Preantral Ovarian Follicles in Serum-Free Culture: Suppression of Apoptosis after Activation of the Cyclic Guanosine 3',5'-Monophosphate Pathway and Stimulation of Growth and Differentiation by Follicle-Stimulating Hormone*

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ABSTRACT

Progression of preantral follicle development is essential to further follicle maturation and ovulation, but there are few models for studying the regulation of preantral follicle survival and growth. We have evaluated preantral follicle survival in vivo and in vitro, and have developed a serum-free rat follicle culture system that can be used to characterize the regulation of preantral follicle growth and differentiation. Analysis of ovarian cell DNA fragmentation during the first wave of follicle growth in the infantile rat indicated negligible apoptosis up to day 16 of age. However, a major increase in apoptosis was found by day 18, a time point associated with the appearance of large antral follicles. In situ analysis confirmed that apoptotic DNA fragments were limited to antral follicles. Culture of individual preantral follicles mechanically dissected from ovaries of 12- or 14-day-old rats in serum-free conditions led to major increases in follicle cell apoptosis, similar to that seen in cultures of antral and preovulatory follicles. In contrast to antral and preovulatory follicles, treatment of preantral follicles with gonadotropins or cAMP analogs did not prevent apoptosis. However, treatment with 8-bromo-cGMP or 10% serum suppressed apoptosis by 75% in cultured preantral follicles. In situ analysis identified granulosa cells as the cell type susceptible to apoptosis regulation. Taking advantage of the ability of the cGMP analog to suppress apoptosis, we evaluated the potential of FSH as a growth factor. In the absence of serum, FSH treatment for 48 h did not affect follicle size compared to controls; however, treatment with the cGMP analog together with FSH increased follicle diameter (13%; P < 0.01) and viable cells (2.4-fold; P < 0.01) compared to control values. Immunoblot analysis further indicated that the inhibin-a content of the cultured follicles was increased by treatment with the combination of FSH and 8-bromo-cGMP, demonstrating the induction of follicle cell differentiation during culture. Therefore, we demonstrated that activation of the cGMP pathway promotes the survival of cultured preantral follicles and that in the presence of a cGMP analog, FSH is a growth and differentiation factor for preantral follicles. The present serum-free follicle culture model system will be useful in further evaluation of the regulation of growth and differentiation of preantral follicles. (Endocrinology 138: 2417–2424, 1997)

OVARIAN FOLLICLES, consisting of an oocyte surrounded by inner layers of granulosa and outer layers of thecal cells, represent the basic functional unit of the ovary. Each follicle develops through primordial, primary, and secondary stages before acquiring an antral cavity. Further growth and differentiation lead to large Graafian or preovulatory follicles, which, under proper conditions, ovulate and transform into corpora lutea (1, 2). The progression of growth and differentiation of preantral follicles in the ovary is crucial to reproductive success, but relatively little is known about the regulation of follicle development before the antral stage. The study of preantral follicles has been difficult due to their slow rate of growth and small size (3). Although culture of mechanically dissected preantral follicles has been described (4–6), individual follicles exhibit variable growth properties. In some studies, follicles were selected for experiments only after demonstrating growth in culture. Although the use of enzymatically dispersed ovaries has also been described (7–12), follicle structures in these cultures did not uniformly include basement membranes and thecal layers, and disruption of the three-dimensional follicular architecture may alter follicle responses to hormonal stimuli. Using these models, studies dealing with a potential stimulatory effect of FSH on preantral follicle growth have been conflicting (4, 7, 8, 13).

Recently, apoptosis has been shown to be the underlying mechanism of follicle atresia (14). Although gonadotropins and several local ovarian factors suppress apoptosis in cultured preovulatory and early antral follicles (15–17), few studies have involved preantral follicles. Because increases in follicle cell number can occur by either increased cell division or decreased cell death, analysis of apoptosis in cultured preantral follicles is also important.

In this study, we have evaluated apoptosis in preantral follicles in vivo in rats as well as investigated the effects of...
activators of the cAMP and cGMP pathways in the regulation of preantral follicle cell apoptosis in vitro. Furthermore, we used the antipapoptotic properties of cGMP analogs to develop a serum-free culture system for intact, mechanically dissected preantral follicles. Using this system, we demonstrated that FSH induces both growth and differentiation of preantral follicles when apoptosis is prevented.

