Involvement of follicular basement membrane and vascular endothelium in blood–follicle barrier formation of mice revealed by ‘*in vivo* cryotechnique’

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Abstract

The molecular sieve with size- and charge selectivity in ovarian follicles, the so-called blood–follicle barrier (BFB), was examined during follicular development under physiological conditions to reveal ovarian structures responsible for the BFB by using our ‘*in vivo* cryotechnique’ (IVCT). Mouse ovary specimens were prepared with different methods including IVCT, immersion, or perfusion chemical fixation and quick-freezing following resection or perfusion. Their paraffin sections or cryosections were stained with hematoxylin–eosin or immunostained for serum proteins with different molecular weights: albumin, immunoglobulin (Ig) G1 heavy chain, inter- α -trypsin inhibitor (α_1 I), fibrinogen, and IgM. Their immunoreactivity was better preserved with IVCT. The immunostaining for albumin was clearly observed in blood vessels, interstitium, and developing follicles, but that of IgG1, α_1 I, or fibrinogen was significantly decreased inside the follicles. IgM was immunohistochemically decreased throughout the interstitium outside blood vessels. The immunoreactivities of IgG1 and IgM, as compared with albumin, were clearly changed along follicular basement membranes and around vascular endothelial cells respectively. These findings indicate that BFB functions throughout follicular development, and the follicular basement membrane and the vascular endothelium could play some significant roles in the permselectivity for such soluble proteins with intermediate and high molecular weight respectively.

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Introduction

Ovarian follicles of living animals have been known to be dynamic structures that undergo a gradual increase in volume during the various phases preceding ovulation, and the follicular development is always accompanied by significant structural changes of perifollicular capillary networks (Macchiarelli 2000). The perifollicular capillary networks are initially formed when the membrana granulosa of secondary follicles has developed into a multilayered follicle cell population. Furthermore, they also develop a single-layered to multilayered thick theca interna, as the secondary follicles grow up to larger or mature Graafian follicles (Kanzaki *et al.* 1982, Kitai *et al.* 1985, Kranzfelder & Maurer-Schultze 1989, Yamada *et al.* 1994, Macchiarelli 2000, Jiang *et al.* 2002). These morphological changes of the follicular microvasculature have shown an urgent need for increased blood supply and nutritional support systems during the follicular development and oocyte maturation. In living animal ovaries, follicular fluid, which contributes to a

microenvironment within the avascular compartment of follicles, is usually produced not only by secretion from the membrana granulosa cells but also by serum protein diffusion from the thecal capillaries during the follicle development (Donahue & Stern 1968, Shalgi *et al.* 1973, Andersen *et al.* 1976). In the last year, a proteomic analysis revealed that a large number of 210 proteins in the human follicular fluid, including transferrin, ceruloplasmin, afamin, hemopexin, haptoglobin, and plasma amyloid protein, were acute inflammatory phase proteins, which actually supports a hypothesis that the common ovulation is comparable to an inflammatory event (Angelucci *et al.* 2006). They also identified a number of important antioxidant enzymes, including catalase, superoxide dismutase, glutathione transferase, paraoxonase, heat shock protein 27, and protein disulfide isomerase, which probably protect the follicle maturation from various toxic injuries and oxidative stresses. Therefore, the follicular fluid is now assumed to nearly resemble the blood plasma, thereby providing the

developing oocytes with the microenvironment that contains some necessary endocrine and non-endocrine growth factors (Angelucci *et al.* 2006). However, several earlier studies have already described definite differences in both protein compositions and concentrations between the follicular fluid and the blood serum, which led to a proposal of the blood–follicle barrier (BFB; Zachariae 1958, Shalgi *et al.* 1973). They have described the BFB as a ‘molecular sieve’ with size selectivity consisting of the capillary endothelium, the subendothelial basal lamina, the theca interna, the follicular basement membrane, and the membrana granulosa; the BFB allowed low-molecular weight proteins (500 kDa) to enter the follicular fluid, but blocked the entrance of higher molecular components or larger particles, such as colloidal gold (1000 kDa) (Shalgi *et al.* 1973, Cran *et al.* 1976). Some subsequent studies have also revealed another charge selectivity and involvement of nitric oxide in the functional mechanism of BFB, and so putative significance of BFB was also proposed (Powers *et al.* 1995, Hess *et al.* 1998, Zhuo & Kimata 2001). However, in spite of such accumulating pieces of evidence about the BFB properties, morphological data of BFB under physiological conditions *in vivo* are still limited, because the conventional morphological studies on BFB have been often made with animal models of artificially induced ovulation under experimental conditions. Furthermore, the significant structures within the follicles that contribute to the BFB during the routine follicular development still remain to be elucidated.

