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Folliculogenesis Is Impaired and Granulosa Cell Apoptosis Is Increased in Leptin-Deficient Mice[1]

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ABSTRACT

Leptin purportedly plays an important role in pubertal development in a number of mammalian species. Adult leptin-deficient (ob/ob) female mice are infertile, but the mechanisms responsible for the reproductive failure have not been fully elucidated. The major objective of the current study was to assess the effects of a leptin deficiency on ovarian folliculogenesis and apoptosis. Beginning at 4 wk of age, control (n = 8) and ob/ob (n = 7) mice were weighed and examined daily for vaginal opening. After 3 wk the mice were killed, and the reproductive organs were weighed. Ovaries were paraffin-embedded for hematoxylin and eosin histology, TUNEL assay, and immunohistochemistry for Fas, Fas ligand (FasL), and proliferating cell nuclear antigen (PCNA). Vaginal opening was delayed, uteri were smaller, and the number of primordial follicles and total number of ovarian follicles were subnormal in ob/ob animals. Leptin-deficient animals also had a higher number of atretic follicles than controls. Granulosa cells (predominantly in preantral and early antral follicles) of ob/ob mice exhibited increased apoptotic activity as documented by TUNEL assay and elevated expression of the apoptotic markers Fas and FasL, compared with that in control animals. Ovarian expression of PCNA, a marker of DNA replication, repair, or both, did not differ between ob/ob and control mice. The data suggest that a leptin deficiency in mice is associated with impaired folliculogenesis, which results in increased follicular atresia. This impairment may be one of the causative components of infertility in leptin-deficient animals.

apoptosis, follicle, granulosa cells, leptin, ovary

INTRODUCTION

Leptin, a 167 amino acid protein produced predominantly by white adipose tissue, is the product of the Ob gene. Female mice with two defective copies of the Ob gene are morbidly obese, infertile, and have a subnormal number of ovarian follicles [1]. When these mice are treated with leptin they lose weight, fertility is restored, and the total number of follicles is normalized. It is not known whether this effect of leptin on folliculogenesis is mediated indirectly via alterations in gonadotropin secretion or directly at the level of the ovary. The leptin receptor is expressed in several ovarian cell types, including granulosa and theca cells, suggesting that leptin may have a direct effect on follicular development and differentiation [2]. Preliminary unpublished observations from our laboratory suggested that there were increased numbers of pyknotic nuclei in the granulosa cell layer of follicles in leptin-deficient mice, perhaps suggesting increased programmed cell death. Thus, increased granulosa cell apoptotic activity may contribute to the subnormal number of ovarian follicles found in leptin-deficient animals, although to the best of our knowledge, until the current study, this had not been previously investigated.

Recent studies have implicated the Fas/Fas ligand (FasL) system as one of the pathways responsible for programmed granulosa cell death, which is an underlying cause of follicular atresia [3, 4]. The Fas antigen is a transmembrane glycoprotein that belongs to the tumor necrosis factor (TNF) and nerve growth factor receptor family [5]. The FasL is a type II transmembrane protein that belongs to the TNF protein family. The binding of FasL to Fas on the cell surface initiates apoptosis in Fas-bearing cells [5–7]. A number of previous reports have implicated Fas and FasL in granulosa cell apoptosis during follicular atresia [3, 4, 8].

The primary objective of this study was to investigate the effect of a leptin deficiency in mice on granulosa cell apoptosis and follicular atresia. Ovarian apoptotic activity was detected by TUNEL assay, and immunocytochemistry was used to determine levels of ovarian expression of the Fas and FasL. Another objective was to assess the effect of a leptin deficiency on cellular proliferation and DNA repair within the murine ovary by immunohistochemical detection of proliferating cell nuclear antigen (PCNA). PCNA, also known as cyclin, is a 36-kDa auxiliary protein for DNA polymerase that functions during DNA replication [9]. This protein has also been located in the sites of DNA repair [10, 11] and has been shown to be necessary for DNA excision repair [12]. The expression of PCNA was assessed in the present study as an indication of the effects of a leptin deficiency on cellular proliferation, repair, or both, within specific cellular components of the developing follicle.