Materials and Methods

Hormones and reagents

Human FSH (ISAPP-1; 8466 IU/mg) was obtained from the National Hormone and Pituitary Distribution Program, NIDDK, NIH (Baltimore, MD). 8-bromo-cAMP (8-br-cAMP), 8-bromo-cGMP (8-br-cGMP), and sodium nitroprusside (SNP) were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant human interleukin-1β (IL-1β) was obtained from Genzyme (Cambridge, MA).

Animals and ovarian dissection

All animal experiments were performed in accordance with NIH and institutional guidelines. Sprague-Dawley rats were obtained from Simonsen (Gilroy, CA) and housed under standard conditions. Animals were anesthetized with CO₂ and killed by cervical dislocation before dissection of ovaries. Apoptotic DNA fragmentation in ovarian cells was assessed in rats at 12, 15, 16, 18, and 21 days of age. Ovaries were snap-frozen or formalin-fixed for DNA fragmentation analysis. Ovaries for follicle cultures were obtained from 12- or 14-day-old rats, immediately placed in warmed culture medium, and dissected.

Preantral follicle culture

The effects of hormones or cyclic nucleotide analogs on apoptosis of preantral follicles were first examined in a 24-h in vitro culture system, as described previously (15). Ovaries were collected from 12-day-old rats, and preantral follicles (160–200 μm in diameter) were dissected microsurgically using fine needles. Twenty follicles per experimental group were cultured in 500 μl Eagle’s MEM (Life Technologies, Grand Island, NY) supplemented with penicillin, streptomycin sulfate, 1-glutamine, and 0.1% BSA (Sigma Chemical Co., St. Louis, MO) in the absence or presence of different hormones or agents. The cultures were performed at 37 °C under 95% O₂ and 5% CO₂. After 24 h, follicles were collected and stored frozen until further analysis.

To assess the growth and survival of individual follicles, a more prolonged culture system was also developed. Preantral follicles of 160- to 170-μm diameter were dissected from the ovaries of 14-day-old rats under a dissecting microscope using fine needles. Follicles were cultured individually in 96-well dishes lined with polycarbonate membranes in 150 μl medium overlaid with 75 μl sterile mineral oil at 37 °C in a moist atmosphere using 9% CO₂ and 5% O₂. After 24 h, follicles were counted and stored frozen until further analysis.

To assess the growth and survival of individual follicles, a more prolonged culture system was also developed. Preantral follicles of 160- to 170-μm diameter were dissected from the ovaries of 14-day-old rats under a dissecting microscope using fine needles. Follicles were cultured individually in 96-well dishes lined with polycarbonate membranes in 150 μl medium overlaid with 75 μl sterile mineral oil at 37 °C in a moist atmosphere using 9% CO₂ and 5% O₂. After 24 h, follicles were counted and stored frozen until further analysis.

Apoptotic DNA fragmentation analysis

Genomic DNA was extracted from ovaries and follicles as previously described (15). Briefly, tissues were digested with 200 μl buffer containing 0.5% SDS, 0.1 M NaCl, 0.05 M Tris, 4 mM EDTA, and 100 μg/ml proteinase K for 4 h at 57 °C. DNA was isolated by chloroform extraction and ethanol precipitation before resuspension in sterile water. After digestion of RNA with 10 μg/ml ribonuclease at 37 °C for 1 h, DNA was isolated after phenol-chloroform extraction and ethanol precipitation. The DNA concentration was estimated by absorbance at 260 nm. A 200-ng aliquot of DNA from each sample was labeled at the 3'-ends with [32P]dideoxy-ATP (3,000 Ci/mmol; Amersham, Arlington Heights, IL) using terminal deoxynucleotidyl transferase (Boehringer Mannheim, Indianapolis, IN). After 1-h incubation at 37 °C, labeled DNA was isolated by phenol-chloroform extraction and precipitated in ethanol with 2% agarose gels by electrophoresis before exposure using Fuji x-ray films (Fuji Photo Film, Minami-Ashigarashi, Japan) at −70 °C. After autoradiography, gels of each lane corresponding to DNA less than 15 kilobases (kb) were cut out and counted in a scintillation counter for quantification of the degree of apoptotic DNA fragmentation.

