Changes in the Localization of Immunoreactive von Willebrand Factor in Microvascular Network of Bovine Ovarian Follicles during Atresia

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Abstract Changes in the localization of von Willebrand factor (vWF) in the follicular microvascular network were examined to determine whether functional modulation of microvasculature occurred in the atretic follicle in cows. Paraffin sections of healthy follicles and various stages of atretic follicles were immunostained with rabbit monoclonal antibody to vWF. The vWF-positive cells were counted in 4 different regions of a follicle from the apical to the basal side. In all types of follicles, immunoreactions for vWF were observed in the endothelial cells of capillaries as well as veins and arteries in the theca interna and externa. In the theca interna, the vWF-positive area was significantly (P<0.05) greater in advanced and late atretic follicles than in healthy and early atretic follicles at all regions except for the apical region of advanced atretic follicles. In the theca externa, vWF-positive area significantly (P<0.05) increased with a progress of atresia only at the apical region of follicles. However, in the late atretic follicles, the theca tissues and blood vessels did not show obvious histological disintegration. These results suggest that the increase of the vWF expression in the endothelial cells occurs before morphological changes of microvasculature appear in the atretic follicles. The functional modulation of microvasculature that is associated with the expression of vWF may be involved in the events of atresia.


Key words: Atresia, Endothelial cell, Follicle, Microvasculature, von Willebrand factor

Non-atretic healthy follicles have a uniformly distributed capillaries, whereas in the atretic follicles microvascular network surrounding avascular area that are occupied by the granulosa layers, oocyte and its investment, changes irregularly19). It is also reported that atretic follicles show a deficient theca layer and a reduction of the capillary network11,24). Recent studies have suggested that regression of atretic follicles is caused by cellular apoptosis and tissue disintegration15,16). Reduction of capillary network leading to the deficiency of blood supply may also be an important factor for the regression of atretic follicles.

It is known that von Willebrand factor (vWF) is a multimeric glycoprotein that is present in the plasma and subendothelium20) and mediates adhesion of platelet to the subendothelium at the sites of vascular injury and disorder29,31). This factor is synthesized exclusively by endothelial cells and megakaryocytes and stored within intracellular granules in both endothelial cells (Weibel-Parade bodies) and platelets (α-granules)6,23,31). Gidding et al.10) found that vWF in the endothelial cells stained by anti-vWF antibody more intensely in veins than in arteries. The expression of
vWF in the endothelial cells was significantly less in the immature blood vessels than in the mature one\textsuperscript{1}. Yamamoto et al.\textsuperscript{35} suggested that vWF in endothelial cells is differentially expressed among different tissues. High concentrations of vWF have been proposed to be a specific marker for endothelial damage\textsuperscript{2,17,33}, suggesting that synthesis and secretion of vWF is largely attributable to the damages of endothelial cells. Detachment of endothelial cells in the regressing blood vessels\textsuperscript{21} may cause the release of more amount of vWF. If the expression of vWF in the endothelial cells is enhanced before disintegration of follicular tissue and/or microvasculature, it may suggest that the changes in the functions of microvasculature is involved in the early events of atresia.

The aim of the present study was to determine whether changes in the localization of vWF in the follicular microvasculature occur during atretic process. The vWF in the thecal layer of healthy and atretic follicles was immunostained using anti-vWF antibody and the number of vWF positive-blood vessels and vWF positive area were compared to both types of follicles.

**Materials and Methods**

**Collection and Preparation of Tissues**

1) **Collection of ovaries**

Ovaries (n = 5) without cystic follicles were collected from mixed-age Holstein-Frisian cows at the slaughterhouses of the Meat Inspection Office in Onomichi city. One of the ovaries (right or left side) was collected from each cow. No macroscopic abnormality was observed in the reproductive tract of any of the cows. The ovaries were fixed with 10% (v/v) formalin in phosphate buffered saline (PBS, pH 7.4) and processed for paraffin sections. The sections (6μm thick) were air-dried on slides treated with 3-aminitriethoxysilane (Sigma Chemical Co., St Louis, MO, USA).