Although the morphology and molecular distribution of animal ovaries have been examined by various preparation techniques, each technique has some limitations for morphofunctional and immunohistochemical analyses of cells and tissues. Among them, the conventional preparation procedures, such as perfusion- or immersion fixation (IM) with chemical fixatives, often produce morphological artifacts caused by anoxia of tissue resection, artificial perfusion pressures with fixatives, and dehydration shrinkage with organic solvents (Kellenberger 1991, Hippe-Sanwald 1993, Shiurba 2001, Li *et al.* 2005, Ohno *et al.* 2006, Zhou *et al.* 2007). For overcoming these problems, our ‘*in vivo* cryotechnique’ (IVCT) has been developed since 1996 to directly clarify functioning morphology and immunodistribution of molecular components in cells and tissues of living animal organs (Ohno *et al.* 1996, Terada *et al.* 2006a). The IVCT is a kind of cryofixation method in which target organs of living animals are directly frozen *in vivo* without tissue resection or perfusion fixation (PF), thus enabling us to capture transient changes of tissue and cell morphology under different hemodynamic conditions and also molecular changes such as rapid phosphorylation of signaling molecules (Ohno *et al.* 2005a, Terada *et al.* 2006b). In addition, it was already reported with our several studies that the IVCT followed by

freeze-substitution very efficiently retained soluble extra- and intracellular molecules within tissues and cells, and their clear localizations or dynamic structural changes *in vivo* were histochemically or immunohistochemically revealed on paraffin-embedded sections (Zea-Aragon *et al.* 2004, Terada *et al.* 2005, Li *et al.* 2006a, Liao *et al.* 2006, Ohno *et al.* 2006).

In the present study, we have morphologically and immunohistochemically examined living mouse ovaries by the IVCT and focused on the actual clarification of their BFB under normal blood circulation. As the immunolocalizations of different serum proteins in blood vessels, interstitium, and ovarian follicles could be clearly detected with the IVCT, the BFB appeared to exist from early stages of normal folliculogenesis in the living mouse ovaries. Also, the follicular basement membranes in addition to the vascular endothelium were suggested to play some significant roles in the formation of BFB against serum proteins, depending partly on their molecular sizes.

Material and Methods

Tissue preparation methods

All animal experiments were performed in accordance with the guidelines by the Animal Care and Use Committee, University of Yamanashi. Each cycle of all mice was checked by the routine vaginal smear examination, and the selected mice at proestrus to estrus stages were always used for further analyses. Ovarian tissues of 20 C57BL/6 mice aged 8–9 weeks, which were anesthetized with inhalation of diethyl ether, were prepared by the following different methods. i) IM and alcohol dehydration (IM–DH): the ovarian tissues of two mice were surgically resected and immersed in 0.1 M phosphate buffer solution containing 2% paraformaldehyde (PFA; pH 7.4) overnight. ii) PF–DH: two mice were transcidentally perfused with the similar buffered 2% PFA, and their ovarian tissues were resected and immersed in the same fixative overnight. iii) Conventional quick-freezing for fresh resected tissues (FQF): the ovarian tissues were surgically removed from two mice and quickly frozen by plunging into the isopentane–propane cryogen (−193 °C), as described before (Ohno *et al.* 2004). iv) PF–QF: two mice were transcidentally perfused with the buffered 2% PFA as described above, and their ovarian tissues were resected and quickly frozen as in FQF. v) IVCT: the isopentane–propane cryogen was poured over the carefully exposed ovaries of 12 mice, as reported before (Ohno *et al.* 2006), and then the completely frozen tissues were removed with a dental electric drill in liquid nitrogen (−196 °C). All the specimens prepared by the methods (i–v) were additionally treated with the conventional DH (i and ii) or the routine freeze-substitution with 2% PFA (iii–v) and embedded in either paraffin wax or optimal cutting

