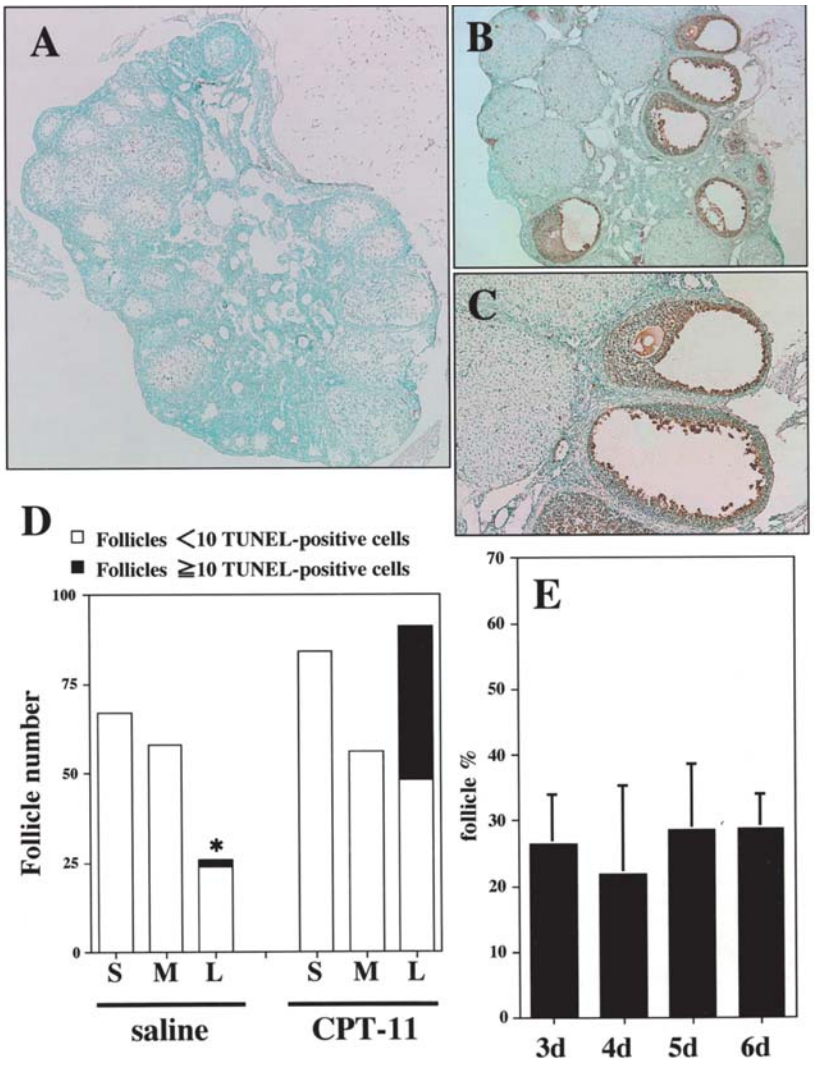

Figure 1. CPT-11-induced specific granulosa cell death in large ovarian follicles *in vivo*. CPT-11 was intraperitoneally administered into 8-week-old female mice. (A) A TUNEL-stained section of ovarian tissue from a mouse injected with PMSG without CPT-11. (B) A TUNEL-stained section of ovarian tissue from a mouse injected with PMSG followed by 100 μg of CPT-11. (C) Higher magnification of part of the image in B. (D) Total numbers of small (S), medium (M) and large (L) follicles and TUNEL-positive follicles in ovarian tissue sections from 6 mice treated with PMSG followed by 100 μg of CPT-11. The asterisk indicates the presence of TUNEL-positive large follicles in mice treated with PMSG without CPT-11. CPT-11 was administered into mice at 48 h after a PMSG injection, and the mouse ovaries were studied immunohistochemically at 48 h after the CPT-11 injection. Follicles with at least 10 strongly TUNEL-positive cells were evaluated as TUNEL-positive follicles. Open bars, numbers of TUNEL-negative follicles containing <10 TUNEL-positive cells per follicle section; black bars, numbers of TUNEL-positive follicles. (E) All ovarian follicles were counted at 3, 4, 5 and 6 days after injection of 5 U of PMSG. The ratio (%) of large ovarian follicles was evaluated as the number of large follicles per total number of ovarian follicles in one ovarian section. Six ovaries were counted. Bars represent the mean ± SD. There were no statistical differences in the large ovarian follicular ratios in the PMSG-treated ovaries.