In situ DNA fragmentation analysis

DNA fragmentation in ovarian or cultured follicle sections was evaluated by nonradioactive labeling of DNA using Apop Tag Kit (Oncor, Gaithersburg, MD). Ovaries or follicles were fixed with 4% buffered formaldehyde for 48 h at room temperature. The tissues were embedded in paraffin and sectioned. Three-micron sections were deparaffinized and incubated with 10 μg/ml proteinase K for 30 min at 37 °C. After washing, sections were treated with 2% H₂O₂ for 5 min to inactivate endogenous peroxidase. The 3'-ends of DNA fragments were labeled with digoxigenin-deoxy-UTP by incubation with terminal deoxynucleotidyl transferase for 1 h at 37 °C. The sections were further incubated with antidigoxigenin antibody conjugated to peroxidase for 30 min at room temperature. DNA fragmentation was visualized by staining with 3',5'-diaminobenzidine. Negative control sections were processed without terminal transferase.

Quantification of viable follicle cells

Cell proliferation was verified using the tetrazolium salt-based Cell Proliferation Kit I from Boehringer Mannheim (18). This assay is based on the ability of living cells to metabolize yellow tetrazolium salt to blue formazan crystals that can be solubilized and quantified spectrophotometrically. At the completion of the experiments, replicates of four follicles from each treatment group were moved to single wells containing 100 μl basal medium. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide labeling reagent was added to the wells and incubated for 4 h at 37 °C under 6.5% CO₂. Solubilization solution was then added for 18 h before determination of absorbance at 560 nm. Absorbance levels were standardized to known quantities of granulosa cells.

Immunoblot analysis of inhibin-α content of cultured follicles

Twenty follicles from each treatment group were collected in Eppendorf tubes at the end of the experiment and kept frozen. Follicles were later thawed in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% SDS, 5 mM EGTA, 0.5 mM MgCl₂, 0.5 mM MnCl₂, and 0.2 mM phenylmethylsulfonylfluoride) and homogenized with a glass rod before sonication at −70 °C. Inhibin-α antigen levels in homogenates were determined using immunoblot analysis as previously described (19). Briefly, electrophoresis was performed in a 10% polyacrylamide gel. Proteins were transferred to nitrocellulose membranes, and immunoblotting was performed with a mouse monoclonal antibody to inhibin-α (Serotec, Oxford, UK), followed by incubation with a biotinylated antimouse secondary antibody and immunofluorescent imaging with the ECL Western System (Amersham). Quantification of autoradiographs was performed using densitometric analysis.

Data analysis

All experiments were repeated at least three times, and a representative autoradiogram is presented where appropriate. Quantitative results obtained from 3- or 4-counting of radiolabeled low mol wt DNA fragments represent the mean ± SEM of combined data from replicate experiments. Statistical significance between mean values was determined by ANOVA followed by t test analysis. Significance was accepted at the 0.05 level.

Results

Granulosa cell apoptosis occurs predominantly in antral follicles of juvenile rats

To determine the pattern of cell death during the first wave of follicle development in infantile and juvenile ovaries, the
fragmentation pattern of ovarian DNA from rats of increasing ages was determined. As shown in Fig. 1A, ovarian internucleosomal DNA fragmentation was very low until day 18 of life and continued to be elevated on day 21. Quantification of low mol wt DNA fragments (<15 kb) after scintillation counting indicated 55% and 180% increases on days 18 and 21 of age, respectively, compared to the low mol wt DNA labeling at 15 days of age (Fig. 1B). In situ analysis of ovarian sections was performed to identify the types of follicles in which DNA fragmentation occurred. As shown in Fig. 2A, at 12 days of age, many follicles had reached the secondary stage, although small antra were beginning to form in a few follicles. There was negligible incorporation of digoxigenin-deoxy-UTP in any of these immature follicles. By 16 days of age (Fig. 2B), slightly larger antra were present within the most advanced follicles, with minimal DNA fragmentation in any of the follicles. By 18 days of age (Fig. 2C), there were medium-sized antral follicles in advanced atresia with heavy staining. By 21 days of age (Fig. 2D), many antral follicles were present in different stages of atresia. At all ages studied, there was minimal staining of preantral follicles of any size.