temperature (OCT) compound, as described previously (Ohno *et al.* 2004, Terada *et al.* 2005). Briefly, in the freeze-substitution, freeze-substitution solution was first prepared by adding 20% (w/v) PFA to pure acetone to finally become 2% PFA, followed by complete dehydration with sufficient amounts of Molecular Sieves 3A (Nacalai Tesque, Kyoto, Japan). The frozen ovarian specimens were incubated in the PFA–acetone solution cooled down at about -80°C in dry ice–acetone for 48 h, and gradually warmed up to room temperature. Thereafter, the specimens were either infiltrated with xylene for the common paraffin embedding or rehydrated with PBS containing 30% sucrose for embedding in OCT compound.

Immunohistochemistry of serum proteins

All paraffin-embedded samples were cut at 3 μm thickness and mounted on glass slides treated with 3-aminopropyltriethoxysilane (Nacalai Tesque). The cut sections were then deparaffinized with xylene and a graded series of ethanol. For histological analyses, some serial sections were routinely stained with hematoxylin–eosin (HE). For immunohistochemical analyses, others were incubated with 1% hydrogen peroxide in the PBS for 1 h and then 5% normal rabbit serum (NRS) in PBS for 1 h. They were immunostained with various primary antibodies in PBS containing 5% NRS at 4°C overnight. The primary antibodies were as follows: goat anti-mouse albumin antibody at dilution of 1:5000, goat anti-mouse immunoglobulin (Ig) G1 heavy chain antibody at dilution of 1:500, and goat anti-mouse IgM antibody at dilution of 1:500. They were all purchased from Bethyl Laboratories (Montgomery, TX, USA). The immunostained sections were then incubated in biotin-conjugated rabbit anti-goat IgG (Vector Laboratories, Burlingame, CA, USA) at room temperature for 1 h. The immunoreaction products were visualized with Vectastain ABC reagent (Vector Laboratories) and metal-enhanced DAB substrate kit (Pierce, Rockford, IL, USA), and additionally fixed with 0.04% osmium tetroxide solution as described before (Ohno *et al.* 2005b). All immunostained sections were counterstained with methylgreen, embedded in glycerol, and observed with a light microscope (BX-61; Olympus, Tokyo, Japan).

For the double immunolabeling study, cryosections at 6 μm thickness and also deparaffinized sections on the glass slides were blocked with 2% gelatin (Sigma) in PBS for 1 h and immunostained with the primary antibodies at 4°C overnight as follows: i) goat anti-mouse albumin antibody at dilution of 1:4000 and rabbit anti-human fibrinogen antibody (Dako Japan, Kyoto, Japan) at dilution of 1:500 or rabbit anti-human inter- α -trypsin inhibitor ($\text{I}\alpha\text{I}$) antibody (Dako Japan) at dilution of 1:400; and ii) goat anti-mouse type IV collagen antibody (Southern Biotechnology Associates, Birmingham, AL, USA) at dilution of 1:50 and rabbit anti-mouse albumin antibody (Bethyl Laboratories) at dilution of 1:4000 or

rabbit anti-mouse IgGfc antibody (Bethyl Laboratories) at dilution of 1:500. They were then incubated with donkey anti-rabbit IgG antibody coupled with Alexa Fluor 488 at dilution of 1:400, donkey anti-goat IgG antibody coupled with Alexa Fluor 546 at dilution of 1:400, and To-pro-3 at dilution of 1:500 (Invitrogen) with 2% gelatin in PBS at room temperature for 1 h. The immunostained cryosections were embedded in Vectashield (Vector Laboratories) and observed with a confocal laser scanning microscope (FV1000; Olympus).

Double fluorescence labeling with periodic acid-Schiff (PAS) and immunostaining

We used the fluorescence emission of PAS staining for fluorescent detection of basement membranes around blood vessels because the PAS-fluorescence emission could be most effective to visualize the PAS-stained glomerular basement membrane (Li *et al.* 2006b). First, 3 μm thick deparaffinized sections were routinely stained for PAS, as described previously (Li *et al.* 2006b). Subsequently, all the sections were incubated with 5% gelatin in PBS for 1 h and then with the goat anti-mouse IgM antibody at 4°C overnight. They were then incubated with the donkey anti-goat IgG antibody coupled with Alexa Fluor 488 and To-pro-3 for 1 h, and embedded in Vectashield. All sections stained with PAS reaction were examined with FV1000 (Olympus), as described before (Li *et al.* 2006b).

