A Thesis

For the Degree of Doctor of Veterinary Medicine

Investigation of Flotillin-1 in the Rat Testis during Development, Spermatogenesis, and Ischemia/Reperfusion Injury

Department of Veterinary Medicine

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General introduction

Flotillin-1, a structural protein of caveolae

The surfaces of most cells are studded with tiny, flask-shaped membrane invaginations called caveolae, which have been reviewed by Anderson (1993) [1]. Caveolae are located in glycosylphosphatidylinositol-anchored membranes and are involved in transcytosis, vesicular trafficking, and signal transduction [1, 2, 20]. The principal constituents of caveolae are caveolins and flotillins, which were identified by Rothberg et al. (1992) [19] and Bickel et al. (1997) [2], respectively (Fig. 1). There are two flotillin isotypes: flotillin-1 and flotillin-2 [10]. Although similar in structure and function, flotillin-1 and flotillin-2 differ in their cellular distribution and specific properties [26]. For example, flotillin-1 is detected mainly in the heart, diaphragm, adipose tissue, skeletal muscle, and lungs, whereas flotillin-2 has a much wider tissue distribution but is virtually absent in skeletal muscle and the diaphragm [26]. Since the discovery of flotillin-1, many investigators have studied its functional roles in various body systems, including the nervous [7, 8], urinary [28], and male reproductive [5] systems.

Possible involvement of flotillin-1 in the testis during development

The testis is the male reproductive organ that produces spermatozoa. Testis maturation is essential for the production of spermatozoa. During testicular development, differentiation and maturation of spermatogenic cells and Sertoli cells occur in the seminiferous tubules [14, 22]. The differentiation schedule of spermatogenic cells in the rat testis is as follows: at postnatal days 6-7, the testis

contains somatic (Sertoli) cells and spermatogonia cells (gonocytes) only; by days 13-14, leptotene spermatocytes appear; by days 17-18, zygotene spermatocytes are present; by days 19-20 and days 22-23, early and late pachytene spermatocytes are seen, respectively; haploid round spermatids first appear at days 24-25, and elongating spermatids first appear by days 30-31; by day 36, elongated spermatids can be found [14]. The functional maturation (differentiation) of Sertoli cells occurs after their proliferation, during the fetal and infantile periods [22]. The maturation of Sertoli cells involves the loss of proliferative ability, the formation of inter-Sertoli cell tight junctions (the blood-testis barrier), and the expression of functions not present in immature Sertoli cells [22]. The series of changes that occur in spermatogenic and Sertoli cells do not occur independently, but arise through cell-cell interactions [6, 22].

In vitro studies of flotillin-1 suggest that it is involved in the process of testis development. López-Casas and del Mazo (2003) found that when cell-cell interactions were disrupted, the levels of flotillin-1 mRNA and protein decreased significantly in an NIH-3T3 culture model [13]. The subsequent increase in the levels of both flotillin-1 mRNA and protein was concomitant with the restoration of these interactions [13]. This indicates that flotillin-1 is involved in cell-cell interactions. In addition, flotillin-1 is involved in neuronal differentiation [12]. Santamaría et al. (2005) found that the levels of prostate overexpressed protein 1 and flotillin-1 oscillated during the cell cycle [21]. Moreover, the depletion of either protein markedly inhibited cell proliferation under basal conditions, whereas the overexpression of either protein strongly induced proliferation [21], implying that flotillin-1 is involved in proliferation. Therefore, it is possible that flotillin-1 is involved in testis development (Fig. 2).

Possible involvement of flotillin-1 in the testis during spermatogenesis

Spermatogenesis occurs in the seminiferous tubules and is a complex process involving mitotic spermatogenic cell division, meiosis, and the process of spermiogenesis [9]. In the seminiferous tubules, spermatogenic cells and Sertoli cells are arranged in defined associations or seminiferous epithelium stages [18]. In rats, these cellular organizations are classified into 14 stages of the seminiferous epithelial cycle [11]. The organization and localization of spermatogenic cells differs in each stage, and the localization and morphology of Sertoli cells changes regularly during the seminiferous epithelium cycle [11, 18]. There is indirect evidence of signal transduction between spermatogenic cells and Sertoli cells, which produce spermatozoa. In particular, Sertoli cells change morphologically and functionally during the seminiferous epithelium cycle. For example, cyclic changes in the volume and surface area are observed for various organelles, including mitochondria, rough endoplasmic reticulum, lipid droplets, Golgi apparatus, and lysosomes, in Sertoli cell cytoplasm [25]. Of these, the formation of lipid droplets and lysosomes is associated with the phagocytosis of residual bodies and apoptotic germ cells during spermatogenesis [4, 27].

Caveolae are frequently found in the Sertoli cell membrane in the embryonic mouse testes [17]. After the discovery of caveolae in Sertoli cells, studies examined the constituents of caveolae—caveolins and flotillins—in the testis [3, 5]. In the adult testis, Sertoli cells and Sertoli glycosphingolipid-enriched fractions were found to lack caveolin, while they were enriched in flotillin-1 [5]. This indicates that flotillin-1 is more involved than caveolin in Sertoli cell signal transduction. In addition, flotillin-1 is related to the formation lipid droplets and lysosomes [7, 19]. In spermatogenesis, the lipid droplets and lysosomes are located in the Sertoli cell cytoplasm and are related to phagocytosis [4, 27]. In general, phagocytosis in Sertoli

cells changes cyclically during spermatogenesis [4, 27]. Therefore, it is possible that the involvement of flotillin-1 in Sertoli cells changes cyclically during the seminiferous epithelium cycle (Fig. 3).

Possible involvement of flotillin-1 in the testis during testicular torsion

Testicular torsion causes damage via twisting and untwisting of the spermatic cord, resulting in ischemia and reperfusion. After testicular torsion, the changes in blood flow cause primary injury to the constituent cells of the testis, and then infiltrating inflammatory cells cause secondary damage [23, 29]. The spermatogonia and preleptotene and leptotene spermatocytes in the seminiferous tubules are easily damaged by testicular torsion, because these cell types are located outside the blood-testis barrier of Sertoli cells and are directly damaged by the changes in blood flow and infiltrating inflammatory cells, as has been demonstrated by the location of apoptotic germ cells in the seminiferous tubules after testicular torsion [24]. The testis is a self-renewing organ, which is controlled by the regular mitosis of spermatogonial stem cells [15]. Therefore, spermatogenesis decreases with the apoptosis of spermatogonial stem cells or spermatogonia after testicular torsion. The histopathologic findings after testicular torsion include the (1) apoptosis of germ cells, (2) infiltration of inflammatory cells in the interstitial space, and (3) activation of Sertoli cells for the phagocytosis of apoptotic germ cells [16, 22, 24].