subjected to microwave treatment at 100˚C for 15 min in citrate buffer (1.8 mM $C_6H_8O_7$, 8.2 mM $Na_3C_6H_5O_7$, pH 6.0) for antigen retrieval. The microwaved sections were then incubated with one of three primary polyclonal rabbit antibodies at 4˚C overnight, washed four times with PBS for 5 min each at RT and incubated with a peroxidase-labeled anti-rabbit secondary antibody (Simple Stain Max-PO Kit; Nichirei Co., Tokyo, Japan) for 30 min at RT. Finally, the sections were washed four times with PBS for 5 min each at RT, incubated with diaminobenzine (DAB) (Dako, Carpinteria, CA, USA) solution comprising 30 mg DAB in 150 ml of 0.05 M Tris-HCl (pH 7.6) containing 4% hydrogen peroxide and counterstained with methyl green (Nacalai Tesque Co. Ltd., Kyoto, Japan) at RT. The three primary antibodies used were anti-mouse cleaved caspase 3 polyclonal rabbit IgG (1:200; Cell Signaling Technology, Beverly, MA, USA), anti-mouse Fas polyclonal rabbit IgG (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-mouse FasL polyclonal rabbit IgG (1:1; Spring Bioscience, Fremont, CA, USA).

In vitro organ culture of normal ovarian tissue fragments. Normal murine ovaries were removed from 8-week-old MCH mice at 48 h after an intraperitoneal injection of 5 U of PMSG, cut in half longitudinally and *in vitro*-cultured in 384-well culture plates (Genetix Ltd., New Milton, Hampshire, UK). The culture medium used was OPTI-MEM (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 5% fetal calf serum (Equitech Bio Inc., Ingram, TX, USA), 100 U/ml of penicillin (Gibco-BRL) and 100 μg/ml of streptomycin (Gibco-BRL). Various doses of recombinant soluble mouse Fas ligand (rec-sm-FasL; R&D Systems, Minneapolis, MN,

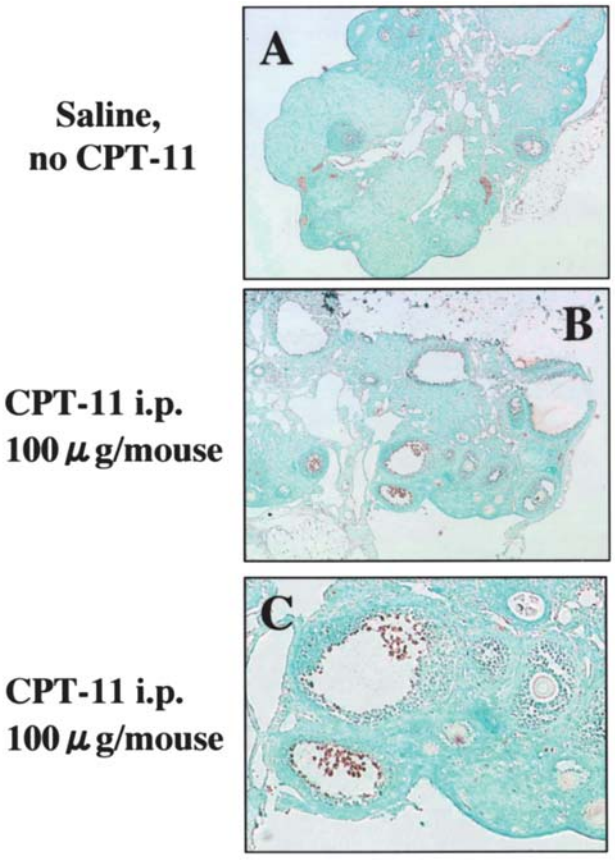

Figure 2. Expression of cleaved caspase 3 in ovarian tissues. CPT-11 or saline was injected intraperitoneally into mice at 48 h after injection of 5 U of PMSG, and the ovaries were resected and analyzed immunohistochemically at 48 h after the CPT-11 or saline injection. (A) Cleaved caspase 3 expression in an ovarian section from a mouse treated with PMSG followed by saline. (B) Cleaved caspase 3 expression in an ovarian section from a mouse treated with PMSG followed by CPT-11. (C) Higher magnification of part of the image in B. Strong immunoreactivity for cleaved caspase 3 is specifically present in large follicular granulosa cells.

USA) were added to the half ovaries and incubated for 6 h at 37˚C under a 5% CO_2/5% O_2/90% N_2 atmosphere. The final concentrations of rec-sm-FasL were 0, 2 and 50 μg/ml. The ovarian fragments were then fixed with 0.2% paraformaldehyde in PBS overnight on ice and embedded in paraffin. The paraffin-embedded tissues were sectioned and stained by the TUNEL assay as described above. TUNEL-positive cells were counted among luteal and granulosa cells. All experiments were performed twice to verify the results. Graphical data are shown as the mean ± SD, and data comparisons were analyzed by the t-test (n=6) and/or one-way ANOVA. Values of $p<0.05$ were determined to indicate a statistically significant difference.