MATERIALS AND METHODS

Animals and Tissue Preparation

All experiments were conducted according to the principles and procedures of the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals.* Four-week-old female C57BL/6J (n = 8) and C57BL/6J-lep(ob) mice (n = 7) were maintained under controlled, standard laboratory conditions for 3 wk. Mice were monitored daily for day of vaginal opening. At the end of the 3-wk period the animals were anesthetized with halothane vapors and decapitated. The ovaries and uteri were immediately removed and weighed. The ovaries were fixed in 10% formalin and processed for paraffin embedding, sectioned (6–8 μm), and mounted on aminoalkylsilane-coated slides (Sigma, St. Louis, MO) for subsequent hematoxylin-eosin, TUNEL, and immunohistochemical analyses.

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In Situ DNA 3′ End Labeling of Apoptotic Cells

Labeling of DNA fragmentation was performed using the ApopTag apoptosis detection kit (Serologicals Corp., Norcross, GA). The in situ terminal deoxynucleotidyl transferase (TdT)-mediated deoxy-UTP-digoxigenin nick end labeling (TUNEL) method was used to localize apoptotic cells in paraffin-embedded, whole ovarian sections. Sections were deparaffinized, hydrated, and pretreated with 20 μg/ml proteinase K (25°C, 15 min), washed with PBS, and soaked with equilibration buffer followed immediately by incubation with TdT reaction mixture in a humidified chamber (37°C, 60 min). The sections were then washed in stop buffer solution (25°C, 10 min), followed by a wash in PBS, and incubated with antidigoxigenin antibody conjugated to a rhodamine reporter molecule in a humidified chamber (25°C, 30 min). Sections were rinsed in PBS and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). For negative control staining, TdT reaction mixture was omitted.

Immunohistochemistry

For Fas, FasL, and PCNA immunohistochemistry, paraffin-embedded whole ovarian sections were deparaffinized, hydrated, and incubated in 0.3% NH_4Cl for 10 min. The sections were washed with PBS and incubated in citric acid buffer (pH = 6) in a steamer for 20 min to facilitate the retrieval of antigen. The sections were washed with labeling buffer (0.2% Triton X-100 in PBS) and blocked with 2% BSA, and incubated for 20 min at 37°C. Sections were then washed in labeling buffer and incubated overnight at 4°C with 0.5 μg/ml rabbit polyclonal immunoglobulin G (IgG) for Fas, FasL, or PCNA primary antibody in labeling buffer (Santa Cruz Biotechnology, Santa Cruz, CA). Sections were rinsed with labeling buffer and incubated with a biotin-SP-conjugated secondary antibody at 25°C for 80 min (0.3 μg/ml anti-rabbit IgG; Jackson ImmunoResearch Laboratories, West Grove, PA). Immediately after incubation with the secondary antibody, sections were rinsed in labeling buffer, streptavidin-conjugated Alexa Fluor 488 was applied, and nuclei were counterstained with 0.5 μg/ml 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) at 25°C for 10 min (Molecular Probes, Eugene, OR), then rinsed in labeling buffer and mounted with Vectashield mounting medium (Vector Laboratories) and examined using an Olympus BX41 microscope equipped with an Optronics MagnaFire digital camera and Prior Proscan motorized-driven stage (Olympus, Melville, NY). The specificity of the antibodies was verified by incubating ovarian sections without primary antibodies.

For digital image capturing, the exposure time was adjusted using sections incubated without the primary antibody to minimize any auto or nonspecific fluorescence recording without compromising the actual signal. The signal obtained after such a background correction was considered an antigen-specific signal. For each image, specific antibody staining was merged with nuclear staining (blue) using Soft Imaging System Software (Soft Imaging System Corp., Lakewood, CO) that caused virtually no pixel shifting during image merger, and resulted in shades of red, green, and blue.

Cytomorphometry

Three slides were selected (at least 8–10 sections apart) from serial sections of an ovary from each ob/ob and control mouse. The slides were stained with hematoxylin and eosin, and the number of follicles at each stage of folliculogenesis (primordial through antral follicle stage) was counted using a light microscope. Follicles were classified as primordial if they consisted of an oocyte surrounded by a single layer of cells and had no zona pellucida present. Follicles were classified as preantral follicles if they were composed of an oocyte surrounded by one or more layers of granulosa cells and a developing or developed zona pellucida, but without a visible antrum. Follicles were identified as an early antral or antral follicle when they were composed of an oocyte surrounded by multiple layers of granulosa cells with a developed zona pellucida and two layers of theca cells, and an antrum was present.