It is possible that flotillin-1 is involved in testicular torsion. Flotillin-1 has been identified in Sertoli cells, which are activated under testicular torsion; flotillin-1 activity in Sertoli cells may be altered after testicular torsion [5]. The principal inflammatory cell type seen after testicular torsion is macrophages, which are involved in phagocytosis and forming lysosomes [16]. Cathepsin D is a lysosomal

protein related to flotillin-1 [7]. Flotillin-1 contributes to the formation of lysosomes in astrocytes and macrophages in experimental autoimmune encephalomyelitis [7]. Therefore, it is possible that flotillin-1 is involved in the activation of macrophages and Sertoli cells after testicular torsion through the formation of lysosomes with cathepsin D.

References

1. **Anderson RG**. Caveolae: where incoming and outgoing messengers meet. Proc Natl Acad Sci USA 1993, 90, 10909-10913.

2. **Bickel PE, Scherer PE, Schnitzer JE, Oh P, Lisanti MP, Lodish HF**. Flotillin and epidermal surface antigen define a new family of caveolae-associated integral membrane proteins. J Biol Chem 1997, 272, 13793-13802.

3. **Casanova MB, Lustig L, Diaz ES, Pellizzari EH, Cigorraga SB, Denduchis B**. Expression of caveolin-1 in rat Leydig cells. Biocell 2006,30, 431-438

4. **Chemes H**. The phagocytic function of Sertoli cells: a morphological, biochemical, and endocrinological study of lysosomes and acid phosphatase localization in the rat testis. Endocrinology 1986, 119, 1673-1681.

5. **Evans WE 4th, Coyer RL, Sandusky MF, Van Fleet MJ, Moore JG, Nyquist SE**. Characterization of membrane rafts isolated from rat sertoli cell cultures: caveolin and flotillin-1 content. J Androl 2003, 24, 812-821.

6. **Griswold MD**. Interactions between germ cells and Sertoli cells in the testis. Biol Reprod 1995, 52, 211-216.

7. **Kim H, Ahn M, Moon C, Matsumoto Y, Sung Koh C, Shin T**. Immunohistochemical study of flotillin-1 in the spinal cord of Lewis rats with experimental autoimmune encephalomyelitis. Brain Res 2006, 1114, 204-211.

8. **Kokubo H, Helms JB, Ohno-Iwashita Y, Shimada Y, Horikoshi Y, Yamaguchi H**. Ultrastructural localization of flotillin-1 to cholesterol-rich membrane microdomains, rafts, in rat brain tissue. Brain Res 2003, 965, 83-90.

9. **de Kretser DM, Loveland KL, Meinhardt A, Simorangkir D, Wreford N**. Spermatogenesis. Hum Reprod 1998, 13, Suppl 1:1-8

10. **Lang DM, Lommel S, Jung M, Ankerhold R, Petrausch B, Laessing U, Wiechers MF, Plattner H, Stuermer CA**. Identification of reggie-1 and reggie-2 as plasmamembrane-associated proteins which cocluster with activated GPI-anchored cell adhesion molecules in non-caveolar micropatches in neurons. J Neurobiol 1998, 37, 502-523.

11. **Leblond CP, Clermont Y**. Definition of the stages of the cycle of the seminiferous epithelium in the rat. Ann N Y Acad Sci 1952, 55, 548-573.

12. **Limpert AS, Karlo JC, Landreth GE**. Nerve growth factor stimulates the concentration of TrkA within lipid rafts and extracellular signal-regulated kinase activation through c-Cbl-associated protein. Mol Cell Biol 2007, 27, 5686-5698.

13. **López-Casas PP, del Mazo J**. Regulation of flotillin-1 in the establishment of NIH-3T3 cell-cell interactions. FEBS Lett 2003, 555, 223-228.

14. **Malkov M, Fisher Y, Don J**. Developmental schedule of the postnatal rat testis

determined by flow cytometry. Biol Reprod 1998, 59, 84-92.

15. **McLean DJ, Russell LD, Griswold MD**. Biological activity and enrichment of spermatogonial stem cells in vitamin A-deficient and hyperthermia-exposed testes from mice based on colonization following germ cell transplantation. Biol Reprod 2002, 66, 1374-1379.

16. **Moon C, Shin T**. Involvement of macrophages in germ cell death in the rat testis with acute experimental testicular torsion. Korean J Vet Res 2004, 44, 329-334.

17. **Nagano T, Suzuki F**. Cell to cell relationships in the seminiferous epithelium in the mouse embryo. Cell Tissue Res 1978, 189, 389-401.

18. **Parvinen M**. Regulation of the seminiferous epithelium. Endocr Rev 1982, 3, 404-417.

19. **Rajendran L, Le Lay S, Illges H**. Raft association and lipid droplet targeting of flotillins are independent of caveolin. Biol Chem 2007, 388, 307-314.

20. **Rothberg KG, Heuser JE, Donzell WC, Ying YS, Glenney JR, Anderson RG**. Caveolin, a protein component of caveolae membrane coats. Cell 1992, 68, 673-682.

21. **Santamaría A, Castellanos E, Gómez V, Benedit P, Renau-Piqueras J, Morote J, Reventós J, Thomson TM, Paciucci R**. PTOV1 enables the nuclear

translocation and mitogenic activity of flotillin-1, a major protein of lipid rafts. Mol Cell Biol 2005, 25, 1900-1911.

22. **Sharpe RM, McKinnell C, Kivlin C, Fisher JS**. Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. Reproduction 2003, 125, 769-784.

23. **Shiraishi K, Naito K, Yoshida K**. Nitric oxide promotes germ cell necrosis in the delayed phase after experimental testicular torsion of rat. Biol Reprod 2001, 65, 514-521.

24. **Turner TT, Tung KS, Tomomasa H, Wilson LW**. Acute testicular ischemia results in germ cell-specific apoptosis in the rat. Biol Reprod 1997, 57, 1267-1274.

25. **Ueno H, Mori H**. Morphometrical analysis of Sertoli cell ultrastructure during the seminiferous epithelial cycle in rats. Biol Reprod 1990, 43, 769-776.