Results

TUNEL-positive cells in ovaries from CPT-11-injected mice. In order to confirm the hypothesis that CPT-11 combination chemotherapy frequently causes ovarian follicular damage in cancer patients, mouse experiments were performed as follows. Clinically therapeutic doses of CPT-11 (2-10 μg/g body weight) were intraperitoneally injected into mice once at 48 h after a PMSG injection, and histopathological examinations of the murine ovaries were carried out after 2-4 days. As a result, many TUNEL-positive cells were detected among large follicular granulosa cells in ovaries from CPT-11-injected mice. On the other hand, there were almost no TUNEL-positive cells in small and medium follicles (Fig. 1A-C). When follicles containing at least 10 TUNEL-positive granulosa cells in an ovarian section were determined as TUNEL-positive follicles, TUNEL-positive follicles were observed in large follicles in CPT-11-injected mice, but not in small and medium follicles (Fig. 1D).

Since TUNEL-positive follicles were only observed in large follicles in CPT-11-injected mice, the effects of PMSG pretreatment on the ratios of large follicles to total ovarian follicles were examined. As a result, the ratios of large follicles showed no significant change from days 3-6 after a PMSG injection and the large follicle ratios remained at ~20-30% (Fig. 1E). These results indicate that CPT-11 injection specifically induces TUNEL-positive follicles among large ovarian follicles without any effect of PMSG pretreatment. Moreover, the results demonstrate that PMSG pretreatment is useful for allowing stable experiments on CPT-11-injected mice to be carried out over several days after a PMSG injection.

Cleaved caspase 3 expression in ovaries from CPT-11-injected mice. To examine whether the CPT-11-induced TUNEL-positive granulosa cells in large ovarian follicles were necrotic or apoptotic cells, immunohistochemical analyses of cleaved caspase 3 expression in ovaries from CPT-11-injected mice were performed. In saline-injected control mice without CPT-11, cleaved caspase 3 expression was detected in a few granulosa cells, but not in corpus luteal or thecal cells (Fig. 2A). In all CPT-11-injected mice, however, strong cleaved caspase 3 expression was found in the granulosa cells of large ovarian follicles. In particular, cells with strong immunoreactivity against cleaved caspase 3 were detected among the granulosa cells of large follicles at 48 h after a CPT-11 injection (Fig. 2B and C). Since the localization of the cleaved caspase 3-expressing cells almost coincided with that of the TUNEL-positive cells, most of the TUNEL-positive cells among the granulosa cells of CPT-11-injected mice were considered to be apoptotic cells, rather than necrotic cells. Therefore, follicles containing at least 10 TUNEL-positive cells within an ovarian section were designated as apoptotic follicles.

Dose-dependent effects of CPT-11 on follicular apoptosis. Next, the dose-dependent effects of CPT-11 on the induction of apoptotic follicles were examined. A few apoptotic follicles were observed in saline-injected mouse ovaries (Fig. 3A-C), which may indicate an atresic process of normal ovarian follicles after PMSG stimulation. Within 24 h after a CPT-11 injection, apoptotic follicles were significantly increased (Fig. 3A). The apoptotic follicle ratio reached 20% after 72 h in the 20 μg CPT-11/mouse group (Fig. 3C), while that in the 100 μg CPT-11/mouse group reached 20% after 48 h (Fig. 3B) and that in the 500 μg CPT-11/mouse group reached >10% after 24 h (Fig. 3A). These results indicate that CPT-11 dose-dependently stimulated the apoptotic process in CPT-11-

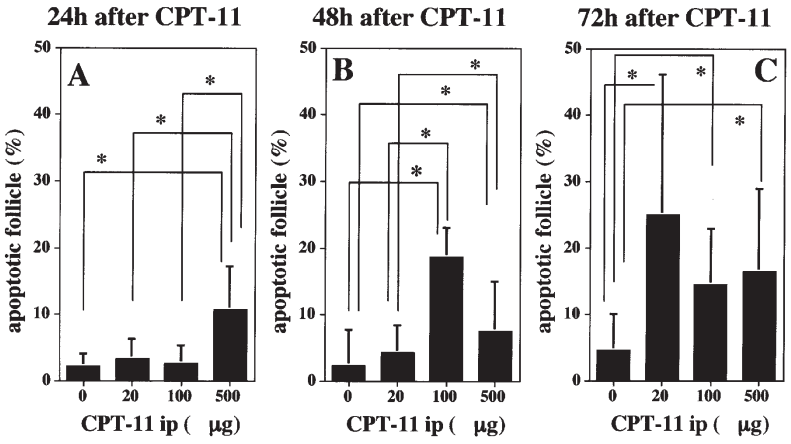

Figure 3. Time- and dose-dependent effects of CPT-11 on follicular apoptosis in murine ovaries. The total numbers of follicles were counted, and the apoptotic follicle ratios (%) were calculated. CPT-11 was intraperitoneally injected into mice (0, 20, 100 or 500 μg/mouse) at 48 h after a PMSG injection. Six ovaries were resected and subjected to TUNEL staining at 24 (A), 48 (B) or 72 h (C) after the CPT-11 injection, respectively. $^{*}p<0.05$.