Results

Immunolocalization of albumin in ovarian follicles

We have first examined the immunolocalization of albumin (Fig. 1b, d, f, h and j) in ovarian tissues prepared with IVCT (Fig. 1a and b), FQF (Fig. 1c and d), PF–DH (Fig. 1e and f), IM–DH (Fig. 1g and h), and PF–QF (Fig. 1i and j) on the serial sections, corresponding to the areas with HE staining (Fig. 1a, c, e, g and i). In the specimens prepared with IVCT, immunoreactivity of albumin was detected in blood vessels (Fig. 1a and b, arrowheads), interstitium (Fig. 1b, thin arrows), and ovarian follicles (Fig. 1b, thick arrows). Immunostaining for albumin was also observed in zona pellucida around the oocyte. The immunolocalization of albumin observed with IVCT was similar to that with FQF (Fig. 1c and d); albumin was clearly immunolocalized in both interstitium (Fig. 1d, thin arrow) and follicles (Fig. 1d, thick arrows) in addition to blood vessels. To the contrary, in the specimens prepared with PF–DH (Fig. 1e and f) or IM–DH (Fig. 1g and h), less immunoreactivity of albumin was detected inside the ovarian follicles (Fig. 1f and h, thick arrows), although previous biochemical studies have already reported large amounts of albumin in the follicular fluid (Shalgi *et al.* 1973, Collins *et al.* 1997). Moreover, by PF–QF, some diffuse immunostaining for

Figure 1 Light micrographs of hematoxylin–eosin (HE) staining (a, c, e, g and i) and immunostaining for serum albumin (Alb; b, d, f, h and j) on serial sections prepared by *in vivo* cryotechnique under normal circulation (N-IVCT, a and b), quick-freezing of resected fresh tissues (FQF, c and d), perfusion fixation (PF–DH, e and f) or immersion fixation (IM–DH, g and h) followed by alcohol dehydration and perfusion fixation followed by quick-freezing (PF–QF, i and j). (a and b) In the specimens prepared by N-IVCT, the immunoreactivity of albumin is detected in blood vessels pointed with black arrowheads in (a) (b, arrowheads), interstitium (b, thin arrows), and ovarian follicles (b, thick arrows). Immunostaining is also observed in zona pellucida. (c–h) The immunolocalization is similar to that in the specimens prepared by FQF (d, thin or thick arrows). In the specimens prepared by PF–DH (e and f) or IM–DH (g and h), the immunoreactivity of albumin decreases inside the ovarian follicles (f and h, thick arrows), as compared with that in the interstitium (f and h, thin arrows). (i and j) By PF–QF, diffuse immunostaining for albumin can be detected inside an oocyte and some granulosa cells (j, arrowheads). O, oocytes. Bars, 50 μm.

albumin could be detected not only in extracellular spaces but also inside cytoplasm of some oocytes and granulosa cells (Fig. 1j, arrowheads). We also examined immunolocalizations of the other serum proteins including IgG1 and IgM and found their decreased immunoreactivity in PF–DH and IM–DH or similar immunolocalization in PF–QF (data not shown). These findings indicated that the immunodistribution of serum proteins *in vivo* were significantly affected during preparation procedures of organ perfusion or chemical fixation followed by DH, and the IVCT would be more useful for immunohistochemical analyses of soluble serum proteins in living mouse ovaries.