26. **Volonte D, Galbiati F, Li S, Nishiyama K, Okamoto T, Lisanti MP**. Flotillins/cavatellins are differentially expressed in cells and tissues and form a hetero-oligomeric complex with caveolins in vivo. Characterization and epitope-mapping of a novel flotillin-1 monoclonal antibody probe. J Biol Chem 1999, 274, 12702-12709.

27. **Wang H, Wang H, Xiong W, Chen Y, Ma Q, Ma J, Ge Y, Han D**. Evaluation on the phagocytosis of apoptotic spermatogenic cells by Sertoli cells in vitro

through detecting lipid droplet formation by Oil Red O staining. Reproduction 2006, 132, 485-492.

28. **Welker P, Geist B, Frühauf JH, Salanova M, Groneberg DA, Krause E, Bachmann S**. Role of lipid rafts in membrane delivery of renal epithelial Na+-K+-ATPase, thick ascending limb. Am J Physiol Regul Integr Comp Physiol 2007, 292, R1328-1337.

29. **Zini A, Abitbol J, Girardi SK, Schulsinger D, Goldstein M, Schlegel PN**. Germ cell apoptosis and endothelial nitric oxide synthase (eNOS) expression following ischemia-reperfusion injury to testis. Arch Androl 1998, 41, 57-65.

Figures

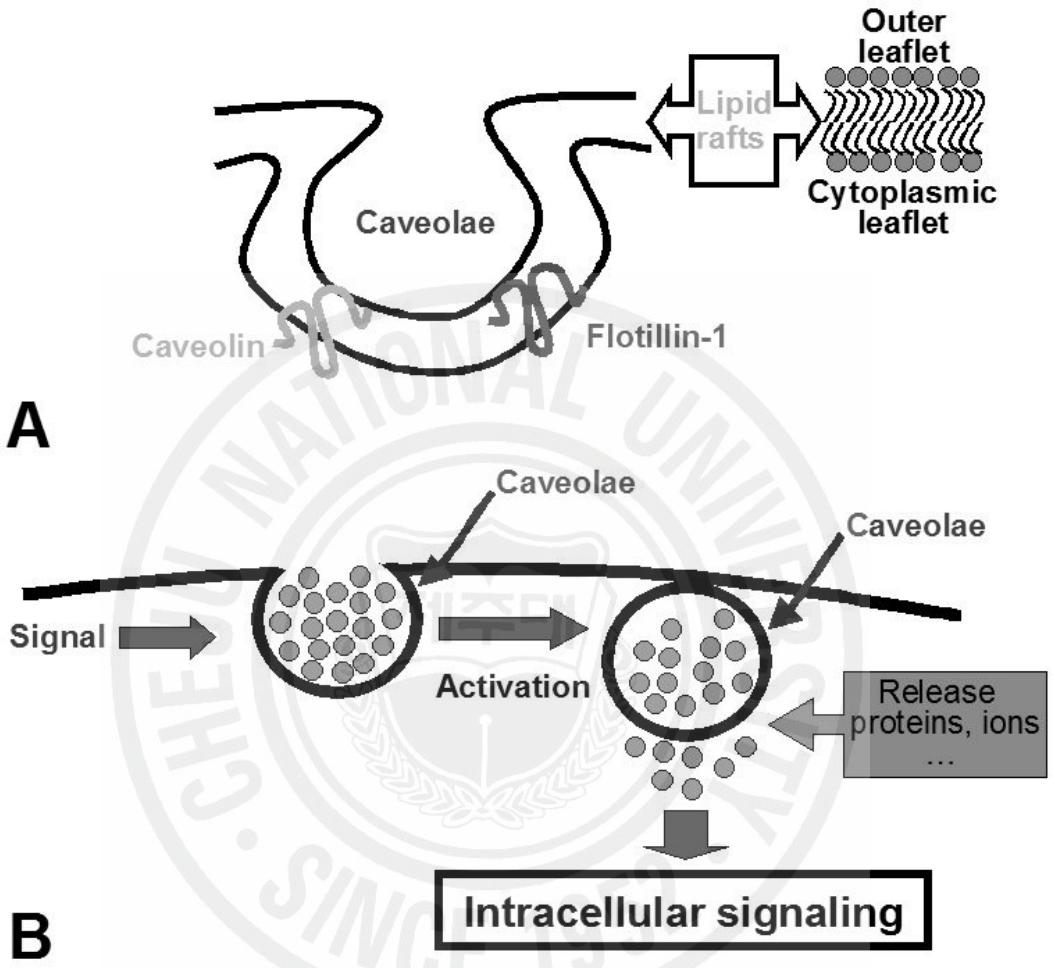

Figure 1. Diagram of caveolae. (A) Caveolae are located in glycosylphosphatidylinositol-anchored membranes (lipid rafts). The principal constituents of caveolae are caveolin and flotillin-1. (B) The involvement of caveolae in intracellular signaling. A signal is transmitted to caveolae, causing their activation. The activated caveolae release proteins and ions into the cytoplasm.