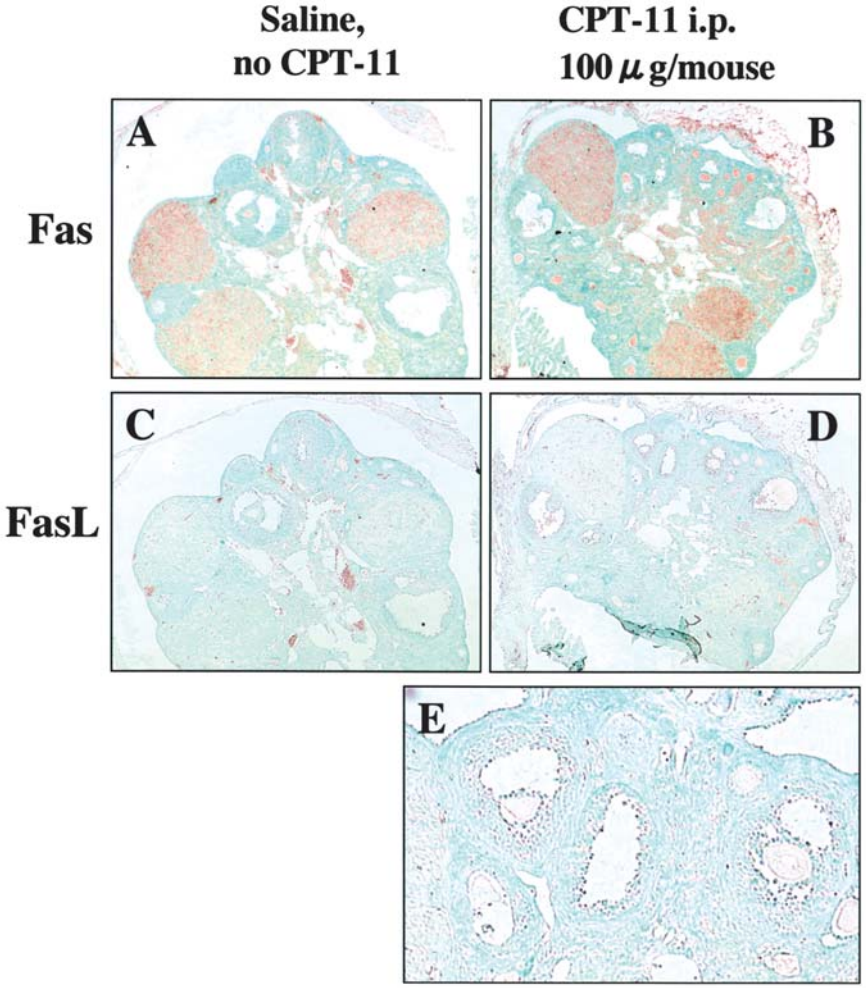

Figure 4. Expression of Fas and FasL in ovaries of CPT-11-treated mice. (A and B) Immunohistochemical analyses of Fas antigen expression in ovaries. (C-E) Immunohistochemical analyses of FasL expression in ovaries. (A and C) Sections of ovarian tissue from a mouse treated with saline at 48 h after a PMSG injection. (B, D and E) Sections of ovarian tissues from a mouse treated with CPT-11 (100 μg) at 48 h after a PMSG injection. E is a magnified view of D.

sensitive ovarian follicles. Although CPT-11 dose-dependently stimulated the apoptotic process in the follicles, the final apoptotic follicle ratios in both the 20 and 500 μg CPT-11/mouse groups were ~20% of the total follicles (Fig. 3C). The dose of 20 μg CPT-11/mouse corresponds to the lowest clinical dose of CPT-11 applied to humans. Even higher doses of CPT-11 did not increase the apoptotic follicle ratios within several days, while only large follicles showed