The number of apoptotic granulosa cells (TUNEL-positive) in each stage of follicle development and number of atretic follicles were quantified in each stained section using imaging technology (ImagePro Plus Software, Media Cybernetics, Silver Spring, MD). Follicles were defined as atretic if they had five or more TUNEL-positive granulosa cells. Cells were considered positive if they stained bright yellow-orange with the TUNEL assay (Fig. 1). Few positive cells were noted in the follicles of control ovaries.

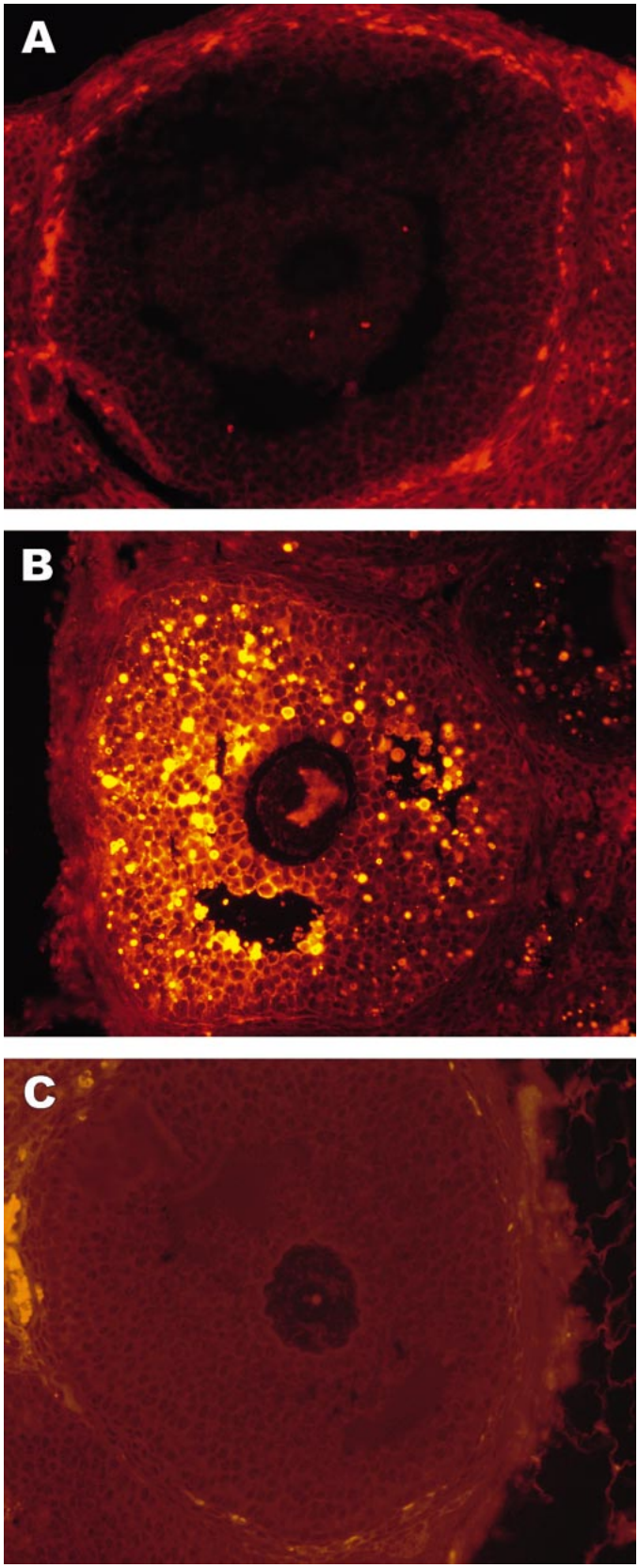

FIG. 1. Representative photomicrographs of TUNEL-stained (bright yellow-orange cells) early antral follicles from control (**A**) and ob/ob (**B**) animals showing that apoptosis was more pronounced in the granulosa cells of the ovary of the ob/ob animal. **C**) A negative control for TUNEL staining (TdT reaction mixture omitted). Magnification ×400.