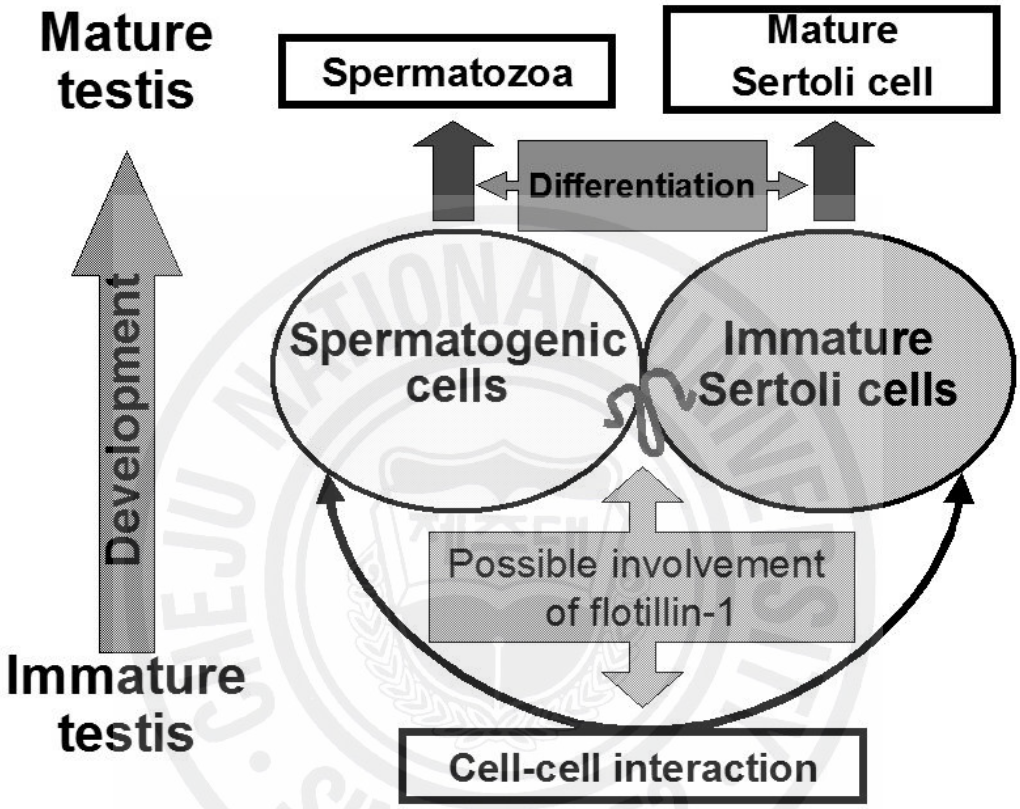

Figure 2. Illustration of the possible involvement of flotillin-1 in the Sertoli cell-spermatogenic cell interaction. Immature Sertoli and spermatogenic cells mature via cell-cell interactions during development. It is possible that flotillin-1 is involved in this process.

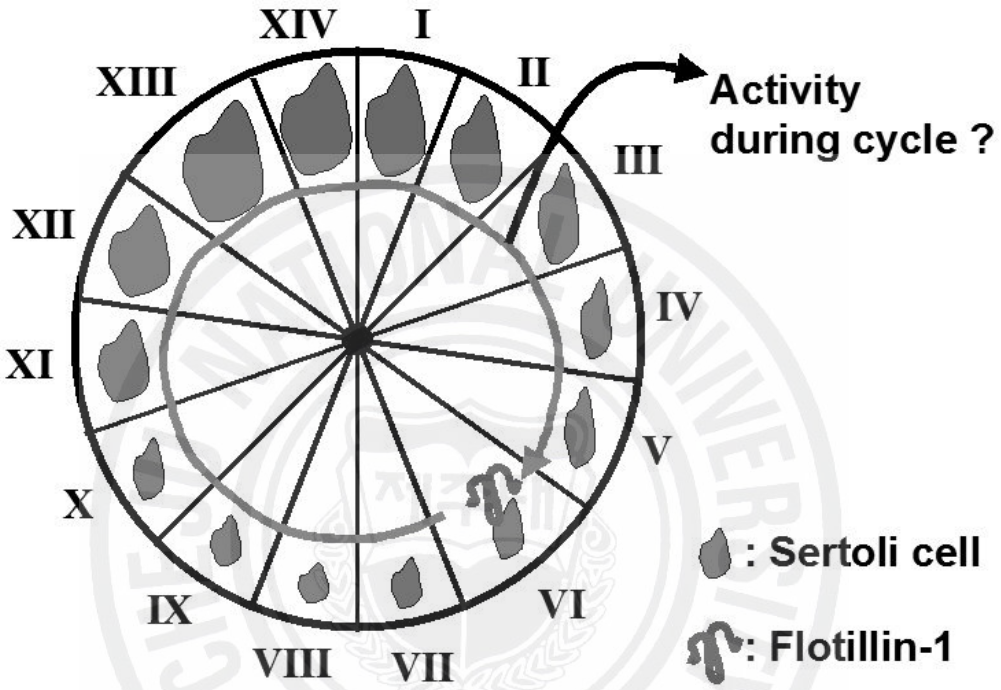

Figure 3. Illustration of the possible change in flotillin-1 activity during the seminiferous epithelium cycle in the rat. Sertoli cells change morphologically and functionally during the seminiferous epithelium cycle. Flotillin-1 is located in Sertoli cells. It is possible that the flotillin-1 activity changes during the seminiferous epithelium cycle.

CHAPTER I

Immunohistochemical Study of Flotillin-1 in the Rat Testis during Development, and Spermatogenesis

I.1. Abstract

The level and cellular localization of flotillin-1, a lipid raft protein, was examined in the testis of rats during postnatal development and spermatogenesis in order to determine if flotillin-1 is involved in testicular development. The testes of rats were sampled on postnatal days 7, 14, 21, 40, and 60, and analyzed by Western blot and immunohistochemistry. Western blot analysis detected flotillin-1 in the testes at days 7 and 14 after birth but the level decreased significantly at postnatal days 21, 40 and 60. At postnatal days 7, 14, 21, and 40, flotillin-1 immunolocalisation was observed mainly in the Sertoli cells. However, there was little flotillin-1 immunolabeling in the spermatogenic cells from the seminiferous tubule of the testes. In the seminiferous tubule of the testes at postnatal day 60, flotillin-1 immunoreactivity in the Sertoli cells varied according to the stages of the spermatogenic cycle; intense immunoreactivity being observed in stages IX-III and less in stages IV-VIII. These results suggest that flotillin-1 participates in the developmental process of Sertoli cells and is involved in the regulation of spermatogenesis.

KEYWORDS: Testis; Flotillin-1; Development; Spermatogenesis; Sertoli cell

I.2. Introduction

Flotillins are structure proteins in the lipid rafts [1] that are particularly enriched in the detergent-insoluble glycolipid-enriched membrane domains involved in both signal transduction and vesicular trafficking [5, 9]. Two isotypes of flotillins have been identified: flotillin-1 and flotillin-2 [1]. Flotillin-1 has been observed in various organs including the brain [8], spinal cord [7], heart [13] and testis [3, 6]. In the adult rat testis, flotillin-1 has been found to be abundant in both Sertoli and peritubular myoid cells but is found in lesser quantities in spermatogenic cells and spermatozoa [3]. Furthermore, there was increased flotillin-1 immunoreactivity reported in Sertoli cells and inflammatory cells in a pathological condition of the testis, such as experimental acute testicular torsion [6]. This suggests that flotillin-1 is involved in the signal transduction of Sertoli cells as well as in the formation and activation of phagolysosomes in normal and pathological conditions of the testis.