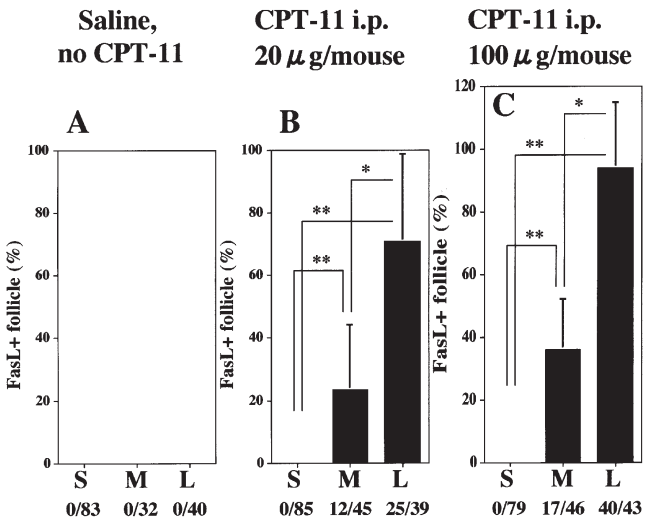

Figure 5. Dose-dependent FasL expression in ovaries from CPT-11-treated mice. FasL-expressing cells were counted immunohistochemically in sections of ovarian tissue resected from CPT-11-injected mice at 48 h after the CPT-11 injection. Follicles with at least 10 strongly FasL-positive cells per ovarian section were designated as FasL-positive follicles. (A) Numbers of FasL-positive follicles in ovarian sections from saline-injected mice. (B) Numbers of FasL-positive follicles in ovarian tissues from mice treated with 20 μg of CPT-11. (C) Numbers of FasL-positive follicles in ovarian tissues from mice treated with 100 μg of CPT-11. The numbers under the columns indicate the numbers of total FasL-positive follicles per total numbers of small (S), medium (M) or large (L) follicles in six ovarian sections. $^{*}p<0.05$, $^{**}p<0.01$.

apoptotic changes. Since large follicles represent ~20-30% of the total follicles, a bolus CPT-11 injection above a certain dose can cause 70-90% of the large follicles to become apoptotic independent of the CPT-11 dose.

Expression of Fas antigen in CPT-11-injected mice. Since several studies have shown that some of the mechanisms for ovarian follicular apoptosis can be induced by Fas-FasL interaction, one of the death signals (24-28), an immunohistochemical study was performed to examine whether CPT-11-induced ovarian follicular apoptosis may be induced by CPT-11-stimulated increases in Fas antigen expression. Even in saline-injected mice without CPT-11, expression of Fas antigen was detected in various types of ovarian cells, including granulosa cells (Fig. 4A and B). In particular, Fas expression was much higher in luteal cells than in granulosa cells. Fas expression was observed in all murine ovaries regardless of CPT-11 injection. The apparent localization of Fas-positive cells did not coincide with that of TUNEL-positive cells. Moreover, in the 100 μg CPT-11/mouse group, Fas expression was not enhanced at 24 h after a CPT-11 injection when the increase in TUNEL-positive cells was detected. These results indicate that the CPT-11-induced granulosa cell apoptosis in large follicles is not caused by enhanced expression of Fas antigen in granulosa cells. Strong immunoreactivity for Fas antigen was observed in all luteal cells, whereas granulosa cells showed moderate immunoreactivity for Fas antigen in most large follicles, and a few large follicles showed very weak immunoreactivity for Fas antigen. These two types of large follicles with apparently different Fas expression levels did not show any histomorphological differences (data not shown).

FasL expression in ovaries from CPT-11-injected mice. An immunohistochemical study was carried out to examine whether the CPT-11-induced follicular apoptosis was caused by induction of FasL expression. As shown in Fig. 4C, saline-injected control mice did not show FasL expression in any ovarian cell types, including granulosa cells. In CPT-11-injected mice, FasL expression was detected in granulosa cells in medium and large follicles, while no FasL expression was found in small follicles (Fig. 4D and E). Follicles or corpora lutea that contained at least 10 FasL-positive cells in one ovarian section were evaluated as FasL-positive follicles or FasL-positive corpora lutea, respectively. In mice injected with 20 μg of CPT-11, ~20% of medium follicles and ~70% of large follicles were classified as FasL-positive follicles (Fig. 5B), while ~40% of medium follicles and ~90% of large follicles were classified as FasL-positive follicles in mice injected with 100 μg of CPT-11. Interestingly, no FasL-positive corpora lutea were found in CPT-11-injected mice (Fig. 4D).

Effects of FasL treatment on organ culture of murine ovaries. The distribution of TUNEL-positive cells in large follicles in CPT-11-injected mice almost coincided with the distribution of FasL-positive cells in large follicles. Therefore, organ culture experiments of murine ovaries were carried out to examine whether FasL could directly induce ovarian follicular apoptosis. In organ cultures of mouse ovaries, similar rates of TUNEL-positive cells were observed in ovarian interstitial cells between the control group (no rec-sm-FasL) and rec-sm-FasL-treated groups (Fig. 6A and B). In untreated ovaries (control group) and ovaries treated with 2 μg/ml of rec-sm-FasL, ~40% of the corpora lutea were classified as TUNEL-positive corpora lutea (containing ≥10 TUNEL-positive cells) and ~25% of large follicles were classified as TUNEL-positive follicles (containing ≥10 TUNEL-positive cells). However, in ovaries treated with 50 μg/ml of rec-sm-FasL, ~80% of the corpora lutea and ~60% of large follicles were classified as TUNEL-positive, indicating that mice treated with 50 μg/ml of rec-sm-FasL exhibited significantly increased numbers of TUNEL-positive cells in the corpora lutea and large follicles compared to mice treated with 2 μg/ml of rec-sm-FasL and untreated mice ($p<0.05$) (Fig. 6C-E). These results demonstrate that direct treatment of ovaries with a high dose of rec-sm-FasL can increase apoptosis in corpus luteal cells and large follicular granulosa cells that constitutively express moderate to high levels of Fas antigen.