Immunohistochemical examination of BFB in combination with IVCT

Next, serial paraffin sections prepared with IVCT were stained with HE or immunostained for three serum proteins including albumin with about 69 kDa molecular weight, IgG1 with about 150 kDa molecular weight, and IgM with about 900 kDa molecular weight, and their immunoreactivities were compared around follicles in different stages. In the present study, the following follicular classification was used: the primordial follicle is composed of a primary oocyte and a single layer of flat follicular cells surrounding the oocyte; the early primary follicle displays a single layer of cuboidal follicular cells that surround the primary oocyte; the late primary follicle displays a stratified layer around the oocyte; the secondary or antral follicle shows more layers of follicular cells, and some antrum appears in the intercellular spaces; the mature or Graafian follicle is characterized by the presence of a large fluid-filled antrum (Ross *et al.* 1989, Gartner & Hiatt 1994). In a late primary

follicle on the serial sections (Fig. 2), all three proteins were clearly immunolocalized in blood vessels (Fig. 2, white arrowheads). In addition, immunostainings for both albumin (Fig. 2b, black arrowhead) and IgG1 (Fig. 2c, black arrowhead) were detected in the interstitium, but IgG1 alone was more weakly immunostained inside the primary follicle (Fig. 2c, black arrow). To the contrary, immunostaining for IgM was decreased in follicles, interstitium, and theca layers, and was hardly detected along the zona pellucida (Fig. 2d, white arrow), although it was strongly observed inside blood vessels of the theca layers (Fig. 2d, white arrowheads). Double fluorescence labeling of IgM with PAS-positive basement membranes around blood vessels is shown in Fig. 6. From these findings, it is suggested that the albumin can easily enter ovarian follicles, but either IgG1 or IgM was blocked around the follicles and the blood vessels respectively.

We also examined these immunohistochemical findings of serum proteins in other stable or developing follicles, such as primordial or antral ones (Fig. 3a–h). The similar immunoreactivity of three serum proteins was observed inside blood vessels around those follicles (Fig. 3a–h, white arrowheads). The albumin immunostaining was detected in them (Fig. 3b and f, black arrows). Moreover, immunostainings for IgG1 and IgM were weakly detected inside those follicles (Fig. 3c, d, g and h, black arrows), and also that for IgM was decreased in the theca layers and interstitium (Fig. 3d and h, black arrowhead), as inside or around the primordial or antral follicles. To the contrary, the stronger immunoreactivity of the three serum proteins was usually observed in the ovulated follicles (Fig. 4a–d), and no significant difference of their immunoreactivity was observed among blood vessels, interstitium, and follicles. This

Figure 2 Serial paraffin sections of late primary follicle in living mouse ovaries prepared by *in vivo* cryotechnique and stained with hematoxylin–eosin (HE, a) or immunostained for albumin (Alb, b), immunoglobulin G1 (IgG1, c), or IgM (d). The areas around blood vessels are twice magnified in the insets. In the late primary follicle, multiple layers of follicular cells surround the oocyte. The strong immunoreactivity of three serum proteins is clearly detected inside blood vessels (a–d, white arrowheads). Although the immunostainings for albumin (b, black arrowhead) and IgG1 (c, black arrowhead) are clearly detected in the theca layers and interstitium of ovary, that for IgG1 is weakly seen inside ovarian follicles (c, black arrow). Another immunostaining for IgM is decreased in the interstitium and theca layers (d, black arrows) and hardly detected in the follicles (d, white arrow), although it is strongly observed inside the blood vessels of theca layers (d, white arrowheads). Bars, 50 μ m.

Figure 3 Serial paraffin sections of primordial (a–d) and antral follicles (e–h) in living mouse ovaries prepared by *in vivo* cryotechnique and stained with hematoxylin–eosin (HE, a and e) or immunostained for albumin (Alb, b and f), immunoglobulin G1 (IgG1, c and g), or IgM (d and h). The areas around blood vessels are twice magnified in the insets. The primordial follicles (b–d, black arrows) are composed of a single layer of flat follicular cells surrounding the oocyte, and antral follicles have more layers of follicular cells and antrum among them. The strong immunoreactivities of three serum proteins are detected inside blood vessels (a–h, white arrowheads). The immunostainings for IgG1 and IgM are weakly seen inside the follicles (c, d, g and h, black arrows), and the IgM immunostaining is also decreased in the theca layers and interstitium (d and h, black arrowheads), as in or around the late primary follicles. Bars, 50 µm.

possible destruction of BFB upon ovulation stimuli has been already reported in the animal ovulation model (Powers *et al.* 1995, Hess *et al.* 1998).