TABLE 1. Effect of a leptin deficiency on body and organ weights, day of vaginal opening, and serum leptin concentration.*

Treatment	Leptin (ng/ml)	Body weight (g)	Ovary (mg)	Uterus (mg)	VO (days)
Control	0.6 ± 0.2	16.9 ± 0.4	8.1 ± 0.2	48.0 ± 6.0	36.6 ± 1.5
Ob/ob	<0.1 ± 0[a]	36.4 ± 0.9[a]	6.3 ± 0.2	18.0 ± 6.0[a]	46.4 ± 0.6[a]

* Values represent means ± SEM. VO, vaginal opening.
[a] Significantly different from the control value, $P < 0.05$.

Hormone Assay

Serum samples were assayed for leptin using a commercially available ELISA kit (Assay Designs, Ann Arbor, MI). All samples were run in duplicate in the same assay. The intra-assay coefficient of variation for the leptin assay was 2.9%. The minimum detection limit for the leptin assay was 5 pg/ml.

Statistical Analysis

One-way ANOVA (control vs. ob/ob) was used to compare the effect of a leptin deficiency on body and organ weights, vaginal opening, and serum leptin concentrations. A one-way ANOVA was also used to compare the effect of a leptin deficiency on the number of follicles at each stage of folliculogenesis, the number of apoptotic cells, and the number of atretic follicles. A *t*-test was used to compare the effect of a leptin deficiency on the percentage of atretic follicles. A Wilcoxon test was also performed to account for the non-normal percentages. Results using the Wilcoxon test were in agreement with the initial *t*-tests. Results are represented as the mean ± SEM.

RESULTS

Leptin, Organ Weights, and Age of Vaginal Opening

The effects of a leptin deficiency on serum leptin, and body and organ weights are shown in Table 1. Body weights of ob/ob animals were double those of control animals ($P < 0.0001$). Uterine weights were significantly reduced in ob/ob animals compared with those of littermate controls (38% of control weights; $P = 0.003$). Ovarian weights in ob/ob animals were lower than control values, but differences did not reach levels of significance. In contrast, the day of vaginal opening was delayed by as much as 10 days in ob/ob animals compared with controls ($P < 0.0001$, Table 1), and the diameter of the vaginal opening of ob/ob mice was substantially smaller than that of control animals. All control animals experienced vaginal opening but only five of seven ob/ob animals attained vaginal opening by Day 49. The two ob/ob mice that had not achieved vaginal opening by Day 49 were not included in the vaginal opening data.

Staging of Folliculogenesis

The number of follicles at each stage of folliculogenesis was subnormal in leptin-deficient mice compared to controls, however, only the differences for the number of primordial follicles ($P = 0.02$) and the total number of ovarian follicles ($P = 0.03$) reached levels of significance (control, primordial follicles = 4.1 ± 0.5; ob/ob, primordial follicles = 2.2 ± 0.5; control, total number of follicles = 12.8 ± 0.7; ob/ob, total number of follicles = 9.4 ± 1.3).

TABLE 2. The number of apoptotic granulosa cells per follicle in leptin-deficient (ob/ob) and control mice.*

Treatment	Preantral	Early antral/Antral
Control	6.3 ± 2.2	3.4 ± 2.0
Ob/ob	28.0 ± 4.9[a]	52.6 ± 19.4[a]

* Values represent means ± SEM.
[a] Significantly different from the control value, $P < 0.05$.

Ovarian Apoptotic Activity

TUNEL-positive cells were confined largely to the granulosa cells of follicles (Fig. 1). There were few TUNEL-positive cells in the theca layers of follicles (Fig. 1). Moreover, the level of apoptosis as indicated by TUNEL assay was much greater in granulosa cells of the follicles of ob/ob ovaries (Fig. 1B) than control ovaries (Fig. 1A) and was confined predominantly to preantral and early antral follicles. The preantral and early antral/antral follicles of leptin-deficient mice had significantly higher numbers of apoptotic granulosa cells per follicle than the follicles from control mice ($P < 0.01$ and $P = 0.02$, respectively; Table 2). Follicles were classified as atretic if they had five or more apoptotic granulosa cells as determined by TUNEL staining. The percentage of follicles that were atretic was significantly greater in leptin-deficient animals (59.4% of preantral, 71.4% of early antral/antral) than controls (18.4% of preantral, 8% of early antral/antral; $P = 0.006$ and 0.007, respectively).