Discussion

We originally noted that patients with gynecologic cancers treated with CPT-11 combination chemotherapy frequently complained of ovarian failure and menopausal malaises. Our clinical endocrinological studies of cancer patients during CPT-11 combination chemotherapy led to the hypothesis that CPT-11 may cause strong granulosa cell damage in ovarian follicles (22). In the present study, we directly proved that CPT-11 induces strong apoptotic injury to granulosa cells in large ovarian follicles using mouse experiments. Intraperitoneal injections of clinical doses of CPT-11 into the mice strictly induced differentiation stage-specific and cell lineage-specific apoptosis in granulosa cells of large ovarian

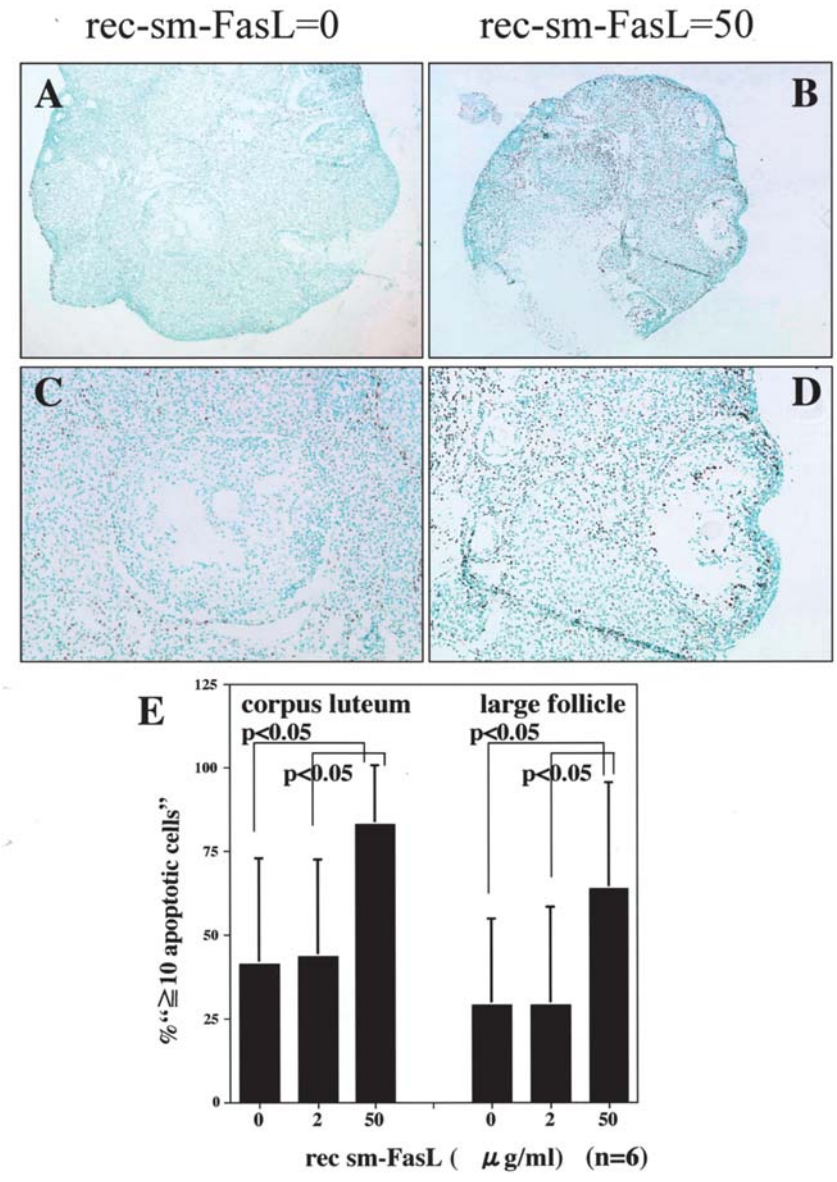

Figure 6. Effects of FasL treatment on normal ovarian tissue fragments. Mouse ovaries were resected at 48 h after a PMSG injection, organ-cultured with rec-sm-FasL at final concentrations of 0, 20 or 50 μg/ml, respectively, and then subjected to TUNEL staining. (A and C) Section of TUNEL-stained ovarian tissue incubated without rec-sm-FasL. (B and D) Section of TUNEL-stained ovarian tissue incubated with 50 μg/ml of rec-sm-FasL. C is a magnified version of A, and D is a magnified version of B. (E) The numbers of TUNEL-positive large ovarian follicles and corpora lutea were counted in 6 murine ovaries after incubation with rec-sm-FasL at 0, 20 or 50 μg/ml, respectively. Follicles and corpora lutea containing at least 10 TUNEL-positive cells per tissue section were determined as TUNEL-positive large ovarian follicles or corpora lutea. The ratio (%) of TUNEL-positive follicles or corpora lutea was calculated as the numbers of TUNEL-positive large follicles or corpora lutea per total numbers of ovarian follicles or corpora lutea in one ovarian section, respectively. Six ovaries were counted. Bars represent the mean ± SD. Rec-sm-FasL treatment significantly increased the numbers of TUNEL-positive follicles and corpora lutea.

follicles. Furthermore, the CPT-11-induced granulosa cell apoptosis was accelerated in a dose-dependent manner. Immunohistochemical studies revealed that Fas antigen, a strong cell surface death receptor, was constitutively expressed in almost all types of ovarian cells in the mice, while FasL, the Fas antigen-specific death inducer, was not expressed in normal ovarian tissues. In CPT-11-injected mouse ovaries, FasL expression was only induced in granulosa cells of large follicles, which were thought to be cell lineage-specific and differentiation-specific phenomena in these granulosa cells. However, Fas antigen expression in ovarian tissues was not significantly enhanced by CPT-11 stimulation. Organ culture experiments with murine ovaries revealed that rec-sm-FasL induced apoptosis in corpora lutea and medium and large follicles where Fas antigen was moderately or highly expressed. These results indicate that Fas antigen is widely and constitutively expressed on normal ovarian cells and acts as a receptor for FasL, a strong apoptosis-inducible molecule. Taking these results together, one mechanism for the ovarian failure frequently complicated in cancer patients treated with CPT-11 combination chemotherapy can be explained as follows. CPT-11 induces specific FasL expression only in granulosa cells of medium and large follicles where Fas antigen is constitutively highly expressed. The induced FasL acts on Fas antigen on granulosa cells in paracrine or autocrine mechanisms, and induces apoptosis in some

