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Intrabursal Administration of the Antiangiopoietin 1 Antibody Produces a Delay in Rat Follicular Development Associated with an Increase in Ovarian Apoptosis Mediated by Changes in the Expression of BCL2 Related Genes[1]

Fernanda Parborell,[3] Dalhia Abramovich,[3] and Marta Tesone[2,3,4]

Instituto de Biología y Medicina Experimental (IBYME)-CONICET,[3] Departamento de Química Biológica,[4] Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires (UBA), 1428 Buenos Aires, Argentina

ABSTRACT

The angiopoietin (ANGPT) receptor (TEK) system plays a crucial role in blood vessel development and regression. To date, no reports have addressed the actions of the anti-ANGPT1 antibody on gonadotropin-stimulated follicular development and atresia in the ovary. Therefore, in this study we specifically investigated whether ANGPT1 plays a critical intraovarian survival role for gonadotropin-dependent folliculogenesis. In particular, we examined the effect of local administration of anti-ANGPT1 antibody on follicular development, apoptosis, and expression of BCL2 protein family members (BAX, BCL2, and BCL2L1), TNFRSF6, and FASLG in ovarian follicles from prepubertal eCG-treated rats. The inhibition of ANGPT1 caused an increase in the number of atretic follicles and a decrease in the number of both antral follicles (AFs) and preovulatory follicles in gonadotropin-treated rat ovaries. Taking into account that follicular atresia is mediated by apoptosis, we analyzed the effect of the antibody against ANGPT1 on programmed cell death. The inhibition of the action of ANGPT1 caused an increase both in the number of apoptotic granulosa cells in AFs and in the spontaneous DNA fragmentation of AFs cultured in serum-free medium. Besides, AFs obtained from rats treated with intraovarian antibodies against ANGPT1 showed both a decrease in BCL2 and an increase in BAX protein levels. Moreover, a reduction in the $BCL2L1_L/BCL2L1_S$ ratio was observed in this group, with a reduction of $BCL2L1_L$ greater than that of $BCL2L1_S$, thus showing that the expression of these antiapoptotic proteins is lower in follicles from treated rats than in those from untreated ones. Our findings suggest that the inhibition of ANGPT1 activity causes an increase in the number of atretic follicles mediated by ovarian apoptosis through an imbalance in the ratio of antiapoptotic to proapoptotic proteins. This could take place through a paracrine effect on granulosa cells mediated by the TEK receptor in theca cells. Therefore, these data clearly indicate that ANGPT1 is necessary for follicular development induced by gonadotropins.

angiogenesis, angiopoietin 1, apoptosis, follicle, ovary

INTRODUCTION

Angiogenesis is a process of vascular growth that is limited mainly to the reproductive system in healthy adult animals. The development of new blood vessels in the ovary is essential to guarantee the necessary supply of nutrients and hormones to promote follicular growth and corpus luteum formation [1, 2]. Nongrowing primordial follicles and slow-growing preantral follicles (PFs) do not present a vascular supply of their own but, rather, rely on vessels in the surrounding stroma [3]. The angiogenic factors promote vessel permeability, favoring the antrum formation and the events inducing follicle rupture. After ovulation, newly formed blood vessels cross the basement membrane between the theca and the granulosa layers and continue a rapid growth to sustain corpus luteum development and function.

While the vascular endothelial growth factor (VEGF) is the main initiator of angiogenesis, the formation and differentiation of a structurally and functionally mature vascular network probably requires the coordinated action of various factors. These include angiopoietins ANGPT1 and ANGPT2, which act via the tyrosine kinase receptor, TEK [4]. Unlike VEGF, ANGPT1 is unable to stimulate endothelial cell proliferation [5], but instead is required for the recruitment of perivascular cells that lead to the maturation and stabilization of newly developed capillaries [4, 6]. ANGPT2 is a natural antagonist of ANGPT1 that opposes the effect of ANGPT1-mediated stabilization by promoting a more plastic state for the capillary endothelium, which can lead to endothelial migration and neovascularization [4]. The ANGPT-TEK system is expressed in the ovarian follicle and corpus luteum from rodents, bovines, and primates [4, 7–10]. Bovine follicles express both *Angpt* and *Tek* mRNAs in the theca interna, whereas granulosa cells express *Angpt1* and *Angpt2* mRNAs but not *Tek* mRNA [9]. Neither *TEK* mRNA nor its receptor protein is expressed by granulosa cells in primate mature follicles. In addition, in situ hybridization studies in rat ovaries showed that *Angpt2* mRNA is absent from PFs and does not become detectable until the preovulatory stage, where its expression is associated with the thecal blood vessels. ANGPT1 is expressed in the thecal layer uniformly during follicular development [4]. In the marmoset, *ANGPT1* mRNA was detected in the theca of tertiary follicles but at a very low level, whereas *ANGPT2* mRNA was not detected in follicles but was present in a subset of stromal vessels [8]. All these findings suggest that ANGPTs achieve a local action by binding to the TEK receptor present both in theca and endothelial cells, supporting the notion that the ANGPT-TEK system plays a physiological role in the theca interna layer of the developing follicle.

So far, the understanding of the role of ANGPTs in ovarian development and apoptosis is still unknown. The molecular mechanism of apoptosis is a matter of an active debate. It is known that ovarian apoptosis takes place to eliminate follicular cells in atretic follicles [11–17]. FSH and LH are the primary survival factors for ovarian follicles; the antiapoptotic effects of these gonadotropins are probably mediated by the production of ovarian growth factors. It has been demonstrated that various

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[2]Correspondence: FAX: 54 011 4786 2564; e-mail: mtesone@dna.uba.ar