2) **Classification of follicles**

Follicles were morphologically classified as healthy or as one of three classes (early, advanced and late stages) of increasing degrees of atresia\textsuperscript{34}. Briefly, healthy antral follicles (approximately 2 to 5 mm in diameter) had an intact granulosa layers that revealed a compact and well organized appearance without degenerated cell and apoptotic bodies. Early atretic follicles (approximately 2 to 5 mm in diameter) had a few minor degenerative changes, such as the presence of a few pyknotic nuclei and/or partial destruction of the basement membrane. Compared to early atretic follicles, advanced atretic follicles were characterized by a greater destruction of follicular structure, more degenerative granulosa cells and/or a noticeable decrease in the number of granulosa cells. Follicles with more severe degenerative changes than advanced atretic follicles were classified as the late atretic follicles. To ascertain the atretic follicles, sections were also stained by terminal deoxynucleotidyl transferase (Tdt)-mediated biotinylated deoxyuridine triphosphates (dUTP) nick end-labeling (TUNEL) because apoptosis is a primary sign of atresia\textsuperscript{12,15,16}.

**Antibodies**

The antibodies used in the study were rabbit monoclonal antibody to human vWF (F3520, Sigma), VECSTAIN ABC-PO kit (Vector Lab. Inc., Burlingame, CA, USA) and alkaline phosphatase-conjugated anti-rabbit IgG (Sigma). The specificity of anti-vWF antibody was examined by western blot as follows.

Theca interna tissue was collected from atretic follicles. The samples were homogenized with homogenization buffer (1% (v/v) NP-40, 0.1% (w/v) sodium deoxycholate, 1% (v/v) phenylmethylsulfonyl fluoride, 0.15 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5) using a Polytron homogenizer (PT 1200; Kinematica AG, Switzerland) on ice. Homogenized tissue samples were centrifuged for 15 min at 6,200 x g (MRX-150; Tomy Seiko Co, Tokyo) and the supernatants were collected. The protein concentrations in each sample were determined using protein assay reagent (Bio-Rad, Hercules, CA).

Antigen sample (2.5 mg/ml) containing vWF was separated by electrophoresis on discontinuous SDS-polyacrylamide gels (4% upper gel and 5% lower gel). After electrophoresis the separated proteins in the lower gel were transferred electrophoretically onto nitrocellulose membrane (Hybond-ECL; Amersham International plc, Amersham) in transfer buffer (25 mM Tris, 192 mM glycine (pH 8.3) and 20% (v/v)
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Fig. 1. Western blots of von Willebrand Factor in theca interna of bovine atretic follicles. Ten micrograms of sample protein were loaded in a lane. Values show molecular weights of von Willebrand Factor specific bands.

Fig. 2. Illustration of 4 different regions in a follicle. Observation was conducted in 4 regions of each follicle, namely, apical, subapical, subbasal and basal regions.

methanol). The membrane was washed with western buffer (0.15M NaCl, 0.5% (v/v) Tween-20 and 0.05% (w/v) BSA, 1% (w/v) casein milk in 20mM Tris-HCl, pH 7.4) for 30 min. The membrane was incubated in antibody solution containing anti-human vWF antibody diluted in western buffer (1:1,200) for 1 h at room temperature. The membrane was washed and incubated with alkaline phosphatase-conjugated anti-rabbit IgG diluted to 1:5,000 in western buffer at room temperature for 1 h. After washing with western buffer, the immunoprecipitates on the membrane were visualized using a mixture of 0.03% nitroblue tetrazolium and 0.03% 5-bromo-4-chloro-3-indonyl phosphate in substrate buffer (0.1M Tris-HCl, pH 9.6, containing 1mM MgCl₂·6H₂O). The molecular weights of immunopositive protein bands were analyzed by a computer assisted software program (Gel-Pro Analyzer ; Media Cybernetics, The Image Experts, ML).

The antibody recognized 225kDa native subunits and two molecular masses (176, 189kDa) of vWF (Fig. 1). These three bands were comparable to the results of human vWF immunoblot that shown 3 specific bands\(^3\). \(^3\)

Immunohistochemistry for vWF and Image Analysis

1) Immunostaining

After being deparaffined with xylene, the sections were rehydrated through a graded series of ethanol, and were washed with PBS. The unmasking treatment was conducted by incubating the sections with 20µg/ml proteinase K (Sigma) in 10mM Tris-HCl, pH 7.4, at 37°C for 20 min. The sections were then incubated for 30 min with 10% (v/v) goat serum, and with 16µg/ml vWF antibody for 45 min. After washing with PBS for 10 min (5 min × 2 times), the immunoreactions on the sections were detected by using a VECSTAIN ABC-PO kit according to the manufacture’s protocol. Briefly, the sections were incubated with biotinylated goat-anti-rabbit IgG for 1 h and with avidin-biotin-peroxidase for 1 h. Immunoreaction products for vWF were revealed by incubating the sections with 3', 3' diaminobenzidine and H₂O₂. Finally, the sections were counterstained with hematoxylin. Control slides were prepared in an identical manner except that the first antibody was replaced with 1µg/ml normal rabbit IgG (Sigma).