During testicular development, signal transduction in Sertoli and spermatogenic cells is important because a variety of cellular changes have been observed in the seminiferous tubules. In addition, a mechanism of signal transduction, the endocytic mechanism, which is distinct from both clathrin and non-clathrin-mediated endocytosis, has been reported in mammalian cells [4]. It was previously reported that amphiphysin I, which is a related protein in clathrin-mediated endocytosis, is activated substantially from days 15 to 25 during the postnatal development of the seminiferous epithelium, and it is localized in Sertoli cells [19]. On the other hand, flotillin-1 is involved in non-clathrin-mediated endocytosis [4] and the signal transduction of Sertoli

cells [3]. However, the temporal profile and possible role of flotillin-1 during testicular development is unknown.

In mature rat testis, the spermatogenic cycles are classified into 14 stages [10]. In spermatogenesis, Sertoli cells relay external signals and provide the factors essential to the differentiation and proliferation of spermatogenic cells [2]. Sertoli cells themselves change functionally and morphologically during the spermatogenic cycle: these cyclic changes have been detected in the mitochondria, rough endoplasmic reticulum, Golgi apparatus, lysosomes and lipid droplets in the Sertoli cells [16]. Flotillin-1 is closely associated with the lipid droplets [12] and lysosomes [6], suggesting that flotilin-1 is involved in the spermatogenic cycle. However, little is known about the differential distribution of flotillin-1 expression according to the cycle of the spermatogenesis in the rat testis.

This study examined the level and localization of flotillin-1 in the rat testis during postnatal development and in the stages of spermatogenic cycle in order to determine if flotilin-1 plays a role in testicular development and spermatogenesis.

I.3. Materials and Methods

Animals

Male Sprague-Dawley rats (Orient Bio, Gyunggi-do, Korea) were bred at our animal facility. The testes (n = 3 samples) at 7, 14, 21, 40 and 60 days after birth were used for Western blot analysis and histology (n = 3/group). The testes at postnatal days 7 to 40 were classified as immature testes, while the testes at postnatal day 60 were classified as mature testes according to a previous study [11, 15]. All the experiments were performed in line with accepted ethical guidelines.

Tissue preparation

The rats were sacrificed under ether anesthesia. The testes were dissected from each group. Samples of the testes were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4), processed by routine protocols and embedded in paraffin wax for immunolabeling experiments. The opposite testis was snap-frozen and stored for immunoblot analysis.

Antibodies

Rabbit polyclonal anti-flotillin-1 (clone H104) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-beta-actin and mouse anti-vimentin were purchased from Sigma (St. Louis, MO) and Neomarkers (Fremont, CA), respectively.

Western blot analysis

The tissues were homogenized in a lysis buffer (40 mM Tris, 120 mM NaCl, 0.1% Nonidet 40, 2 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin). After incubation for 60 min in an ice-bath, the homogenates were centrifuged at 14,000 rpm for 20 min, and the supernatant was harvested. For the immunoblot assay, supernatant containing 20 μg of protein was loaded into individual lanes of 10% sodium dodecyl (lauryl) sulfate-polyacrylamide gels, separated by electrophoresis under standard conditions and electroblotted onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH). The residual binding sites on the membrane were blocked by incubation with 5% nonfat milk in Tris-buffered saline (TBS; 10 mM Tris-HCl, pH 7.4, and 150 mM NaCl) for 1 h at room temperature (RT). Subsequently, the membrane was incubated for 2 h at RT with the rabbit polyclonal anti-flotillin 1 antobody diluted 1:1,000. The membranes were washed three times in TBS containing 0.1% Tween 20 before being incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Vector, Burlingame, CA) (1:2000 dilution) for 1 h at RT. The membranes were developed using a chemiluminescent substrate (WEST-one Kit; iNtRON Biotech), employed according to the manufacturer's instructions, and exposed to Agfa medical X-ray film (Agfa Gevaert, Mortsel, Belgium). After imaging, the membranes were stripped and reprobed using the anti-beta-actin antibody (Sigma) using a similar protocol to that described above. The optical density (per mm2) of each band was measured using a scanning laser densitometer (GS-700, Bio-Rad, Hercules, CA), and the values are presented as the mean ± SEM. The ratios of the density of each flotillin-1 band relative to that of the beta-actin band were compared using Molecular Analyst software (Bio-Rad).

The data was analyzed using one-way ANOVA followed by a

Student-Newman-Keuls post hoc test for multiple comparisons. In all cases, $p < 0.05$ was considered statistically significant.

Immunohistochemistry

6 μm-thick paraffin wax embedded tissue sections were deparaffinized and rehydrated by routine protocols and heated with citrate buffer (0.01 M, pH 6.0) in a microwave oven for 3 min. They were then incubated in 0.3% hydrogen peroxide in methyl alcohol for 20 min to block the endogenous peroxidase activity. After three washes with PBS, the sections were incubated with 10% normal goat serum (Vector ABC Elite kit, Burlingame, CA) diluted in PBS, which was followed by incubation with rabbit polyclonal flotillin-1 (1:400 dilution) for 1 h at RT. After three washes in PBS, the sections were incubated with biotinylated goat anti-rabbit IgG (Vector) (1:200 dilution) for 45 min at RT. After three washes in PBS, the sections were incubated with the avidin-biotin peroxidase complex (Vector), prepared according to manufacturer's instructions, for 45 min at RT. The peroxidase reaction was developed using a diaminobenzidine substrate kit (Vector) prepared according to manufacturer's instructions for 5 min.

To examine the phenotype of flotillin-1 immunoreactive cells, double immunofluorescence was applied using the cell-type-specific markers, including vimentin for Sertoli cells. First, the sections, prepared as described previously, were incubated with rabbit anti-flotillin-1 (1:200 dilution) overnight at 4°C, followed by fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG (Sigma), diluted 1:50 for 1h at RT. The slides were then incubated with mouse anti-vimentin (1:200 dilution) overnight at 4°C, followed by tetramethyl rhodamine isothiocyanate (TRITC)-labeled goat anti-mouse IgG

(Sigma), diluted 1:50 for 1h at RT.

The lipofuscin autofluorescence was reduced or eliminated using the following procedures: washing the sections three times for 1 h each wash in PBS at room temperature, briefly dipping them in distilled H_2O, treating them with 10 mM $CuSO_4$ in ammonium acetate buffer (50 mM CH_3COONH_4, pH 5.0) for 20 min, briefly dipping them again in distilled H_2O, and then returning them to PBS. The double immunofluorescence-labeled specimens were examined using an FV500 laser confocal microscope (Olympus, Tokyo, Japan).