Preantral follicles undergo apoptosis in vitro

To monitor preantral follicle development in vitro, we evaluated apoptosis of preantral follicles in culture using a 24-h culture system developed previously for antral and preovulatory follicles (15). Preantral follicles (160–200 μm) were dissected from ovaries of 12-day-old rats. As shown in Fig. 3, A and B, negligible DNA fragmentation was found in preantral follicles at the beginning of culture (0 h), but a substantial increase in internucleosomal DNA fragmentation was found after 24 h in serum-free culture. The onset of apoptosis could be suppressed by 65% with the addition of 10% FCS to the culture medium. However, treatment with either FSH (100 ng/ml) or 8-br-cAMP analog (5 mM) failed to suppress apoptosis in preantral follicles (Fig. 3).

8-br-cGMP promotes preantral follicle survival in culture

Because previous data (17) indicated that apoptosis of preovulatory follicles can be suppressed by treatment with cGMP analogs and agents that stimulate endogenous cGMP production (IL-1β and SNP), we evaluated the effect of treatment with a cGMP analog, IL-1β, and SNP on preantral follicles. As shown in Fig. 4, A and B, treatment with 8-br-cGMP (5 mM) suppressed apoptosis of preantral follicles in culture by 76%. In contrast, IL-1β (250 ng/ml) and SNP (0.5 mM) were less effective, suppressing apoptosis in these follicles by 19% and 29%, respectively. Histological analysis of sections from cultured preantral follicles (Fig. 5) demonstrated that the anatomical integrity of the follicles was maintained over 24 h in culture. Pyknotic nuclei can be seen in granulosa cells after 24 h of culture (Fig. 5B), but not in follicles before culture (Fig. 5A). In situ staining of cultured follicles for DNA fragmentation indicated that apoptosis occurred in the granulosa cells of follicles cultured for 24 h without serum (Fig. 5C), and treatment with 8-br-cGMP markedly reduced apoptosis (Fig. 5D). No staining was seen in the negative control group when terminal transferase was deleted from the reaction (Fig. 5E).

FSH promotes preantral follicle growth only when apoptosis is suppressed

Taking advantage of the ability of 8-br-cGMP to suppress apoptosis and to allow better monitoring of follicle growth, we further evaluated the independent and combined effects of the cGMP analog and FSH on the survival of preantral follicles cultured individually. After 48 h of culture, morphological analysis demonstrated widespread granulosa cell atresia in follicles grown in control medium (data not shown). In contrast, treatment with either 8-br-cGMP (5 mM) or 5% rat serum protected the cultured follicles from apoptotic demise. Again, treatment with FSH (100 ng/ml) alone was ineffective in preventing apoptotic demise (data not shown).

We also used the 48-h culture system to evaluate follicle growth. Follicles cultured in control medium became slightly larger (3.6% increase in diameter; from 164.7 ± 0.9 to 170.7 ± 1.85 μm) over the 48-h incubation (Fig. 6A). Although treatment with FSH or 8-br-cGMP did not significantly alter this growth.
growth pattern, combined treatment with FSH and 8-br-cGMP increased follicle diameter by 13% to 188.5 ± 1.76 μm (P, 0.01). Because earlier studies had determined that optimal follicle growth in mice was obtained by treatment with 5% serum and FSH (5), we also included a group treated with 5% serum from hypophysectomized rats plus FSH in which a 23% increase in follicle diameter (P, 0.01) was observed. Compared to the control, treatment with serum alone resulted in only a modest increase in follicle growth that was not statistically significant (P > 0.05; data not shown).

To verify that the increased size of cultured follicles represented an increase in the number of viable follicular cells, we used a tetrazolium salt metabolic assay (18) to quantitate the relative changes in viable cell number in follicles from different treatment groups. As shown in Fig. 6B, treatment with FSH together with the cGMP analog or 5% serum re-