Immunocytochemistry for Fas, FasL, and PCNA

Fas and FasL expression within the ovary was confined largely to the granulosa cells of preantral and early antral follicles, and the intensity of the staining was of much greater magnitude in the ovaries of ob/ob animals (Fig. 2, B and E) than in the ovaries of control mice (Fig. 2, A and D). The staining pattern for Fas and FasL had a punctated appearance in the granulosa cells of these animals. We found it interesting that the oocytes of preantral and early antral follicles of ob/ob mice exhibited substantial Fas and FasL expression (Fig. 2, B and E). Much less Fas and FasL expression was evident in the oocytes of control mice (Fig. 2, A and D). There was little evidence of Fas and FasL staining within the theca cells of the follicles of either control or ob/ob ovaries.

PCNA expression occurred predominantly within the granulosa cell nuclei of preantral and early antral follicles, where it was co-localized with the DAPI stain (Fig. 3). PCNA staining also appeared to be more intense in the granulosa cell layers closest to the oocytes (data not shown). There appeared to be no differences in PCNA expression between the follicles of the ovaries of ob/ob and control animals (compare Fig. 3, A and B). The granulosa cells of primordial and very small primary follicles exhibited less PCNA expression than more mature follicles (data not shown). PCNA expression was also present in the oocytes of follicles of both ob/ob and control animals, and the level of PCNA expression did not appear to differ between the two groups (Fig. 3, A and B).

DISCUSSION

In the current study, ob/ob animals had delayed vaginal opening, subnormal uterine weights, and fewer primordial follicles and total numbers of ovarian follicles. The leptin deficiency was also associated with impaired folliculogenesis as indicated by increased follicular atresia (confined