We also examined immunolocalizations of I α I (with 220 kDa molecular weight) or fibrinogen (with 340 kDa molecular weight) in developing ovarian follicles on cryosections prepared with IVCT (Fig. 5), because previous studies indicated that distributions of serum proteins with middle-sized molecules were largely affected by BFB in mouse ovaries (Hess *et al.* 1998). In our findings, immunoreactivities of I α I, fibrinogen, and albumin were clearly detected in the interstitium and theca layers of ovaries (Fig. 5a, b, d–h, arrows), but immunoreactivities of both I α I and fibrinogen were more

weakly detected inside the ovarian follicles, as compared with the albumin immunoreactivity (Fig. 5a, d, e and h, arrowheads).

We further examined the soluble and structural components crucial for BFB around blood vessels and ovarian follicles by using double immunofluorescence labeling of albumin (Fig. 6a, d and e) or IgGfc (Fig. 6f, i and j) and type IV collagen, a major structural component of basement membranes (Fig. 6b and g). We also performed another immunofluorescence labeling for IgM (Fig. 6k, n and o) with PAS-fluorescence emission (Fig. 6l, n and o) in living mouse ovaries prepared by IVCT. The PAS-fluorescence emission in the tissue specimens has been already used for the clear visualization of glomerular

Figure 4 Serial paraffin sections of ovulated follicles in living mouse ovaries prepared by *in vivo* cryotechnique and stained with hematoxylin–eosin (HE, a) or immunostained for albumin (Alb, b), immunoglobulin G1 (IgG1, c), or IgM (d). The strong immunoreactivities of the three serum proteins are clearly detected not only inside the blood vessels (white arrowheads) but also outside the blood vessels in the ovulated follicles (black arrows). Bars, 50 µm.

basement membranes of living mouse kidneys (Li *et al.* 2006b). The immunoreactivity of albumin in the ovarian follicles, theca layers, and interstitium was almost similarly detected in living mouse ovaries (Fig. 6a; black arrow and black arrowhead), which was bordered by the type IV collagen-immunopositive basement membrane (Fig. 6b, d and e, white arrowheads). Although another immunostaining for IgGfc was clearly detected in the theca layers and interstitium of living mouse ovaries (Fig. 6f; black arrow), it was more weakly detected inside ovarian follicles (Fig. 6f, i and j, black arrowhead), and the basement membrane immunopositive for type IV collagen (Fig. 6f, g, i and j, white arrowheads) bordered the immunoreactivity changes of IgGfc (Fig. 6f and i, black arrowheads). The immunoreactivity of IgM was detected exclusively in blood vessels around the theca interna (Fig. 6k and o, black arrowheads), but it was not detected in the ovarian follicles and interstitium (Fig. 6k, black arrow). At higher

magnification, the immunolocalization of IgM was mostly restricted along endothelial cells of the blood vessels surrounded by PAS-positive basement membranes (Fig. 6o; white arrowheads). From these findings, it is suggested that the BFB plays some selective functions throughout the follicular development until ovulation at the final stage of follicles. Furthermore, the *in vivo* permeability of large molecules, such as IgM, is blocked at the endothelial layers of blood vessels in living mouse ovaries, but the follicular basement membranes are playing some crucial roles for efficient inhibition in permselectivity, which means selectivity of molecular permeation, of middle-sized molecules, such as serum IgG.

Discussion

As initially described over 40 years ago, the BFB has remained to be a biological and clinical subject for

Figure 5 Double immunofluorescence labeling of albumin (b, d, f and h, red) and IαI (a and d, green) or fibrinogen (e and h, green) in antral follicles of living mouse ovaries on cryosections prepared with *in vivo* cryotechnique. The cellular nuclei are labeled with To-pro-3 (c, d, g and h, blue). Although the immunostainings for IαI, fibrinogen, and albumin are detected in the theca layers and interstitium of living mouse ovaries (a, b, d, e, f and h, arrows), the IαI and fibrinogen immunostainings are weakly seen inside the follicles, as compared with those for albumin (a, d, e and h, arrowheads). F, ovarian follicle. Bars, 20 µm.