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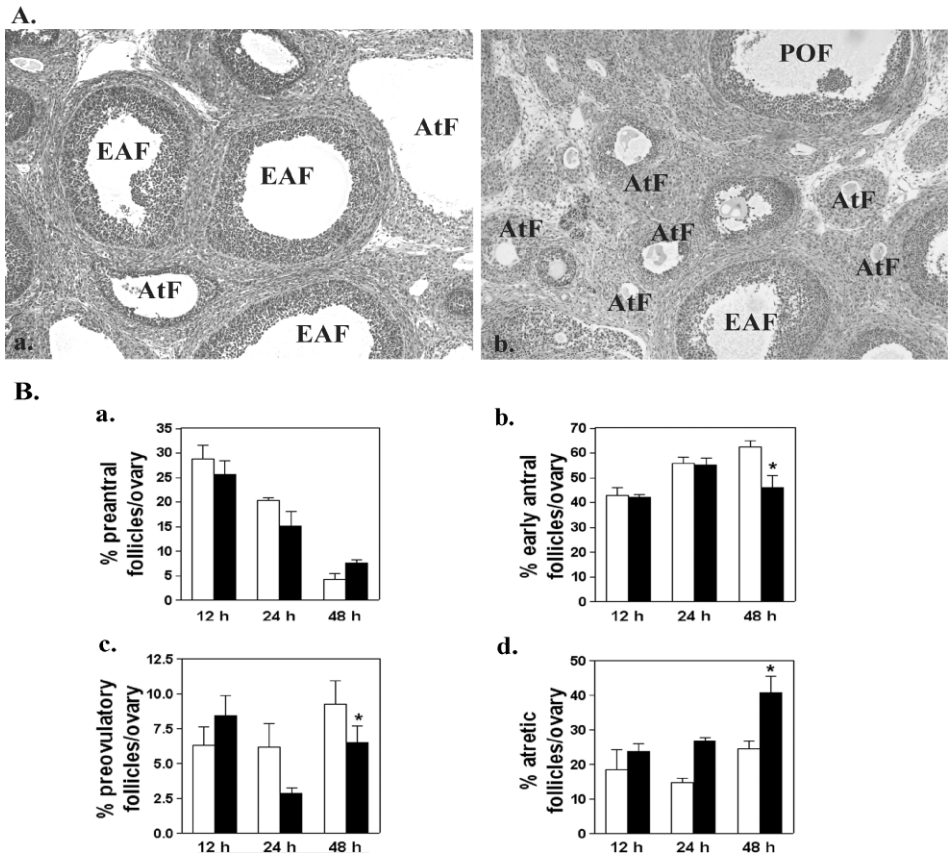


FIG. 1. Time course of ANGPT1 inhibition on follicle growth in ovaries from gonadotropin-stimulated rats. **A**) Representative fields of 100× ovarian sections. Control ovary (a). ANGPT1 Ab-treated ovary (b). AtF, atretic follicle. **B**) Preantral follicles (a). Early antral follicles (b). Preovulatory follicles (c). Atretic follicles (d). Prepubertal rats were injected with 10 ng ANGPT1 Ab under the bursa of one ovary. The contralateral ovary served as a control and was injected with normal goat IgG. Subsequently, 25 IU eCG was administered. The ovaries were removed at 12, 24, or 48 h after injection and prepared for histology. Data are expressed as mean ± SEM. Open bars, control; solid bars, anti-ANGPT1 Ab. $^*P < 0.05$; n = 6.

growth factors and cytokines (IGF1, EGF, TGFA, FGF2, FGF7, interleukin 1B) prevent apoptosis in antral follicles (AFs) [18–22]. Many of these reports use preovulatory follicles obtained from equine CG (eCG)-treated prepubertal rats and describe both a significant degree of apoptosis within 24 h of incubation in serum-free medium and its prevention in the presence of FSH or growth factors. In addition, several molecules, including BCL2 [23, 24], BCL2L1 (also known as BCLX) [25], BAX [23], caspases [26, 27], TNFRSF6 (also known as FAS) and FAS ligand (FASLG) [28, 29], and the inhibitor of apoptosis proteins (IAPs) [30], have been reported to be directly involved in the regulation of ovarian apoptosis.

However, to date, no reports have addressed the actions of the anti-ANGPT1 antibody on gonadotropin-stimulated follicular development and atresia in the ovary. Therefore, in this study, we specifically investigated whether ANGPT1 plays a critical intraovarian survival role for gonadotropin-dependent folliculogenesis. In particular, we examined the effect of local administration of anti-ANGPT1 antibody on follicular development, apoptosis, and expression of BCL2 protein family members (BAX, BCL2, and BCL2L1), TNFRSF6, and FASLG in ovarian follicles from prepubertal eCG-treated rats.

MATERIALS AND METHODS

Materials and Reagents

Polyclonal ANGPT1/4 antibody (sc-9360; Santa Cruz Biotechnology, Santa Cruz, CA) was raised in goats immunized against a peptide mapping at the c-terminus of the mature chain of ANGPT4 of human origin and detects ANGPT1 of mouse, rat, and human. Purified goat immunoglobulin G (IgG; sc-2028; Santa Cruz Biotechnology) was obtained from normal goat sera.

Equine CG (Novormon) was provided by Syntex SA (Buenos Aires, Argentina). Polyclonal primary antibodies for BAX (sc-493), BCL-2 (sc-492-G), BCL-X (sc-634), FAS (sc-715), and FAS-L (sc-956) were purchased from Santa Cruz Biotechnology; β-actin antibody was from Abcam (ab6276); and polyclonal von Willebrand factor antibody was from DakoCytomation (A0082). Anti-rabbit and anti-goat secondary antibodies conjugated with horseradish peroxidase were purchased from Sigma-Aldrich. Anti-mouse secondary antibody conjugated with horseradish peroxidase was purchased from R&D Systems. All other chemicals were of reagent grade and were obtained from standard commercial sources.