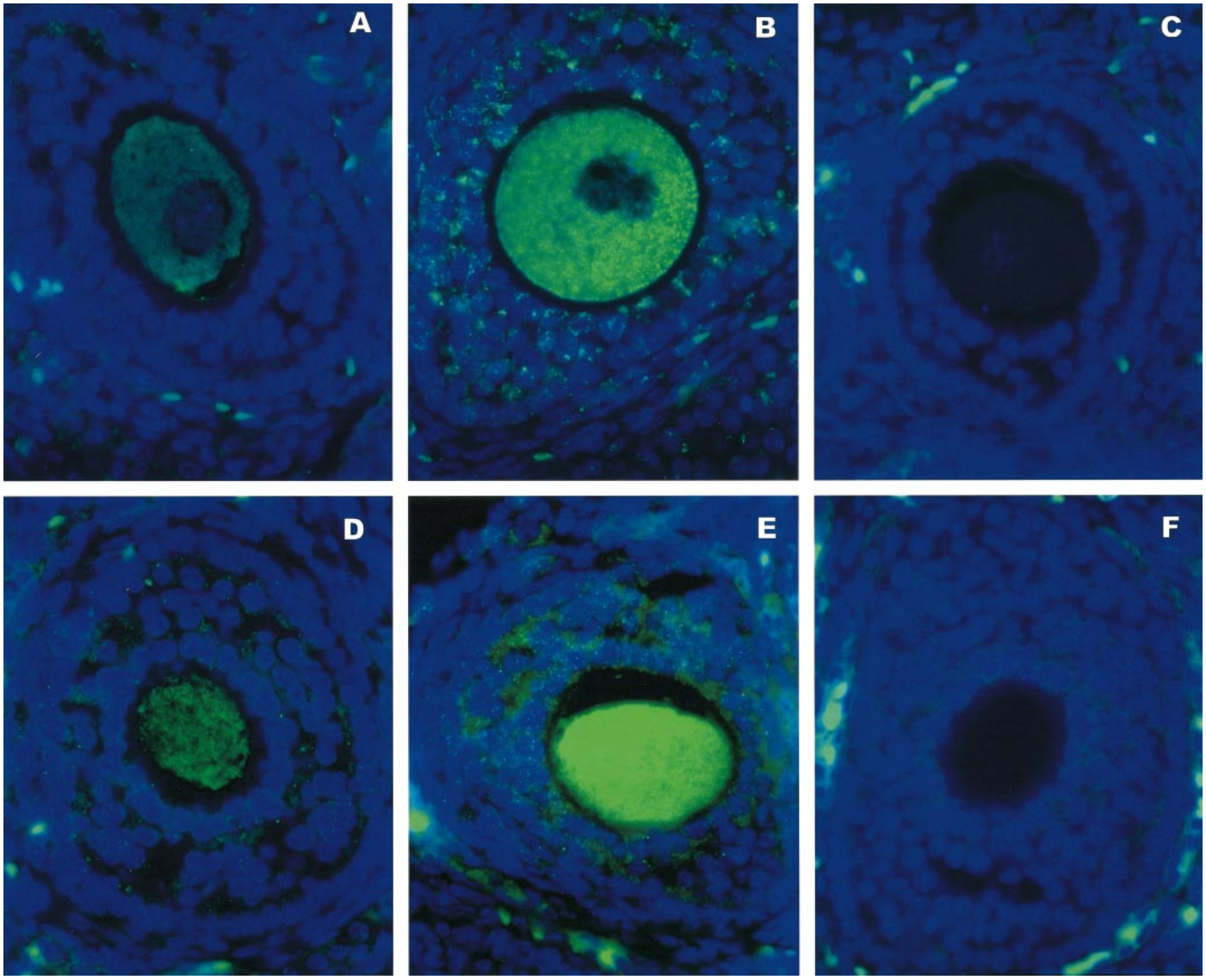

FIG. 2. Fas (**A** and **B**) and FasL (**D** and **E**) expression (green) in preantral follicles from a representative control (**A** and **D**) and an ob/ob (**B** and **E**) animal. The nuclei were counterstained with DAPI (blue). Fas and FasL expression was elevated within the granulosa cell layer of the follicles of ob/ob mice. Fas and FasL expression was also evident in oocytes, particularly those from ob/ob mice. **C**) and **F**) illustrate negative controls for Fas and FasL staining (primary antibodies omitted), respectively. Magnification ×1000.

largely to preantral and early antral follicles). Follicles from ob/ob animals exhibited increased apoptosis and elevated expression levels of Fas and FasL in granulosa cells, suggesting that the Fas/FasL system was one of the signaling pathways involved in mediating the effect of the leptin deficiency on folliculogenesis in these mice.

Mice (male and female) possessing spontaneous mutations of either the Ob or the leptin receptor genes are infertile, hyperphagic, and obese [1, 13, 14]. The infertility in these animals results at least in part from a deficiency of gonadotropin secretion, most likely as consequence of reduced or an altered pattern of GnRH secretion [15, 16]. When leptin-deficient mice were given leptin replacement, gonadal weights increased, serum levels of FSH and LH rose, and fertility was restored [1, 17]. In confirmation of earlier work [1, 17], pubertal development was retarded (delayed vaginal opening and reduced uterine weights) in ob/ob mice in the current study.

A major finding of the current study is the deleterious effect that a leptin deficiency has on folliculogenesis. The number of follicles present in the ovary of ob/ob animals was reduced and there was evidence of increased granulosa cell apoptosis and follicular atresia in these animals compared to control values. Follicles from ob/ob mice also exhibited increased granulosa cell Fas and FasL expression, suggesting that this system is one of the apoptotic pathways involved in mediating the effect of the leptin deficiency on folliculogenesis. To the best of our knowledge, the current study is the first to assess the effects of a leptin deficiency on ovarian follicle apoptosis and atresia and the first to report a potential role for the Fas/FasL system in this process. In an earlier study [1], leptin-deficient mice were treated with recombinant human leptin for 14 days. Leptin administration increased ovarian and uterine weights, and circulating levels of LH and FSH did not reach significance. In these animals there was also an increase in the number of primary follicles and the total number of ovarian follicles compared with those of saline-treated ob/ob animals. Therefore, the current study confirms previous findings in which leptin-deficient mice exhibited impaired folliculo-