Figure 6 Double immunofluorescence labeling of type IV collagen (b, d, e, g, i and j, red) and albumin (a, d and e, green) or immunoglobulin G (IgGfc, f, i and j, green) and immunofluorescence staining for IgM (k, n and o, green) with periodic acid-Schiff staining fluorescence emission (PAS, l, n and o, red) around antral follicles of living mouse ovaries prepared by *in vivo* cryotechnique. The cellular nuclei are labeled with To-pro-3 (c–e, h–j and m–o, blue), and the areas represented by rectangles in (d, i, and n) are highly magnified in (e, j, and o) respectively. (a–e) The immunoreactivities of albumin are similarly detected inside the follicle (a, d and e, black arrowheads) and in the theca layers and interstitium (a, d and e, black arrows). The border between them is marked by the basement membrane (a, b, d and e, white arrowheads). (f–j) Although the immunostaining for IgGfc is clearly detected in the interstitium of ovaries (f, i and j; black arrows), it is weakly detected inside the antral follicle (f, i and j, black arrowheads). The basement membrane (f, g, i and j, white arrowheads) borders the strong immunoreactivity of IgGfc. (k–o) The immunoreactivity of IgM is clearly seen in the blood vessels of theca interna (k, n and o, black arrowheads), while it is not detected inside the antral follicle (k, black arrow). The immunolocalization of IgM is mostly restricted inside the endothelial cells in the blood vessels surrounded by PAS-positive basement membranes (o, white arrowheads). Bars, 10 µm.

developing ovarian follicles and some ovarian diseases. The microscopic analyses with non-physiological tracers, i.e. colloidal gold and Evans Blue dye, led to a suggestion that BFB acting like a molecular sieve was permeable only for exogenous proteins under 500 kDa in molecular size (Cran *et al.* 1976). Later, almost 10 years ago, Hess *et al.* (1998) provided their physiological evidence for the actual existence and regulation mechanism of BFB in an animal ovulation model,

indicating that the BFB would be charge- and size selective in living mouse ovaries. However, the detailed structural properties of BFB and also how it affects the passage of serum proteins *in vivo* have remained unclear in past decades. Although a number of biochemical and morphological studies have been reported to describe such BFB existence, the present study is the first to demonstrate immunohistochemically the *in vivo* permeability of BFB against endogenous serum proteins

with different molecular sizes during follicular development and also the histological layers responsible for the actual barrier functions of BFB.

In our studies, serum proteins with low molecular sizes, such as albumin, were abundantly immunolocalized in ovarian follicles at different developing stages, but those with intermediate molecular sizes, such as IgG1, α_2 I, and fibrinogen, were more weakly detected in the ovarian follicles. To the contrary, the high molecular weight of IgM was almost totally blocked at the basement membrane of the ovarian follicles. These molecular differences in immunolocalizations of serum proteins undoubtedly support a previous idea that the molecular sizes are responsible for the BFB selectivity (Hess *et al.* 1998). Additionally, in the present study, the follicular basement membrane of living mouse ovaries was found to play some functional roles in the permselectivity for serum proteins, such as IgG1, with the intermediate molecular sizes. On the other hand, endothelial cells of the blood vessels in ovaries were responsible for blocking the passage of serum proteins, such as IgM, with high molecular sizes. Since intensities of immunostaining for different molecules depend on nonlinear saturation of different antibodies, it should be noted that relative contribution of these different structures to BFB selectivity remained unclear in the present study. However, as different intensities of immunostaining on single histological sections depend on the amounts of antigens, the relative immunoreactivity difference of each molecule among various structures of ovary, observed on the single sections in the present study, suggested significant involvement of both follicular basement membranes and endothelial cells in BFB. It is generally accepted that endothelial cells of blood vessels in other organs usually control the passage of soluble serum components and circulating cells from the blood vessels to tissue parenchyma, and also that intercellular junctions closely mediate both adhesion and communication between adjoining endothelial cells (Bazzoni & Dejana 2004, Orlova *et al.* 2006). The junctional complexes are generally composed of tight junctions, adherens junctions, and gap junctions, and the previous studies already showed that the tight junctions were more important for the strict control of endothelial permeability (Aijaz *et al.* 2006, Bazzoni 2006). The expression and organization of these junctional complexes usually depend on the type of blood vessels and the permeability requirements of functioning organs (Madara 1989). For instance, such intercellular junctions are well developed in the central nervous system, forming the widely known 'blood-brain barriers' (Wolburg & Lippoldt 2002), whereas post-capillary venules, which allow dynamic trafficking of circulating cells and soluble proteins, often display poorly organized tight junctions (Dvorak *et al.* 1995). To the contrary, the intercellular communication between endothelial cells in living mouse ovaries is