I.4. Results

Flotillin-1 protein levels in testis during postnatal development

In order to determine if the amount of flotillin-1 had changed developmentally in the testes, the level of flotillin-1 in the testes was analyzed semiquantitatively by Western blotting.

Flotillin-1 was strongly detected in the testis at days 7 (density value, 0.98 ± 0.01 OD/mm2; n = 3 rats) and 14 (0.93 ± 0.04; n = 3 rats) after birth. The level then decreased significantly at postnatal days 21 (0.59 ± 0.04; n = 3 rats; $p < 0.05$ vs. postnatal day 7 and 14) and 40 (0.56 ± 0.07; n = 3 rats; $p < 0.05$ vs. postnatal day 7 and 14). The level of flotillin-1 was lowest in the mature testes (at day 60 after birth; 0.41 ± 0.05; n = 3 rats; $p < 0.05$ vs. postnatal day 7, 14, 21, and 40). This is illustrated in Fig. 1.

Localization of flotillin 1 in rat testis

Immunohistochemistry of immature rat testis

At postnatal days 7–40, flotillin-1 immunoreactivity was observed mainly in the Sertoli cells but there was some labeling of the spermatogenic and Leydig cells in the testes, as seen in Fig. 2.

At postnatal day 7 (Fig. 2A) and 14 (Fig. 2B), the seminiferous tubule consisted mainly of Sertoli cells and gonocytes. Flotillin-1 immunoreactivity was detected mainly in the membranes of Sertoli cells but not in the gonocytes. At postnatal day 21 (Fig. 2C), the spermatocytes accumulated in the lumen. Flotillin-1 immunoreactivity was detected mainly in the membrane of the Sertoli cells but not in the spermatocytes. At postnatal day 40 (Fig. 2D), the diameter of the tubule continued to increase and

elongated spermatids appeared. Flotillin-1 immunoreactivity was detected mainly in the cytoplasmic processes of Sertoli cells.

Immunohistochemistry of adult rat testis

In the seminiferous tubule of the adult rat testis (at postnatal day 60), flotillin-1 labelling was observed in the Sertoli cells, peritubular myoid cells, and elongating spermatids, as seen in Fig. 3. In the Sertoli cells, flotillin-1 was differentially localized in the seminiferous tubules according to the different stages of the spermatogenic cycle of the seminiferous epithelium.

At stage II/III of the cycle (Fig. 3A), flotillin-1 immunoreactivity was detected in the adluminal compartment of the Sertoli cells, in which localization was near the head of the elongate spermatids.

At stage V/VI of the cycle (Fig. 3B), the elongated spermatids had moved to the basal area of the seminiferous tubule. Flotillin-1 immunolabeling was prominent in the perinuclear and supranuclear region of Sertoli cells.

At stage VII of the cycle (Fig. 3C), elongate spermatids were moved to the lumen. Flotillin-1 immunoreactivity was located in the basal area of the Sertoli cells.

At stage VIII of the cycle (Fig. 3D), the pattern of flotillin-1 localization matched that of stage VII of the cycle, in which the Sertoli cells were immunopositive. Furthermore, flotillin-1 immunoreactivity was observed in the residual bodies.

At stage X of the cycle (Fig. 3E), flotillin-1 immunoreactivity was detected in the apical region of Sertoli cells, in which localization was close to the head of the elongating spermatids. Furthermore, the elongating spermatids were weakly immunopositive for flotillin-1.

A large number of meiotic spermatocytes were detected in the tubule at stage XIV of the cycle (Fig. 3F). The flotillin-1 immunoreaction was detected in the adluminal compartment of Sertoli cells, in which localization was near to the head of the elongate spermatids.

In summary, there was a high level of flotillin-1-immunoreactivity in Sertoli cells in stages IX-III, and a low level in stages IV-VIII.

The vessels and Leydig cells were also immunopositive for flotillin-1 in the interstitial tissue among the tubules (Fig. 3).

Double immunofluorescence labeling of flotillin-1 and vimentin in testis

Double labeling was performed to observe the co-localization between flotillin-1 and vimentin, illustrated in Fig. 4A-I.

The immunoflurescent localization of flotillin-1 in the testis during development showed was similar to that observed using single immunoperoxidase labeling (Fig. 4A, D, and G). Vimentin immunoreactivity was detected mainly in the perinuclear, supranuclear and apical regions of the Sertoli cells at postnatal days 7 (Fig. 4B), 21 (Fig. 4E), and 60 (Fig. 4H), as described in a previous report [21]. Some of the flotillin-1 immunoreactivity was colocalized in the vimentin-positive-Sertoli cells at postnatal days 7 (Fig. 4C), 21 (Fig. 4F), and 60 (Fig. 4I).

I.5. Discussion

This study examined the immunolocalisation of flotillin-1 in the testis during postnatal development, as well as during the various stages of the spermatogenic cycle. Flotillin-1 was localized mainly in the Sertoli and peritubular myoid cells with lesser quantities being observed in the spermatogenic cells in the adult rat testis. The immunohistochemical findings of the adult testis are largely consistent with the previous report of Evans et al. [3]. However, flotillin-1 immunolocalisation in the testis during postnatal development and in the various stages of the spermatogenic cycle has not previously been reported.

Postnatal development in the rat testis requires signaling pathways that can regulate the precise balance between cell survival, proliferation and differentiation [20]. In the early phase of testicular development, there are a greater number of Sertoli cells in the rat testis [18]. In addition, flotillin-1 participates in the cell proliferation associated with the prostate overexpressed protein 1 [14]. The immunoblot analysis presented here revealed high levels of flotillin-1 at postnatal day 7 and this was maintained until postnatal day 14, after which the level decreased. In this phase, flotillin-1 immunoreactivity was observed mainly in the Sertoli cells. This suggests that flotillin-1 immunoreactivity mirrors the proliferation of Sertoli cells. Therefore, flotillin-1 may play a role in testicular maturation, possibly in the proliferation of Sertoli cells.