In Vivo Treatment and Superovulation

General care and housing of rats was carried out at the Instituto de Biología y Medicina Experimental (IByME) in Buenos Aires, Argentina. Prepubertal rats were from our colony. Immature female Sprague-Dawley rats (21–23 days) were anesthetized with ketamine HCl (80 mg/kg; Holliday-Scott, Buenos Aires, Argentina) and xylazine (4 mg/kg; König Laboratories, Buenos Aires, Argentina). The ovaries were exteriorized through an incision made in the dorsal lumbar region. Rats then received 1, 10, or 100 ng of antibody (Ab) for angiopoietin 1/4 in 5 μl of saline under the bursa of one ovary (ANGPT1 Ab ovary). The contralateral ovary was injected with the same volume and concentration of normal goat IgG (control ovary). An additional control was designed in which one ovary was injected with 10 ng ANGPT1 Ab previously adsorbed with the ANGPT1 peptide (sc-9360 P; Santa Cruz Biotechnology), and the contralateral ovary was injected with normal goat IgG. No significant differences were found in the number of follicles at any stage between both ovaries. Therefore, normal goat IgG was used as control. After injection, ovaries were replaced and the incision closed with skin adhesive. Rats then were injected subcutaneously with 0.1 ml eCG (25 IU/rat) and killed 12, 24, or 48 h after surgery by CO_2 asphyxiation. The ovaries were removed and cleaned of adhering tissue in culture medium for subsequent assays.

The experimental protocols were approved by the Animal Experimentation Committee of the IBYME.

Ovarian Morphology

The ovaries were removed and immediately fixed in 4% neutral-buffered formalin for 12 h and then embedded in paraffin. To prevent counting the same follicle twice, 4-μm step sections were mounted at 50-μm intervals onto microscope slides according to the method described by Woodruff et al. [31]. To count the number of different stages of follicles per ovary section, a set of slides was stained with hematoxylin and eosin, and another set was

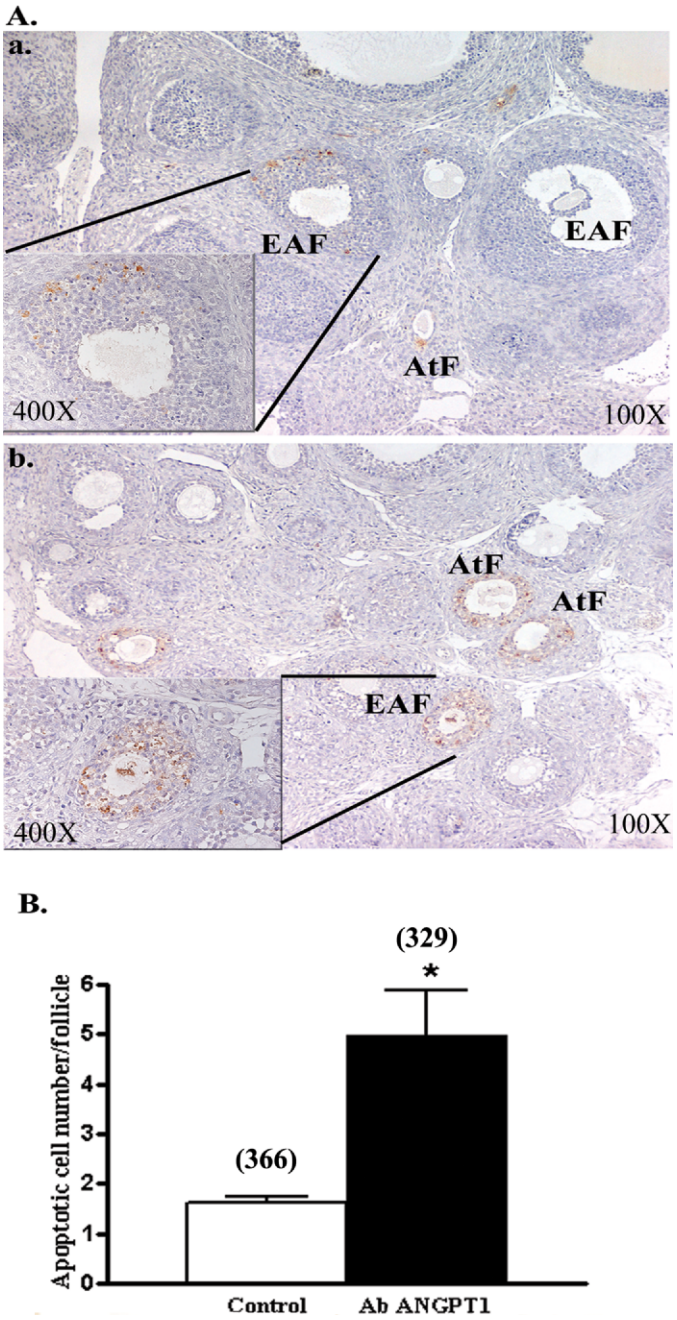


FIG. 2. **A**) Representative fields of ovarian sections labeled by the TUNEL technique. Control ovary (a); ANGPT1 Ab-treated ovary (b). A portion of each section shows the detail observed at high magnification. AtF, atretic follicle. **B**) In situ DNA fragmentation analysis (TUNEL technique). The number of apoptotic cells was determined by counting labeled cells in randomly selected fields (original magnification ×400) of AFs. Data were expressed as the apoptotic cell number per follicle ± SEM. The number of follicles analyzed is shown between parentheses. Three sections per ovary were analyzed (five ovaries per group). *$P < 0.01$ paired Student *t*-test; n = 5.

immunostained with the TUNEL technique. Follicles were classified as either PFs or early antral follicles (EAF) according to the presence or absence of either an antrum or preovulatory follicles (POFs). An atretic follicle was defined as the follicle that presented more than 10 pycnotic nuclei per follicle; in the smallest follicles, the criterion for atresia was a degenerate oocyte, precocious antrum formation, or both [32, 33]. The number of PFs, EAFs, POFs, and atretic follicles was determined in three ovarian sections from each ovary (three sections per ovary; six to eight ovaries per dose or time).

Follicle Isolation

The individual ovarian follicles were dissected from the ovary under a stereoscopic microscope as previously described [18, 34]. Briefly, healthy AFs (300–450 μm in diameter) from eight ovaries per group were frozen and used for biochemical assays. A pool of isolated follicles from each ovary was frozen, and the results obtained from each pool were considered as a single datum.