not well understood in terms of molecular permeability against soluble serum proteins. Although one previous study clarified the endothelial blockage of middle-sized serum proteins, which was controlled by nitric oxide in ovaries (Powers *et al.* 1995), the present study showed abundant immunolocalizations of such middle-sized serum proteins in the ovarian interstitium and indicated that the ovarian endothelial cells possess much less strict selectivity, which more efficiently blocks the serum proteins with large molecular sizes. This morphofunctional discrepancy may be partly explained by the difference of tissue preparation methods, because we frequently detected the modified immunoreactivity of serum proteins within some blood vessels of ovarian tissues prepared by IM-DH or PF-DH. The components and structures of intercellular communication between vascular endothelial cells of ovaries, which regulate the limited passage of larger molecules, would be revealed at an electron microscopic level in the future studies with IVCT.

On the other hand, it is generally accepted that the basement membrane of blood vessels in some animal organs can act as a molecular sieve with pore sizes determined by the charge and spatial arrangement of its components, such as type IV collagen, laminin, and heparan sulfate proteoglycans (Holmquist *et al.* 2004, Farquhar 2006). In the previous studies, it was already shown that certain basement membranes around blood vessels prevented leakage of large serum proteins (Ohlson *et al.* 2000, Maina & West 2005). At the glomerular capillary loops of kidneys, the basement membranes prevented filtration of certain serum proteins for urine production. They also permitted gaseous diffusion without passage of serum proteins through the blood capillaries of animal lungs. Our findings indicated a molecular sieve function of follicular basement membranes in living mouse ovaries, which would regulate some metabolic exchanges between the inside and outside of the ovarian follicles during their development. Indeed, it was already reported that the follicular basement membrane might seal the extracellular matrix in the developing ovarian follicles during their rapid volume expansion (Irving-Rodgers & Rodgers 2000). However, in the present study, the permselectivity of follicular basement membranes was found to be different from that around the blood vessels. The functional difference may be attributed to their distinctive components. As the basement membrane with different components exhibits more complicated ultrastructural features (Takami *et al.* 1991, Xu & Ling 1994), the follicular basement membrane is a specific structure that has to be dramatically changed during the follicular development and ovulation (Zamboni 1974, Rodgers *et al.* 2003). Our finding that the BFB was functioning at an early stage of folliculogenesis also supports the significant involvement of basement membranes as the BFB, which are continuously

enclosing the ovarian follicles at all development stages, although some of their components would be changed at different stages (Rodgers *et al.* 1999).

It was previously reported that there was no I α I in mouse follicular fluid (Hess *et al.* 1998), although our findings suggest its presence in the mouse follicles. This discrepancy may be due to the difference in probes used and experimental procedures, because Hess *et al.* used fluorescence-labeled extrinsic proteins that were injected 3 h prior to fixation; the diffusion of I α I from blood vessels into the follicles, which would be driven by the osmotic potential of large glycosaminoglycans and proteoglycans (Clarke *et al.* 2006), may take longer time in living mouse ovaries. Another possibility would be the difference of tissue preparation methods, because it is sometimes difficult to visualize the *in situ* distribution of serum soluble molecules with the conventional chemical fixation. Actually, we failed in our immunohistochemical visualization of I α I in the tissue specimens prepared with the common IM (data not shown). Other reports on porcine and bovine ovaries also suggested the presence of I α I in their follicles, supporting our immunohistochemical findings (Nagyova *et al.* 2004, Clarke *et al.* 2006).

In conclusion, the morphology of living mouse ovaries and immunolocalization of soluble serum proteins were precisely revealed by the IVCT, as compared with the conventional preparation methods. The different immunolocalizations of serum proteins represent the size selectivity at two different sites of BFB, and the follicular basement membrane would be responsible for the permeability of serum proteins with intermediate molecular sizes, whereas the vascular endothelial cells would be involved in the permselectivity of those with larger molecular sizes.

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