2) Analysis of vWF-immunopositive area

Sections were examined under a light microscope and the populations of vWF positive blood vessels and vWF-positive area in the tissues were examined under an image analyzer with a computer system (Mac Aspect, Mitani Co., Fukui, Japan). Four follicles from different ovaries were selected randomly and examined in each group of follicles. Observation was conducted in 4 different regions in each follicle, namely, apical, subapical and subbasal (regions approximately 1/3 and 2/3 the distance from the apical region towards the basal region, respectively), and basal regions (Fig. 2). Analysis was made within the area of approximately 10,000 to 52,000µm², and was repeated 5 times in each region of a follicle.
number of positive blood vessels and positive area in 1 region of a follicle was obtained by the average of 5 counts. Frequencies of vWF-positive blood vessels and vWF-positive area were expressed as the number of positive vessels and as the positive area in 20,000 $\mu\text{m}^2$.

**Statistical Analysis**

Differences in the frequency of vWF-positive blood vessels and vWF-positive area among different regions of the follicle as well as among different types of follicles were analyzed by one-way ANOVA, followed by Duncan’s multiple range test\(^7\). A probability of $P<0.05$ was considered to be statistically significant.

**Results**

In the healthy follicles, immunoreactions for vWF
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Fig. 5. Light micrographs of the bovine late atretic follicle immunostained for anti-von Willebrand factor. (a) apical region. (b) basal region. Arrowheads show examples of vWF-positive blood vessels. TI: theca interna, TE: theca externa. Bars = 50 μm.

Fig. 6. Differences in number of von Willebrand factor-positive blood vessels in the theca interna of bovine healthy and atretic follicles. □, Healthy follicles; ■, Early atretic follicles; □, Advanced atretic follicles; ■, Late atretic follicles. Bars represent the mean ± SEM (n = 4). Values with different letters (a, b) within each region of a follicle are significantly different (P < 0.05).

 were observed in the endothelial cells of capillaries as well as veins and arteries in the theca interna and externa (Fig. 3a, b). Some of the vessels in these tissues were negative for vWF immunoreactions. In the early and advanced atretic follicles, degenerated or apoptotic cells were observed in the granulosa layers, whereas the theca layers and blood vessels did not show degenerated structure (Fig. 4a, b). However, an increased number of blood vessels were stained positive for vWF in the theca layers of both apical and basal regions of the advanced atretic follicles. In the late atretic follicle, the theca tissues and blood vessels did not show significant structural disintegration, but a large number of blood vessels reacted for vWF-antibody (Fig. 5a, b).

Figure 6 shows the number of vWF-positive blood vessels in the theca interna of healthy and atretic follicles. In all regions of a follicle, the numbers of
vWF-positive blood vessels were lower in the healthy follicle than in each type of atretic follicles. The numbers of vWF-positive blood vessels showed a tendency to increase as the atretic stages proceeds, and there were significant (P<0.05) differences in the number between healthy and late atretic follicles at the apical and basal regions. Advanced atretic follicle at the basal regions also showed a significantly greater number of vWF-positive blood vessels than that in healthy follicle at the same region.

The vWF-positive area in the theca interna of healthy and atretic follicles is shown in Fig. 7. The area stained positive for vWF was small in the healthy follicles, whereas it was markedly increased with a progression of atresia at all regions. The area was significantly (P<0.05) greater in advanced and late atretic follicles than in healthy and early atretic follicles at all regions except for the apical region of advanced atretic follicles. The area stained positive for vWF in the advanced atretic follicle was significantly (P<0.05) greater at the basal region than that at the apical region.