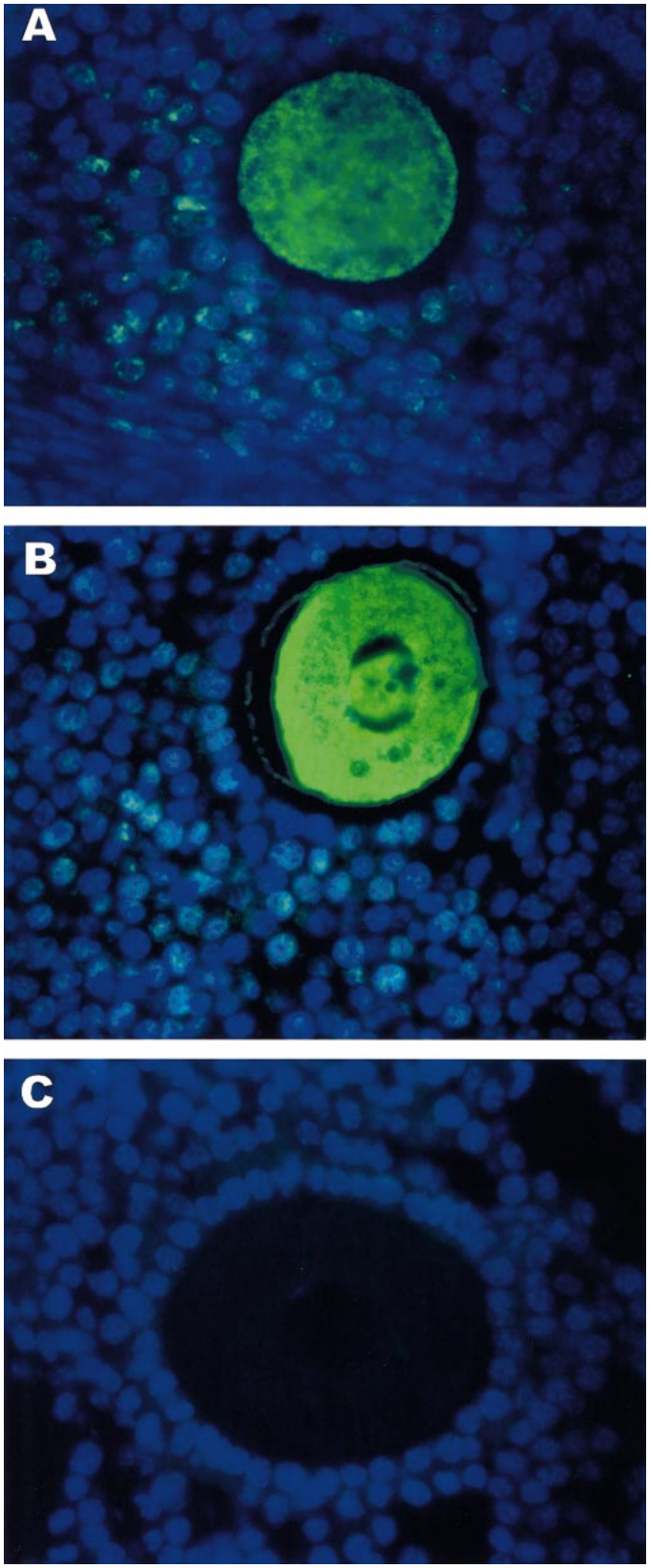

FIG. 3. PCNA expression within the granulosa cells of early antral follicles of a control (**A**) and an ob/ob animal (**B**). There did not appear to be any differences in PCNA expression within the ovarian follicles of ob/ob and control mice. Oocytes from both control and ob/ob mice also showed PCNA expression. **C**) A negative control for PCNA staining (primary antibody omitted). Magnification ×1000.

genesis, but also extends the previous work to document elevated apoptosis in the granulosa cells of follicles of leptin-deficient mice and the potential involvement of the Fas/FasL system in mediating this programmed cell death.

Although the underlying mechanism or mechanisms of the leptin deficiency on granulosa cell apoptosis and follicular atresia is not known, it is likely these effects are at least partially the result of the hypogonadotropic-hypogonadal condition of the leptin-deficient animal. We suggest that the subnormal levels of gonadotropin and sex steroid secretion exhibited in ob/ob mice may induce granulosa cell apoptosis, comparable to gonadotropin withdrawal-induced apoptosis. It is known that gonadotropins are the primary survival factors that inhibit granulosa cell apoptosis in ovarian follicles [18, 19]. Alternatively, leptin may also have a direct effect on ovarian folliculogenesis, because leptin and its receptors are present within the murine ovary, including the oocyte [2].

A number of studies (both in vivo and in vitro) have reported that leptin directly modulates ovarian function, but for the most part, the concentration of leptin employed in these studies was relatively high. Leptin inhibited insulin-induced progesterone and estradiol production from cultured bovine granulosa cells [20], and reduced LH-induced estradiol production by cultured human granulosa cells [21]. Administration of leptin (30 μg at 3-h intervals for 15 h) to immature, gonadotropin-primed rats also reduced the number of oocytes ovulated by more than 67% [22]. These studies suggest that leptin is capable of directly altering ovarian function, but the physiological significance of these studies is questionable because of the high levels of leptin employed. We believe it is more likely that the increased granulosa cell apoptosis and follicular atresia in ob/ob animals in the current study results primarily from the hypogonadotropic condition of the leptin-deficient mouse, particularly because ob/ob females respond to equine chorionic gonadotropin treatment with a normal increase in uterine weight, and the ovaries of ob/ob animals transplanted into control females ovulated, and when the host females were mated with control males, fertilization of ova of the transplanted ovaries was observed [23–25].