The cytoplasmic components of Sertoli cells during the spermatogenic cycles appear to furnish some insights into the functional roles of Sertoli cells in spermatogenesis [16]. The present study revealed that flotillin-1

immunoreactivity varies in the cytoplasm of Sertoli cells during the various stages of the spermatogenic cycles. There is consensus that flotillin-1 is associated with the formation lipid droplets [12]. In the Sertoli cells of the adult testis, it was reported that the formation of lipid droplets is associated with the phagocytosis of residual bodies and apoptotic germ cells [17]. The lipid droplets accumulate markedly after spermiation, decrease after stage XIV of spermatogenesis, and remain at low levels in stages IV-VIII [16]. Interestingly, the accumulation of lipid drops in Sertoli cells during the spermatogenic cycle correlated with the flotillin-1 immunoreactivity. Furthermore, a previous study reported that flotillin-1 is closely related to the formation lysosomes in Sertoli cells [6]. This suggests that flotillin-1 is a lipid droplet protein that is actively involved in the formation and activation of phagolysosomes, resulting in the clearance of residual bodies and apoptotic germ cells in Sertoli cells during spermatogenesis.

In conclusion, these results suggest that the lipid raft protein flotillin-1 is involved in the proliferation process of Sertoli cells after birth, as well as in the formation of lipid droplets in the Sertoli cells under spermatogenesis in mature rats.

I.6. References

1. **Bickel PE, Scherer PE, Schnitzer JE, Oh P, Lisanti MP, Lodish HF**. Flotillin and epidermal surface antigen define a new family of caveolae-associated integral membrane proteins. J Biol Chem 1997, 27, 13793-13802.

2. **Delfino F, Walker WH**. Hormonal regulation of the NF-kappaB signaling pathway. Mol Cell Endocrinol 1999, 157, 1-9.

3. **Evans WE 4th, Coyer RL, Sandusky MF, Van Fleet MJ, Moore JG, Nyquist SE**. Characterization of membrane rafts isolated from rat sertoli cell cultures: caveolin and flotillin-1 content. J Androl 2003, 24, 812-821.

4. **Glebov OO, Bright NA, Nichols BJ**. Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells. Nat Cell Biol 2005, 8, 46-54.

5. **Hooper NM**. Detergent-insoluble glycosphingolipid/cholesterol-rich membrane domains, lipid rafts and caveolae (review). Mol Membr Biol 1999, 16, 145-156.

6. **Jeong CW, Kim H, Kim S, Kim SH, Moon C, Shin T**. Immunohistochemical study of flotillin-1 in rat testis with ischemia/reperfusion injury. Cell Biol Int 2007, 31, 609-614.

7. **Kim H, Ahn M, Moon C, Matsumoto Y, Koh CS, Shin T**. Immunohistochemical study of flotillin-1 in the spinal cord of Lewis rats with experimental autoimmune encephalomyelitis. Brain Res 2006, 1114, 204-211.

8. **Kokubo H., Lemere CA, Yamaguchi H**. Localization of flotillins in human brain and their accumulation with the progression of Alzheimer's disease pathology. Neurosci Lett 2000, 290, 93-96.

9. **Lang DM, Lommel S, Jung M, Ankerhold R, Petrausch B, Laessing U, Wiechers MF, Plattner H, Stuermer CAO**. Identification of reggie-1 and reggie-2 as plasmamembrane-associated proteins which cocluster with activated GPI-anchored cell adhesion molecules in non-caveolar micropatches in neurons. J Neurobiol 1998, 37, 502-523.

10. **Leblond CP, Clermont Y**. Definition of the stages of the cycle of the seminiferous epithelium in the rat. Ann N Y Acad Sci 1952, 55, 548-573.

11. **Malkov M, Fisher Y, Don J**. Developmental schedule of the postnatal rat testis determined by flow cytometry. Biol Reprod 1998, 59, 84-92.

12. **Rajendran L, Le Lay S, Illges H**. Raft association and lipid droplet targeting of flotillins are independent of caveolin. Biol Chem 2007, 388, 307-314.

13. **Rybin VO, Grabham PW, Elouardighi H, Steinberg SF**. Caveolae-associated proteins in cardiomyocytes: caveolin-2 expression and interactions with caveolin-3. Am J Physiol Heart Circ Physiol 2003, 285, H325-332.

14. **Santamaria A, Castellanos E, Gomez V, Benedit P, Renau-Piqueras J, Morote J, Reventos J, Thomson TM, Paciucci R**. PTOV1 enables the nuclear translocation and mitogenic activity of flotillin-1, a major protein of lipid rafts. Mol Cell Biol 2005, 25, 1900-1911.

15. **Tsai-Morris CH, Aquilano DR, Dufau ML**. Cellular localization of rat testicular aromatase activity during development. Endocrinology 1985, 116, 38-46.

16. **Ueno H, Mori H**. Morphometrical analysis of Sertoli cell ultrastructure during the seminiferous epithelial cycle in rats. Biol Reprod 1990, 43, 769-776.

17. **Wang H, Wang H, Xiong W, Chen Y, Ma Q, Ma J, Ge Y, Han D**. Evaluation on the phagocytosis of apoptotic spermatogenic cells by Sertoli cells in vitro through detecting lipid droplet formation by Oil Red O staining. Reproduction 2006, 132, 485-492.

18. **Wang ZX, Wreford NG. De Kretser DM**. Determination of Sertoli cell numbers in the developing rat testis by stereological methods. Int J

Androl 1989, 12, 58-64.

19. **Watanabe M, Tsutsui K, Hosoya O, Tsutsui K, Kumon H, Tokunaga A**. Expression of amphiphysin I in Sertoli cells and its implication in spermatogenesis. Biochem Biophys Res Commun 2001, 287, 739-745.

20. **Yan W, Kero J, Suominen J, Toppari J**. Differential expression and regulation of the retinoblastoma family of proteins during testicular development and spermatogenesis: roles in the control of germ cell proliferation, differentiation and apoptosis. Oncogene 2001, 20, 1343-1356.

21. **Zhu LJ, Zong SD, Phillips DM, Moo-Young AJ, Bardin CW**. Changes in the distribution of intermediate filaments in rat Sertoli cells during the seminiferous epithelium cycle and postnatal development. Anat Rec 1997, 248, 391-405.