TUNEL Technique

For immunohistochemical quantification of apoptosis, formalin-fixed tissue sections were processed for in situ localization of nuclei exhibiting DNA fragmentation by the TUNEL technique [35] using an apoptosis detection kit (Apoptag plus peroxidase in situ Apoptosis detection kit; Chemicon International Inc.) as previously described [32]. The 4-μm-thick tissue sections were deparaffinized and digested for 15 min at room temperature with 20 μg/ml proteinase K (Gibco). Endogenous peroxidase was quenched with 3% hydrogen peroxide in phosphate-buffered saline (PBS). The labeling reaction was carried out by incubating tissue sections with buffer containing digoxygenin-dUTP prior to incubation with TdT for 1 h at room temperature. Tissues were then incubated for 30 min with a peroxidase-conjugated antidigoxygenin monoclonal antibody, and apoptotic cells were visualized as positively immunostained structures after reaction with diaminobenzidine (DAB). Negative controls included TdT omission. Sections were counterstained with hematoxylin. The number of apoptotic cells was determined by counting labeled cells from follicles in 400× microscopic fields (three sections per ovary; five ovaries) and expressed as the apoptotic cell mean per follicle.

Immunohistochemistry

Ovaries from both the control and the ANGPT1 Ab-treated group were immediately fixed in 4% neutral-buffered formalin for 12 h and then embedded in paraffin. Five-micrometer step sections were mounted at 50-μm intervals onto microscope slides to prevent counting the same follicle twice, according to Woodruff et al. [31]. Tissue sections were deparaffinized in xylene and rehydrated by graduated ethanol washes. Endogenous peroxidase activity was blocked with hydrogen peroxide in PBS solution, and nonspecific binding was blocked with 2% bovine serum albumin overnight at 4°C. Sections were incubated with rabbit polyclonal anti-von Willebrand factor (1:100) overnight at 4°C. After washing, the slides were incubated with biotinylated anti-rabbit IgG and after 30 min with avidin-biotinylated horseradish peroxidase complex (Vectastain ABC system; Vector Laboratories, Burlingame, CA). Protein expression was visualized with DAB staining. The reaction was stopped with distilled water, stained with hematoxylin, and dehydrated before mounting with mounting medium (Canada Balsam Synthetic; Biopack). For the negative controls, primary antibody was omitted.

To perform this study, six randomly selected fields were analyzed from each ovarian section (six sections per ovary; five ovaries). The percentage of endothelial cell area was quantified using the National Institutes of Health Image J program [36].

DNA Isolation and Fragmentation Analysis

Cellular DNA was extracted from 10 healthy AFs per ovary. Follicles were incubated for 24 h under serum-free conditions at 37°C in 500 μl DMEM:F12 (1:1) containing 10 mM HEPES, supplemented with fungizone (250 μg/ml) and gentamicin (10 mg/ml; five ovaries/group), and gassed with 95%O_2-5% CO_2 at the start of culture. This model has the advantage of keeping the integrity of the follicle. In addition, the incubation in serum-free conditions for 24 h allows the exhibition of the typical apoptotic DNA ladder: presence of internucleosomal fragments of 180-bp multiples. The follicles from each culture were homogenized in a buffer containing 100 mM NaCl, 4 mM EDTA, 50 mM Tris-HCl, and 0.5% SDS, pH 8, and proteinase K (100 μg/ml) at 55°C for 4 h to facilitate membrane and protein disruption. After incubation, samples were cooled for 30 min on ice in 1 M potassium acetate and 50% chloroform to initiate protein precipitation and centrifuged at 9000 × *g* for 8 min at 4°C. Supernatants then were precipitated for 30 min in 2.5 volumes of ethanol at −70°C and centrifuged for 20 min at 5000 × *g* at 4°C. Finally, samples were extracted in 70% ethanol and resuspended in water. DNA content was incubated for 1 h with RNase (10 μg/ml) at 37°C and measured by reading the absorbance at 260 nm. DNA samples (4 μg) were electrophoretically separated on 1.9% agarose gels containing ethidium bromide (0.4 μg/ml) in TBE buffer (0.089 M Tris:HCl, 0.089 M boric acid, 2mM EDTA, pH = 8). Within each agarose gel, equal amounts of DNA were loaded into each well. DNA was visualized in an ultraviolet (302 nm) transilluminator and photographed with a Polaroid camera system. Densitometric analysis of low-molecular weight (<15

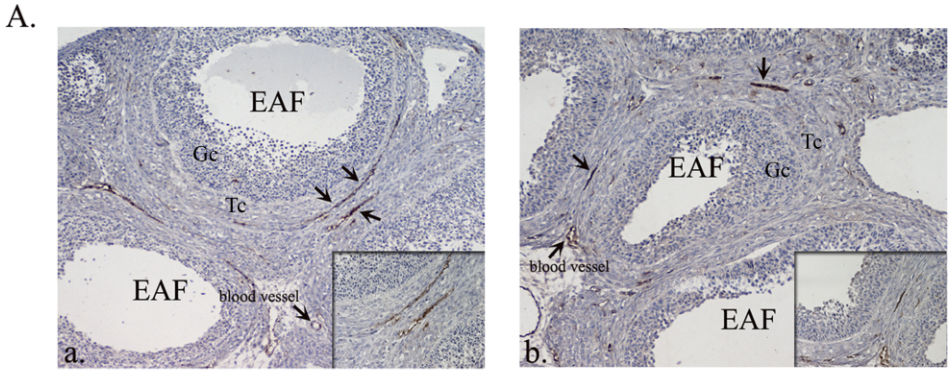
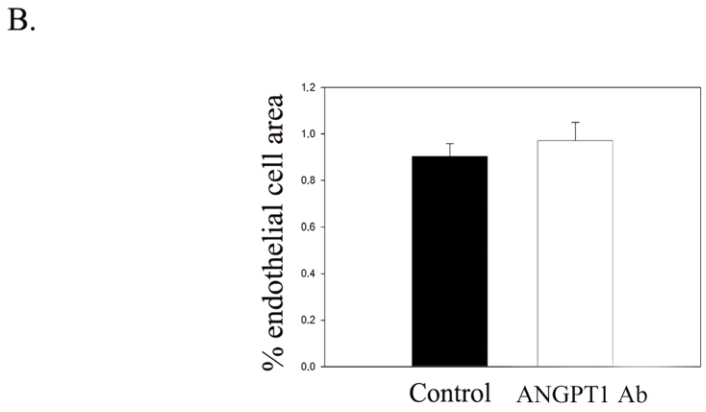