The current data implicating the Fas/FasL system in the increased levels of granulosa cell apoptosis and follicular atresia in leptin-deficient mice are not too surprising given that other studies [3, 4, 26] have implicated this system in follicular atresia resulting from gonadotropin withdrawal. Kim et al. [3, 4] reported that Fas/FasL expression increased in granulosa cells during gonadotropin withdrawal-induced apoptosis in the ovaries of rats. In this context, leptin may normally play an indirect antiapoptotic role by increasing levels of gonadotropin, which may in turn, act as a survival factor to regulate Fas/FasL expression in granulosa cells. A study by Almog et al. [27] demonstrated that 21-day-old female rats treated with leptin experienced advanced puberty, increased granulosa cell proliferation, and reduced granulosa cell apoptosis and follicular atresia. They also reported that after leptin administration, serum levels of FSH and LH were increased in these animals. In the current study it appears that cell death proteins, Fas and FasL, are upregulated in the presence of reduced gonadotropin and gonadal hormone secretion in leptin-deficient mice. Although we did not directly measure circulating gonadotropin and ovarian hormone levels in the present study, the retarded development of the reproductive system (delayed vaginal opening and subnormal ovarian and uterine

weights) of ob/ob animals is indicative of suppressed levels of gonadotropin and ovarian hormone secretion.

Another interesting finding of the current study was that both the Fas antigen and FasL were expressed within the oocyte of preantral and early antral follicles of ob/ob mice. Far less positive staining for Fas and FasL was evident in the oocytes from control mice. It has been reported that murine oocytes of follicles undergoing atresia express the Fas antigen (Fas mRNA) [28]. In the human ovary, follicular atresia appears to proceed in two stages; an earlier stage involving degenerative changes in the oocyte (predominates in preantral follicles), and a second stage (most evident in antral follicles) in which there are degenerative changes in granulosa cells [29]. Thus, the observed higher levels of expression of Fas and FasL within the oocytes of follicles of ob/ob mice in the present study may be indicative of Fas- and FasL-mediated cell death.

In the current study, PCNA expression was very prominent in the nuclei of the granulosa cells of preantral and antral follicles, and the levels of its expression did not appear to differ in magnitude between control and leptin-deficient mice. This may suggest that although follicles of ob/ob animals underwent follicular atresia at a faster rate, DNA synthesis within the granulosa cells did not appear to be compromised as a result of the leptin deficiency. Alternatively, PCNA staining observed in the granulosa cells of atretic follicles in ob/ob mice may be indicative of DNA repair and not necessarily DNA synthesis. Chapman and Wolgemuth [30] also observed PCNA staining in granulosa cells of atretic follicles in mice, suggesting that granulosa cells undergoing apoptosis may also express PCNA. In the current study, PCNA staining was also observed in the oocytes of follicles in both ob/ob and control mice. Several other studies have reported similar observations in follicles from both mice and rats [30, 31]. The reason for the presence of PCNA within growing oocytes remains unknown, although no new DNA synthesis is occurring in the growing oocyte. It has been suggested that DNA repair may be activated in growing oocytes [31]. Thus, long-lived cells such as oocytes may be more susceptible to DNA damage than rapidly proliferating cells, and as a result, the PCNA in the oocyte may be indicative of DNA repair rather than synthesis.

Taken as a whole, data from the current study suggest that a leptin deficiency in female mice is associated with impaired folliculogenesis and increased follicular atresia, and that the Fas/FasL system may be involved in mediating the increased levels of apoptosis observed in the granulosa cells of follicles of ob/ob mice. At the present time, it is not clear whether the effect of leptin deficiency on folliculogenesis results predominantly from the gonadotropin-deprived state of the ob/ob animal or whether leptin also has a direct modulating effect on folliculogenesis, and in the absence of leptin this also contributes to the impairment of follicle development.

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