Fig. 2. In situ analysis of DNA fragmentation in ovaries of juvenile rats. Formalin-fixed paraffin sections of whole ovary mounts from rats of different ages were labeled with digoxigenin-deoxy-UTP at free DNA ends. Incorporated nucleotides were detected by antidigoxigen in antibody conjugated to peroxidase. A, Ovaries from rats at 12 days of age contained primordial follicles (PmF) and many secondary follicles (2F) with some beginning to form early antra (eAF). DNA fragmentation was negligible. B, Ovaries from rats at 16 days of age were predominantly composed of secondary follicles (2F), but some early antral follicles (eAF) were present. There was minimal staining in all follicles at this age. C, By 18 days of age, medium-sized atretic antral follicles with darkly stained granulosa cells were present (AtF). Staining was also present within the antra of some follicles. D, In the ovaries of 21-day-old rats, large heavily stained antral follicles were present in different stages of atresia. Bar = 125 μm. There was essentially no detectable staining of preantral follicles at any age.
sulted in more than a 2-fold increase \((P < 0.01)\) in viable cells per follicle, whereas treatment with FSH or the cGMP analog alone had no effect on viable cell number compared with the control value.

**FSH promotes preantral follicle differentiation**

Earlier studies have demonstrated increases in inhibin-\(\alpha\) messenger RNA and protein content with increasing follicle development (20). To monitor the progression of differentiation of follicles in culture, we evaluated the inhibin-\(\alpha\) protein content of cultured follicles by immunoblot analysis. As shown in Fig. 7, treatment with FSH in combination with either 5% serum or 8-br-cGMP resulted in an increase in the follicular inhibin-\(\alpha\) content over that in control follicles. In contrast, treatment with the cGMP analog or FSH alone did not increase the inhibin-\(\alpha\) content (data not shown). Overall, the relative quantity of inhibin-\(\alpha\) was consistent with the increase in size of the follicles.

**Discussion**

We have established a model in which preantral follicles can be cultured under serum-free conditions with minimal apoptosis. We have also demonstrated the ability of FSH to promote the growth and differentiation of preantral follicles and have shown that preantral follicles differ from antral follicles both in their \textit{in vivo} propensity to undergo apoptosis as well as in the regulation of their survival \textit{in vitro}.

Previous histological investigations revealed minimal atresia or apoptosis of granulosa cells within preantral follicles \textit{in vivo} (21–23), although the large preantral follicles that occur after diethylstilbestrol stimulation of immature rats do undergo apoptosis after estrogen withdrawal (24). However, biochemical studies of preantral follicles are difficult because most analyses use preparations of whole ovaries containing larger follicles and corpora lutea that mask changes in smaller follicles. Our use of juvenile rat ovaries overcomes this problem because at given ages, follicles have...
reached a predictable maximal stage of development. Additionally, these animals have not received exogenous hormone treatment. Using this model, we have demonstrated that based on fractionation of total ovarian DNA and on in situ analysis of individual follicles, apoptosis occurs widely in antral follicles and is minimal in preantral follicles. This is consistent with earlier studies in which the major wave of atresia was observed in early antral follicles 300–350 μm in diameter (21).

In vitro, however, apoptosis of preantral follicles can be demonstrated when follicles are grown in the absence of serum. In contrast to its effect on larger rat follicles (16) or in hamster preantral follicles (22), FSH or cAMP was ineffective in preventing rat preantral follicle atresia in vitro. We have demonstrated that activation of the cGMP pathway prevents preantral follicle cell death in vitro based on both DNA fragmentation and in situ analysis. In contrast, treatment with IL-1β or SNP is less effective in preventing preantral follicle apoptosis compared with their potent antiapoptotic effect in preovulatory follicles (17). These data suggest that IL-1β and SNP may be less effective in activating the cGMP pathway in preantral follicles or that they may also activate pathways independent of cGMP that increase apoptosis, as has been demonstrated in smooth muscle cells in culture (25). IL-1β has also been reported to have both cytotoxic and morphogenetic effects on whole ovarian dispersates in culture (26). It seems likely that the cGMP pathway is regulated by multiple mechanisms within small follicles, possibly with both endogenous ovarian factors as well as serum factors serving to prevent the wide scale follicle loss seen with larger follicles. The physiological stimulators of guanyl cyclase and the in vivo implications of these findings are currently unknown.