FIG. 3. Von Willebrand factor immunostaining in ovaries from control and ANGPT1 Ab-treated rats. **A**) Vessel networks marked with anti-von Willebrand factor antibody within the theca layer and blood vessels. **a**) Control ovary. **b**) ANGPT1 Ab-treated ovary. Solid arrows indicate a positive immunoreaction. EAF, early antral follicle; Gc, granulosa cell; Tc, theca cell. Original magnification ×100; insets X400. **B**) The percentage of endothelial cell area was determined by counting labeled cells in randomly selected fields (original magnification X100) of ovarian sections (five sections per ovary, six to eight ovaries per group). Data are expressed as mean ± SEM.



kb) DNA was performed with an Image Scanner (Genius) using the software program Scion Image for Windows (Scion Corp., Worman's Mill, CT). Quantitative results obtained by densitometric analysis of the low-molecular weight DNA fragments represent the mean ± SEM of three independent gel runs.

Western Blots

Follicles were isolated 48 h after injection and were immediately frozen at −70°C until protein extraction. A total of 100 follicles per ovary were resuspended in five volumes of lysis buffer (20 mM Tris-HCl, pH 8, 137 mM NaCl, 1% Nonidet P-40, and 10% glycerol) supplemented with protease inhibitors (0.5 mM phenylmethylsulphonyl fluoride, 0.025 mM *N*-CBZ-L-phenylalanine chloromethyl ketone, 0.025 mM *N*-*p*-tosyl-lysine chloromethyl ketone, and 0.025 mM L-1-tosylamide-2-phenyl-ethylchloromethyl ketone) and homogenized with an Ultra-Turrax (IKA Werk) homogenizer. Samples were centrifuged at 4°C for 10 min at 10 000 × *g*, and the resulting pellets were discarded. Protein concentration in the supernatant was measured by the Bradford assay. After boiling for 5 min, 40 μg protein was applied to a 12% SDS-polyacrylamide gel, and electrophoresis was performed at 25 mA for 1.5 h. The resolved proteins were transferred for 2 h onto nitrocellulose membranes. The blot was preincubated in blocking buffer (5% nonfat milk, 0.05% Tween-20 in 20 mM TBS (4mM Tris:HCl, 100 mM NaCl, pH = 8) for 1 h at room temperature and incubated with appropriate primary antibodies (FAS 1:500, FASLG 1:500, BAX 1:200, BCL2 1:500, BCL2L1 1:200 in TBS) in blocking buffer overnight at 4°C. Then, it was incubated with anti-rabbit or anti-goat secondary antibodies conjugated with horseradish peroxidase (1:1000), and finally detected by chemiluminescence and autoradiography using x-ray film. The density in each band was normalized to the density of the actin B band that was used as an internal control.

Quantification for Western Blot Assay

Equal amounts of protein were loaded for all samples, and both groups in one experiment were loaded on the same gel. For quantification, a screening was performed on blots with x-ray film using different times of exposure to optimize the signal. The levels of protein were compared and analyzed by densitometric studies using Scion Image for Windows (Scion). Optical density data are expressed as arbitrary units ± SEM (n = 8).

Data Analysis

Data are expressed as the mean ± SEM. Representative gels are shown in the figures. Statistical analysis was performed using paired or unpaired Student *t*-test. Values of $P < 0.05$ were considered significant.

RESULTS

Morphological and TUNEL Studies

The aim of the first study was to evaluate the effects of the inhibition of angiopoietin 1 on follicular development and apoptosis in the rat ovary. To inhibit ANGPT1, an antibody raised against ANGPT1 and ANGPT4 was administered for different periods of time. The time course effects of the Ab on follicle growth are shown in Figure 1. Rats were treated with intrabursal injections of different dilutions of the Ab in saline solution (1, 10, or 100 ng/ovary), and the ovaries were removed 12, 24, or 48 h later, processed, and analyzed as described above. Histological ovarian slides were stained with hematoxylin and eosin to determine the number of different follicle stages (Fig. 1A). Injection of 10 ng of the Ab did not change the number of PFs (Fig. 1B, a); however, it significantly decreased the number of AFs (control: 62.48% ± 2.42%; ANGPT1 Ab: 46.00% ± 4.78%; $P < 0.01$; n = 6) and POFs (control: 9.30% ± 1.64%; ANGPT1 Ab: 6.56% ± 1.14%; $P < 0.05$; n = 6) compared with the control group 48 h after surgery (Fig. 1B, b and c). Furthermore, it significantly increased the number of atretic follicles (control: 24.53% ± 2.44%; ANGPT1 Ab:

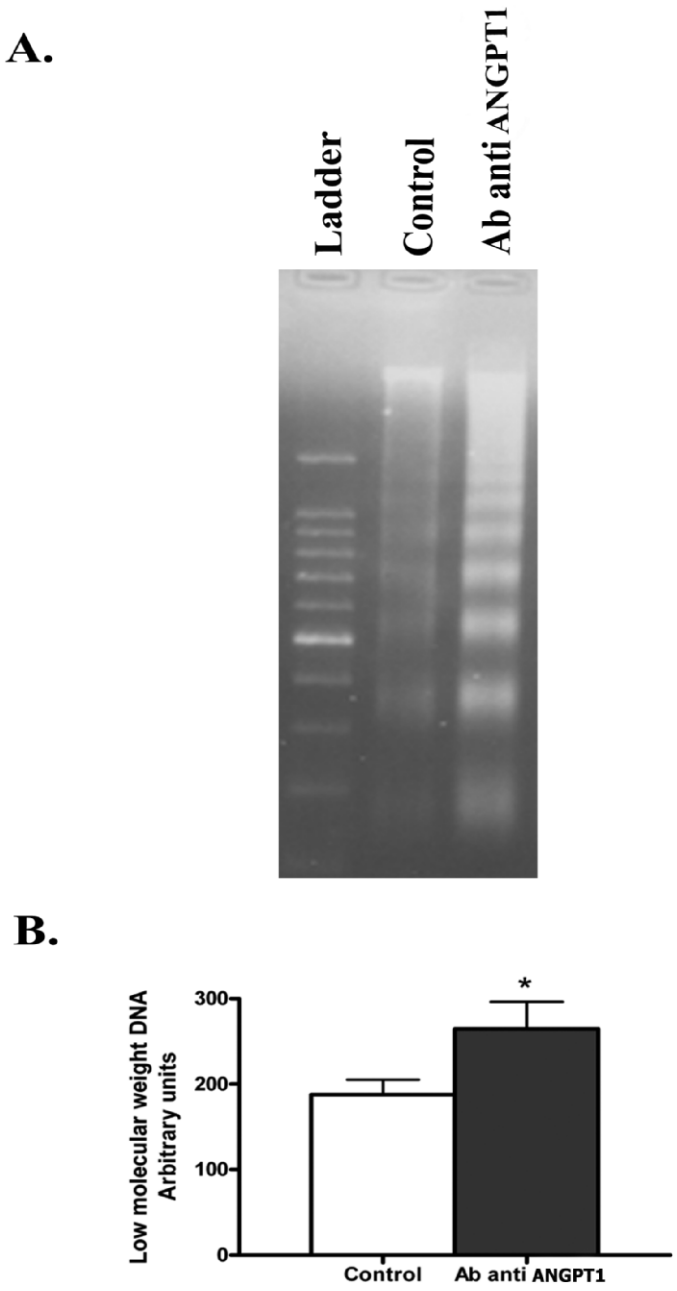


FIG. 4. Effect of ANGPT1 Ab injection on DNA fragmentation in cultured AFs. **A**) Agarose gel showing DNA fragmentation. **B**) Quantitative estimation of DNA cleavage. Animals were injected under the bursa of one ovary with 10 ng ANGPT1 Ab. The other ovary was injected with normal goat IgG and used as a control. At 48 h after surgery, AFs were isolated and cultured for 24 h in serum-free medium. A total of 4 μg follicular DNA extracted from each culture was analyzed by ethidium bromide staining. Low-molecular weight DNA (<15 kb) from the gel was examined to calculate apoptotic DNA fragmentation. Data points represent the mean ± SEM of three independent experiments. *$P < 0.05$.

40.75% ± 4.89%, $P < 0.05$, n = 6; Fig. 1B, d). No significant differences were found at any stage of follicles 12 or 24 h after injection. Similar results were obtained when injecting 100 ng Ab/ovary. Additionally, there were no changes when 1 ng Ab/ovary was injected (data not shown). Therefore, 10 ng and 48 h treatment were used for the following assays.

As granulosa cells of the follicle die by apoptosis during the process of atresia [12, 37], the TUNEL technique was performed on histological ovarian slides from ovaries obtained 48 h after injection of ANGPT1 Ab. Figure 2A shows representative fields of a control and a treated ovary. Specific staining was observed in granulosa cells, and ANGPT1 Ab treatment caused a 3-fold increase in the number of apoptotic cells in AFs (control: 1.62 ± 0.13 apoptotic cells/follicle; ANGPT1 Ab: 4.97 ± 0.9 apoptotic cells/follicle; $P < 0.01$, n = 5) as shown in Figure 2B.

Endothelial Cell Density

In order to evaluate the endothelial cell density 48 h after ANGPT1 Ab treatment, histological ovarian slides were immunostained using an antibody raised against von Willebrand factor (Fig. 3). No significant differences were found in the percentage of endothelial cell area between control and ANGPT1 Ab-treated ovaries 48 h after injection (control: 0.91% ± 0.05%; ANGPT1 Ab: 0.97% ± 0.07%; $P > 0.05$). In addition, there were no apoptotic cells within the endothelium.

Agarose Gel Electrophoresis and Quantitation of DNA Fragmentation

The levels of DNA fragmentation were measured in AFs obtained from control and treated ovaries 48 h after surgery (Fig. 4A; lane 1: control, lane 2: ANGPT1 Ab). Antral follicles cultured in serum-free medium showed spontaneous onset of apoptotic DNA fragmentation. Follicles obtained from ANGPT1 Ab-treated ovaries showed a significant increase (41%) in the spontaneous onset of apoptotic DNA fragmentation (Fig. 4B; control: 187 ± 15 arbitrary units, ANGPT1 Ab: 264 ± 32 arbitrary units; $P < 0.05$). DNA fragmentation was minimal in freshly isolated AFs (data not shown).

Levels of Apoptosis-Related Proteins in AFs

Figure 5 shows the follicular contents of BCL2, BAX, BCL2L1s, $BCL2L1_L$, TNFRSF6, and FASLG proteins measured by Western blotting from follicles isolated 48 h after ANGPT1 Ab injection. The injection of ANGPT1 Ab significantly decreased the levels of BCL2 (control: 0.35 ± 0.04; ANGPT1 Ab: 0.26 ± 0.03; $P < 0.05$, n = 8; Fig. 5a) and increased the levels of BAX protein (control: 0.46 ± 0.06; ANGPT1 Ab: 0.67 ± 0.03; $P < 0.05$, n = 8; Fig. 5b). The $BCL2L1_L$/BCL2L1s ratio was significantly diminished in follicles obtained from ANGPT1-treated ovaries (control: 0.5 ± 0.06; ANGPT1 Ab: 0.4 ± 0.04; $P < 0.05$, n = 8), with a reduction of $BCL2L1_L$ greater than that of $BCL2L1_S$ (Fig. 5c). No changes in the levels of TNFRSF6 or FASLG were observed after the treatment (Fig. 5, d and e).

DISCUSSION

The results reported here represent the first demonstration that in vivo intrabursal administration of a neutralizing antibody against ANGPT1 produces an increase in ovarian apoptosis in eCG-treated rats and that the variations observed in the expression of BCL2L1, BAX, and BCL2 are involved in this effect.