granulosa cells where FasL expression is highly induced. The CPT-11-induced granulosa cell apoptosis decreases the serum estradiol level and secondarily increases the serum FSH and LH levels, as shown for the ovarian failure in cancer patients treated with CPT-11 combination chemotherapy. This is the first study to confirm that Fas/FasL-induced apoptosis is one of the main molecular mechanisms involved in anticancer drug-induced ovarian failure. Therefore, it may be possible to develop molecular targeting therapies to protect young cancer patients against ovarian damage caused by anticancer chemotherapy.

Fenwick and Hurst examined the uptake of bromodeoxyuridine (BrdU), a cell proliferation marker, and caspase 3 expression, an apoptosis executor, in mouse ovaries. In their experiments, BrdU uptake was detected in granulosa cells of large follicles, but was hardly detected in small follicles (29). Caspase 3 expression was observed in oocytes and granulosa cells in large follicles that were thought to be undergoing follicular atresia, but not in small follicles (29). These results suggest that most small follicles are in the resting stage when cell proliferation is suspended and are much less sensitive to apoptotic stimuli. The authors also suggested that granulosa cells in large follicles exhibit high cell proliferation activity and high sensitivity to some apoptotic inducers. CPT-11, a strong topoisomerase I inhibitor that induces cancer cell death by acting specifically at the DNA synthesis stage (7), is considered to act on large follicular granulosa cells with especially high cell proliferation activity and cause selective granulosa cell apoptosis and ovarian failure in cancer patients.

Several apoptotic mechanisms for ovarian follicles have been reported, including a pathway via death receptors on the cell surface such as Fas antigen (24-28) and tumor necrosis factor receptor (30-32), a pathway via mitochondrial activation regulated by BCL-2, BAX, and p53 (33-36) and a pathway accompanied by endoplasmic reticulum breakdown (37,38). In any apoptotic pathway, the final executor of apoptosis is activated cleaved caspase 3. Caspase 3 expression in granulosa cells is regulated by gonadotropin and upregulated during granulosa cell apoptosis and follicular atresia, accompanied by increased expression levels of activated caspase 8 and activated caspase 9 (39). Expression of activated caspase 3 was found to be increased in granulosa cells in large follicles in CPT-11-injected mice where TUNEL-positive cells were increased, while activated caspase 3 was not expressed in corpus luteal cells and thecal cells where Fas antigen was highly expressed. These results indicate that CPT-11 specifically activates caspase 3 in granulosa cells of large follicles.