In the theca externa, the number of vWF-positive
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Table 1. Summary of the numbers of von Willebrand factor (vWF)-positive blood vessels and vWF-positive area in the theca interna and externa of bovine healthy and atretic follicles

<table>
<thead>
<tr>
<th>Follicular type</th>
<th>Theca interna</th>
<th>Theca externa</th>
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<tbody>
<tr>
<td></td>
<td>Number of</td>
<td>vWF-positive area</td>
</tr>
<tr>
<td></td>
<td>vWF-positive blood vessels</td>
<td>Apical</td>
</tr>
<tr>
<td>Healthy</td>
<td>+ 1)</td>
<td>+</td>
</tr>
<tr>
<td>Early atretic</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Advanced atretic</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Late atretic</td>
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1) Numbers of vWF-positive blood vessels and vWF-positive area were expressed. ± = small, + = moderate, ++ = large.

vessels at apical region was significantly (P<0.05) greater in the late atretic follicles than in the healthy follicles (Fig. 8). However, there was no significant difference in the frequency of vWF positive blood vessels among follicles in subapical, subbasal and basal regions.

Figure 9 shows the vWF positive area in the theca externa of healthy and atretic follicles. In healthy and early atretic follicles the positive area was significantly (P<0.05) greater in the basal region than in the apical region. At the apical region, vWF positive area significantly (P<0.05) increased with a progress of atresia (healthy and late atretic follicles, P<0.05).

The data in Fig. 6–9 are summarized in Table 1. Late atretic follicles had the highest values in all parameters among all types of follicles. The numbers of vWF-positive blood vessels and vWF-positive area in both the theca interna and externa were greatest in late atretic follicles.

Discussion

The present findings are that the vWF-positive regions in the microvasculature and the frequencies of vWF positive blood vessels increased in the theca layers with a progress of follicular atresia.

During the regressive process of blood vessels, the endothelial cells are detached from their basement membrane21). Thrombosis or hemostasis needs vWF at the site where subendothelium emerges. However, it is also reported that vWF increases with maturation of vessels, even if they are healthy31). The current study showed that vWF area in the theca interna layer of healthy follicles was greater in the basal region than in the apical region. Microvasculature in the follicular wall develops with the follicular growth, which was probably stimulated by angiogenic factor produced in the follicular tissue27,28). A greater population of vWF at the basal region of follicles may suggest that blood vasculature may be more matured in the basal than in the apical region in the healthy follicle.

The frequency of vWF-positive blood vessels and vWF positive area in the theca interna were greater in advanced and late atretic follicles than in healthy ones at both apical and basal regions. Also, those in the theca externa were greater in the late atretic follicle than in the healthy ones at the apical region. In contrast, morphological observation did not show significant disintegration of thecal tissues and blood vessels in atretic follicles. It is reported that vWF is increased in the endothelial cells where blood vessels are undergoing disorder or damage2,33). Thus, the thecal microvasculature is likely to be functionally disordered before morphological changes occur. Imbalance of many factors such as gonadotropins, sex steroids and cytokines has been suggested to be responsible in the induction of atresia22,37). The present
results suggest that changes in the blood supply that is caused by the functional disorder of microvasculature system may be also a significant factor that occurs in the atresia. Decrease in cell proliferation and increase of apoptosis have been shown in the early atretic process. The present results on the changes in vWF distribution are temporally well associated with these features of follicular cell functions.

The changes of vascularization are dependent upon the secretion of the angiogenic factors from ovarian tissue. Vascular endothelial growth factors (VEGF) are reported to be one of the major angiogenic factors in the ovary, and the production of which declines in the atretic follicles. Furthermore, atretic follicles have lower competence to produce estradiol than do the healthy follicles. Blood vessels possess the receptors for the ovarian steroid such as 17β-estradiol and progesterone, and 17 β-estradiol stimulates the endothelial cell proliferation. Moreover, estrogen is involved in the acute angiogenesis during the growth of several types of cancer. These results strongly suggest that endothelial proliferation and angiogenesis was attenuated by the decline of 17β-estradiol production in the atretic follicles. Thus, decline of intraovarian factors regulating the angiogenesis such as VEGF and estrogen may be one of the factors responsible for the microvasculature disorder with increase in vWF.

In conclusion, it was found that the expression of vWF was enhanced in the endothelial cells of the advanced and late atretic follicles compared to that in the healthy follicles, whereas visible disintegration of thecal tissue and microvasculature were not detected in the advanced and late atretic follicles. These results suggest that the increase of the vWF expression in the endothelial cells occurs before morphological changes of microvasculature appear in the atretic follicles. The functional modulation of microvasculature that is associated with the expression of vWF may be involved in the events of atresia.

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