The inhibition of ANGPT1 caused an increase in the number of atretic follicles and a decrease in the number of both AFs and preovulatory follicles (POFs) in gonadotropin-treated rat ovaries. Taking into account that follicular atresia is mediated by apoptosis, we analyzed the effect of antibody against ANGPT1 on programmed cell death. We found that the inhibition of the action of ANGPT1 caused an increase in the number of apoptotic granulosa cells in AFs and in the spontaneous DNA fragmentation of AFs cultured in serum-

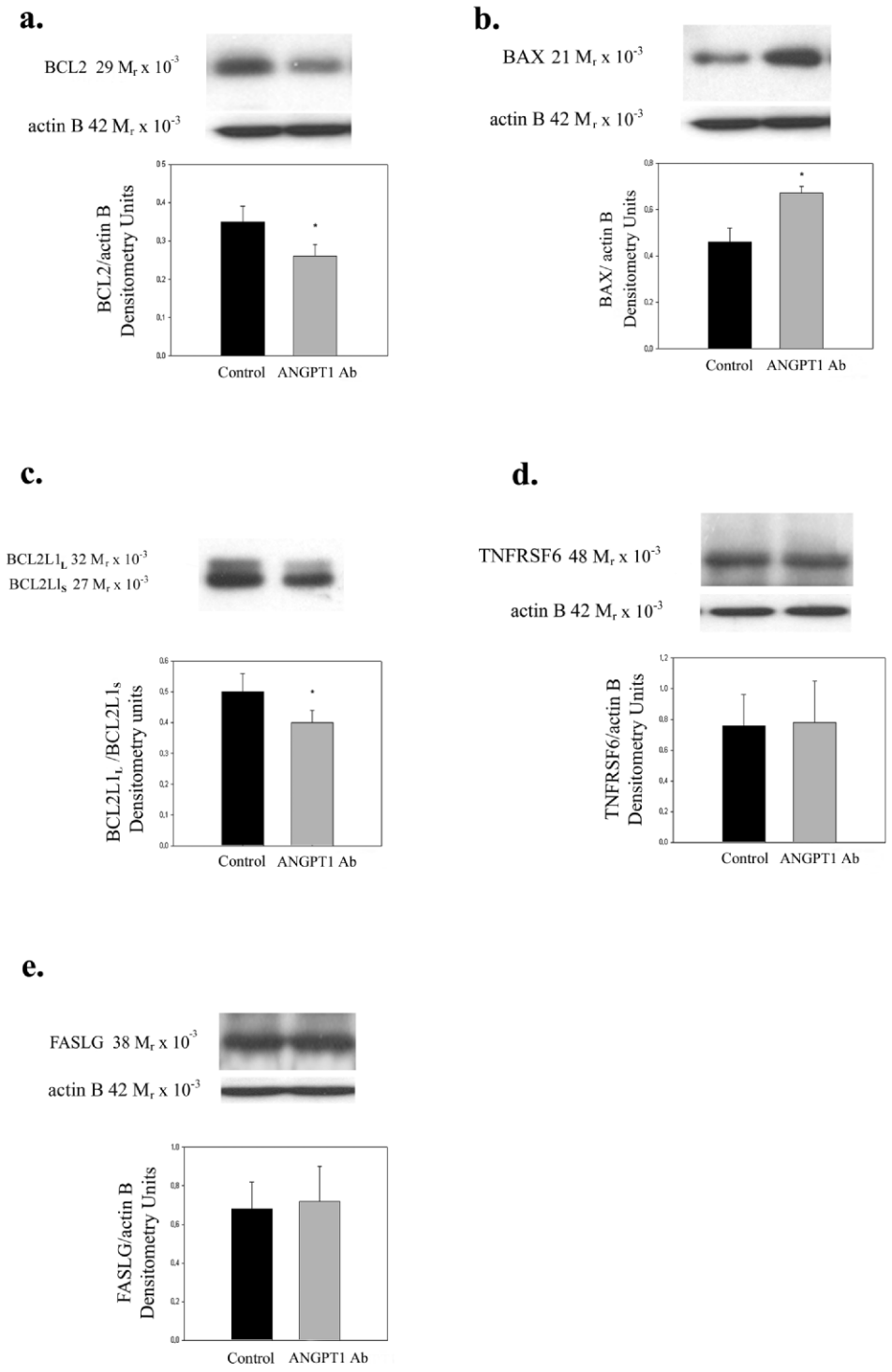


FIG. 5. Effect of ANGPT1 Ab treatment on proapoptotic and antiapoptotic protein content of antral follicles. **a**) Upper panel: representative immunoblot of BCL2 protein content in antral follicles from control and ANGPT1 Ab-treated rats. Levels of BCL2 protein were significantly decreased after ANGPT1 Ab treatment. Lower panel: densitometric quantification of BCL2 content. Bars represent mean ± SEM normalized to actin B. After homogenization, proteins were extracted and subjected to 12% SDS-PAGE and transferred onto nitrocellulose membranes. BCL2 protein was visualized by using an anti-BCL2 antibody (n = 8; $*P < 0.05$). **b**) Upper panel: representative immunoblot of BAX protein content in antral follicles from control and ANGPT1 Ab-treated rats. BAX protein was visualized by using an anti-BAX antibody. Levels of BAX protein were significantly increased after ANGPT1 Ab treatment. Lower panel: densitometric quantification of BAX content. Bars represent mean ± SEM normalized to actin B (n = 8; $*P < 0.05$). **c**) Upper panel: representative immunoblot of BCL2L1 protein content in antral follicles from control and ANGPT1 Ab-treated rats. BCL2L1 protein was visualized using an anti-BCL2L1 antibody that recognizes both isoforms. The $BCL2L1_L/BCL2L1_S$ ratio was significantly decreased after ANGPT1 Ab treatment. Lower panel: densitometric quantification of BCL2L1 content. Bars represent mean ± SEM (n = 8; $*P < 0.05$). **d**) Upper panel: representative immunoblot of TNFRSF6 protein content in antral follicles from control and ANGPT1 Ab-treated rats. TNFRSF6 protein was visualized using an anti-TNFRSF6 antibody. Levels of TNFRSF6 protein were not significantly different between control and ANGPT1 Ab groups. Lower panel: densitometric quantification of TNFRSF6 content. Bars represent mean ± SEM normalized to actin B (n = 8). **e**) Upper panel: representative immunoblot of FASLG protein content in antral follicles from control and ANGPT1 Ab-treated rats. FASLG protein was visualized using an anti-FASLG antibody. Levels of FASLG protein were not significantly different between control and ANGPT1 Ab groups. Lower panel: densitometric quantification of FASLG content. Bars represent mean ± SEM normalized to actin B (n = 8; $P < 0.05$).