The regulation of preantral follicle survival is distinctly different from that in antral and preovulatory follicles. Kinetic studies have shown that follicles grow more slowly in

Fig. 5. In situ DNA fragmentation analysis of cultured preantral follicles. Sections were made of formalin-fixed, paraffin-embedded follicles cultured as described in Fig. 4. DNA fragments were end labeled with digoxigenin-dUTP. A, Hematoxylin and eosin staining of a follicle fixed at 0 h of culture. The oocyte (Oo) is surrounded by multiple layers of granulosa cells (Gc) and a thinner thecal layer (Tc). B, Hematoxylin and eosin staining of follicles cultured for 24 h in serum-free medium. Pyknotic nuclei (arrowheads) in granulosa cells can be seen. C, In situ analysis of DNA fragmentation in follicles cultured for 24 h as described in B. Strong staining of granulosa cells is apparent. D, In situ analysis of DNA fragmentation in follicles cultured with 8-br-cGMP for 24 h. Minimal staining is seen. E, Negative control of follicle cultured for 24 h in serum-free conditions demonstrating lack of staining when terminal transferase was deleted from the reaction.

CULTURE OF PREANTRAL FOLLICLES

Fig. 6. FSH stimulation of the growth of preantral follicles in culture. A, Diameters of individual preantral follicles were measured over 48 h in culture. Treatment groups include serum-free medium alone (control) and with or without 8-br-cGMP (5 mM) and/or FSH (100 ng/ml). For comparison, some cultures were also treated with FSH plus 5% rat serum. Numbers in parentheses represent the number of follicles cultured per group. B, Quantification of cells present per follicle at the end of the 48-h culture, as determined by the tetrazolium salt assay. For each treatment group, five or six groups of four follicles were assayed. *, P < 0.01 compared to the control.

Fig. 7. Immunoblot analysis of inhibin-α antigen content in cultured preantral follicles. Protein was extracted from 20 follicles/treatment group after culture for 48 h with control (basal), FSH (100 ng/ml) plus serum (5%), or FSH plus 8-br-cGMP (5 mM).
the early phases of development than in later stages (3). As follicles within a cohort enlarge, the time required for granulosa cells to double in number decreases. During this rapid growth, the enlarging follicles are highly dependent on gonadotropin activation of the protein kinase A pathway. The withdrawal of gonadotropins in vivo or in vitro results in atresia of larger follicles (14, 27, 28). In contrast, smaller, slow growing follicles, although responsive to gonadotropins under the appropriate conditions, do not depend upon gonadotropins to prevent granulosa cell death in vitro, suggesting that the apoptosis of preantral follicles is regulated differently from that of larger follicles, and this difference may be related to their different growth rates.

Preantral follicles have been cultured after mechanical dissection or enzymatic dispersion (3–13, 22). Although serum-free cultures of cumulus-enclosed oocytes has been described in the mouse (12), these systems were primarily designed to evaluate oocyte maturation, and 50–90% of complexes are not recovered at the end of the initial incubation. Serum-free culture of preantral follicles with intact basement membrane and theca has not been described in the rat or mouse. The present serum-free culture system allows the evaluation of endocrine or paracrine-autocrine factors that may affect the growth and differentiation of preantral follicles. Culturing intact follicles further allows analysis of the entire follicular unit, with interaction of oocyte, granulosa, basement membrane, and thecal components. Once loss of granulosa cells by apoptosis is prevented, a more accurate assessment of mitogenic factors can be performed.

We have demonstrated that the addition of FSH to medium containing the cGMP analog results in increased follicular diameter and cell number of preantral follicles as well as enhances the progression of differentiation, as demonstrated by increased inhibin-α protein production. This is consistent with earlier studies that suggested a role for gonadotropins in preantral follicle development (29–31), and our recent in vitro studies that demonstrate increased growth of preantral follicles in juvenile rats treated with FSH (32).

FSH may also be a growth and differentiation factor for human preantral follicles. The present serum-free culture system may be useful in evaluating human follicle growth and differentiation. If FSH affects human preantral follicles as in the rat, there could be important implications for both fertility management and infertility treatment.

Small ovarian follicles have not been studied to the extent of larger antral and preovulatory follicles. We have described a system in which rat preantral follicles can be cultured under serum-free conditions, thereby providing an in vitro model to evaluate these small follicles. Clearly, the regulation of preantral follicle survival and growth differs from that of more mature follicles, and further studies are needed to clarify the regulation of early folliculogenesis.

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