Fas antigen, a type I transmembrane protein, is a member of the TNF receptor family and acts as a trimeric cell surface death receptor. Binding of Fas with FasL rapidly triggers apoptosis of cells via the intracellular part of the receptor, which contains a death domain (40,41). FasL, a death factor usually secreted by activated T cells, causes apoptosis of virus-infected cells and cancer cells among others, and plays important roles in the maintenance of immunotolerance and bio-homeostasis (42,43). Regarding the relationship between the Fas/FasL system and ovarian follicular apoptosis, several studies have shown that the Fas/FasL system is involved in the induction of follicular apoptosis and follicular atresia. The initial interaction of Fas on oocytes and FasL on granulosa cells has been reported to induce follicular atresia (25). Furthermore, the interaction of Fas and FasL on granulosa cells was reported to induce follicular apoptosis (26), and the initial interaction of Fas and FasL on oocytes was reported to affect granulosa cell viability and induce follicular apoptosis (27). Our present immunohistochemical analyses of murine ovarian tissues revealed that Fas antigen is constitutively and widely expressed on various types of ovarian cells in addition to granulosa cells. Our organ culture experiments on normal murine ovaries treated with rec-sm-FasL further demonstrated that FasL is able to induce apoptosis in Fas-expressing ovarian cells. These results indicate that Fas antigen expressed widely on normal ovarian cells acts as a functional death receptor and that the localization of selective cell death in ovaries can be caused by FasL expression. FasL expression was especially induced in medium and large follicular granulosa cells in the ovaries following injection of CPT-11. It is highly possible, therefore, that the induced FasL binds to the Fas antigen constitutively highly expressed on granulosa cells in large follicles and triggers follicular apoptosis.

Although FasL-positive follicles (follicles containing at least 10 FasL-positive granulosa cells per ovarian section) were detected in ~90% of large follicles and ~40% of middle follicles in CPT-11-injected mouse ovaries, apoptotic follicles (follicles containing at least 10 TUNEL-positive granulosa cells per ovarian section) were only detected in large follicles. Most large ovarian follicles in mice injected once with CPT-11 were apoptotic, and only a few large follicles were not apoptotic. These results may indicate that follicular apoptosis cannot be induced by FasL expression alone. Since incubation of ovarian fragments with 50 μg/ml of rec-sm-FasL, but not 2 μg/ml of rec-sm-FasL, in organ culture experiments induced significant increases in TUNEL-positive cells only among corpus luteal cells and large follicular granulosa cells, which constitutively express high levels of Fas antigen, apoptosis can be induced in large follicular granulosa cells in a dose-dependent manner by locally produced FasL. The immunohistochemical finding that some large follicles expressed apparently lower levels of Fas antigen than most large follicles may be one of the reasons why a single injection of CPT-11 into mice could not induce apoptotic follicles among all large follicles. As shown in Fig. 3C, single injections of CPT-11 above a certain dose induced the same numbers of apoptotic follicles in murine ovaries irrespective of the actual CPT-11 dose. These findings suggest that the frequent repetitive administrations of CPT-11 in clinical cancer chemotherapy can increase apoptotic follicles in the ovaries of the patients and delay their recovery from ovarian dysfunction after cessation of the CPT-11 combination chemotherapy. If a high dose of CPT-11 is administered to a cancer patient aged ~50 years, CPT-11-induced follicular apoptosis can rapidly decrease the serum estradiol level and rapidly increase the serum gonadotropin level in the patient. In fact, premenopausal cancer patients aged ~50 years often complained of estrogen-deprivation menopausal malaises (22). If more than a certain dose of CPT-11 is repeatedly administered to young female cancer patients, almost all CPT-11-sensitive granulosa cells will enter apoptosis and almost all functional ovarian follicles will be lost, finally

leading to severe premature ovarian failure. Considering our previous clinical observations (22) and present experimental results, young female cancer patients who hope for a future pregnancy need to be informed that CPT-11 combination chemotherapy can frequently induce ovarian failure.

In summary, CPT-11 can induce selective and specific apoptosis in granulosa cells in large ovarian follicles. One of the main mechanisms of this CPT-11-induced granulosa cell apoptosis is apoptotic signaling initiated by interaction of Fas antigen in granulosa cells and CPT-11-induced FasL on granulosa cells. The fact that the Fas/FasL system plays a main role in anticancer drug-induced specific granulosa cell apoptosis suggests the possibility of developing molecular targeting therapies involving regulation of Fas/ FasL signaling that may be applied to the prevention or treatment of irreversible ovarian damage induced by certain anticancer drug chemotherapies.

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