free medium. Ovarian follicles cultured in these conditions constitute a model currently used to examine the pathways that regulate apoptosis in the follicle [12, 25]. Our findings indicate that DNA isolated after the incubation showed the typical apoptotic DNA fragmentation pattern and reveal that in vivo ANGPT1 antibody treatment sensitizes granulosa cells to undertake programmed cell death. These data suggest that ANGPT1 may inhibit granulosa cell apoptosis and, consequently, reduce follicular atresia.

The present results, together with our previous work [38], indicate that angiogenic factors have an important role in the processes of follicular selection, dominance, and atresia mediated by apoptosis. Angiogenesis is regulated independently within each individual follicle and, depending on the extent of the vascular plexus and permeability of vessels, it can thus control the supply of large-molecular weight tropic factors, precursors, and lipids. Besides, follicular atresia is associated with inadequate development and/or regression of the thecal vasculature in most species studied [8, 39]. Therefore, this would indicate that follicular vasculature could be intimately involved in follicular development. However, our results show no difference in the vascular density area after ANGPT1 antibody administration, thus suggesting a possible direct effect of ANGPT1 on the theca cells mediated by its

TEK receptor, and that this interaction may consequently produce a decrease in granulosa cell apoptosis.

A number of studies have focused on the role of angiopoietins in vascular development in vivo by using mouse molecular genetic approaches. One of them [40] has described the effect of overexpression of ANGPT2 during embryo development, and a second one has analyzed the effect of the deletion of ANGPT2 on vascular development [4]. Besides, in this last work, Maisonpierre et al. [4] compared the temporal and spatial expression patterns on ANGPT1, ANGPT2, and VEGF in the rat ovary, and proposed that these substances act in concert to either promote or inhibit vascular development.

Until now, only one study in the ovary of the rhesus monkey has shown direct evidence for a role of angiopoietins in this tissue. Xu and Stouffer [41] have demonstrated that local injection of ANGPT2 into the preovulatory follicle is able to block ovulation, whereas the same dose of ANGPT1 is not. It has been proposed that ANGPT2 inhibits the action of ANGPT1 at the level of their common receptor and that the correct balance between these factors is essential for maintaining the integrity of the preovulatory follicle. However, it is not known whether this is a specific angiolytic effect, resulting in a disruption of tissue remodeling, or if ANGPT2 affects the hormone-producing cells directly.

Although several intracellular molecules, including BCL2 [23, 24], BCL2L1 [25], BAX [23], caspases [26, 27], and the inhibitor of apoptosis proteins [30], have been implicated to be directly involved in the regulation of ovarian apoptosis, several studies have suggested that TNFSR6 antigen (TNFSR6) and FAS ligand (FASLG) may be central in the induction of follicular atresia [42, 43]. In the present study, the TNFSR6/FASLG protein levels were not affected by neutralizing the antibody against ANGPT1. These results suggest that the other pathway, related to mitochondria protein regulation, could be involved in this process. In this regard, members of the *bcl2* gene family have been described as the main participants in the cascade of events that either activate or inhibit apoptosis [44]. The BCL2-related proteins can be separated into antiapoptotic and proapoptotic members, and the balance between these counteracting proteins presumably determines cell fate [45]. In our experimental model, AFs obtained from rats treated with intraovarian antibody against ANGPT1 showed a decrease in BCL2 and an increase in BAX protein levels. Moreover, a reduction in the $BCL2L1_L/BCL2L1_S$ ratio was observed in this group, with a reduction of $BCL2L1_L$ greater than that of $BCL2L1_S$, showing that the expression of these antiapoptotic proteins is lower in follicles from treated rats.

ANGPT/TEK signaling is considered to participate in angiogenic processes of endothelial tissues. However, it is becoming evident that ANGPT/TEK signaling also plays an important role as an antiapoptotic system to maintain a dedifferentiated phenotype of other cell types, such as neurons and hematopoietic and granulosa cells [38, 46, 47]. The data presented here support these findings.

In summary, our results suggest that the inhibition of ANGPT1 activity causes an increase in the number of atretic follicles mediated by ovarian apoptosis through an imbalance in the ratio of antiapoptotic to proapoptotic proteins. The mechanism could be through a paracrine effect on granulosa cells mediated by the TEK receptor in theca cells. Accordingly, we observed by immunohistochemistry studies that ANGPT1 protein and its receptor TEK are only present in theca and endothelial cells during follicular development (Rodriguez Celin et al., unpublished results). This leads us to think that ANGPT1 would act not only as an "angiogenic" factor but as a "survival" factor in developing follicles. These results suggest that ANGPT1 would act on its receptor TEK in theca cells, producing the release of an unknown factor. Consequently, this factor would operate in a paracrine way in the adjacent granulosa cells through a mechanism not yet explained, thus causing the inhibition of apoptosis in these cells. Therefore, our data indicate that ANGPT1 is a necessary requirement for follicular development induced by gonadotropins.

A better understanding of the mechanisms of follicular angiogenesis and its regulation may lead to therapies for controlling inappropriate follicle development related to either a decreased or an enhanced angiogenesis. The development of specific agonists or antagonists of angiogenic molecules, together with their application in animal models, presents novel opportunities to corroborate their possible physiological role.

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