I.7. Figures

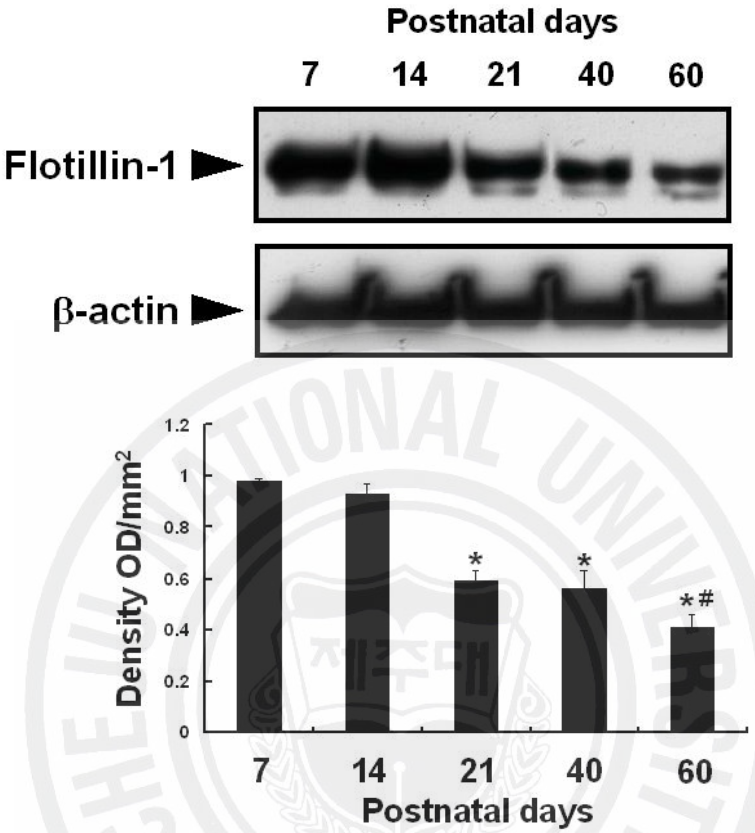

Figure 1. Western blots labeled for flotillin-1 and beta-actin: flotillin-1 levels decreased progressively with the postnatal age in the testis of rats. The arrowheads indicate the positions of flotillin-1 (approximately 45 kDa) and beta-actin (45 kDa).

Bar graph: densitometric data analysis (mean ± S.E., n = 3 rats/group). The relative levels of flotillin-1 were calculated after normalization to the beta-actin bands from three different samples. *, $p < 0.05$ vs. postnatal day 7 and 14; #, $p < 0.05$ vs. postnatal day 21 and 40.

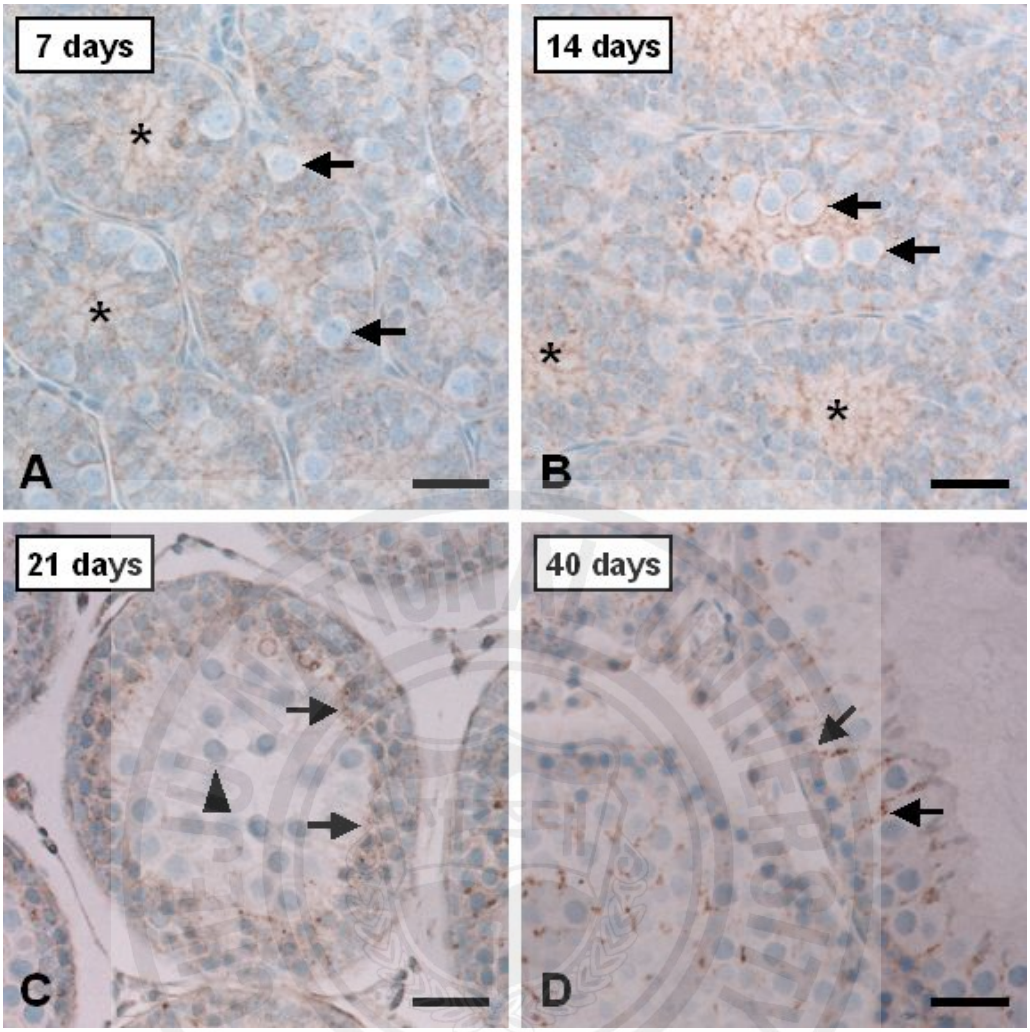

Figure 2. Immunolocalisation of flotillin-1 in the rat testis at postnatal days 7 (A), 14 (B), 21 (C), and 40 (D). At postnatal days 7 (A) and 14 (B), in the cytoplasm of the Sertoli cells that fill the lumen completely, flotillin-1 immunoreactivity was mainly detected in the membrane of the Sertoli cells (asterisks), but not in gonocytes (arrows). At postnatal day 21 (C), flotillin-1 immunoreactivity was clearly observed in the membrane of the Sertoli cells that surrounded the differentiating spermatogenic cells (arrows), but not in the spermatocytes (arrowhead). At postnatal day 40 (D), flotillin-1 immunoreactivity was observed in the supranuclear region of Sertoli cells (arrows). Counterstained with hematoxylin. Scale bars = 30 μm.

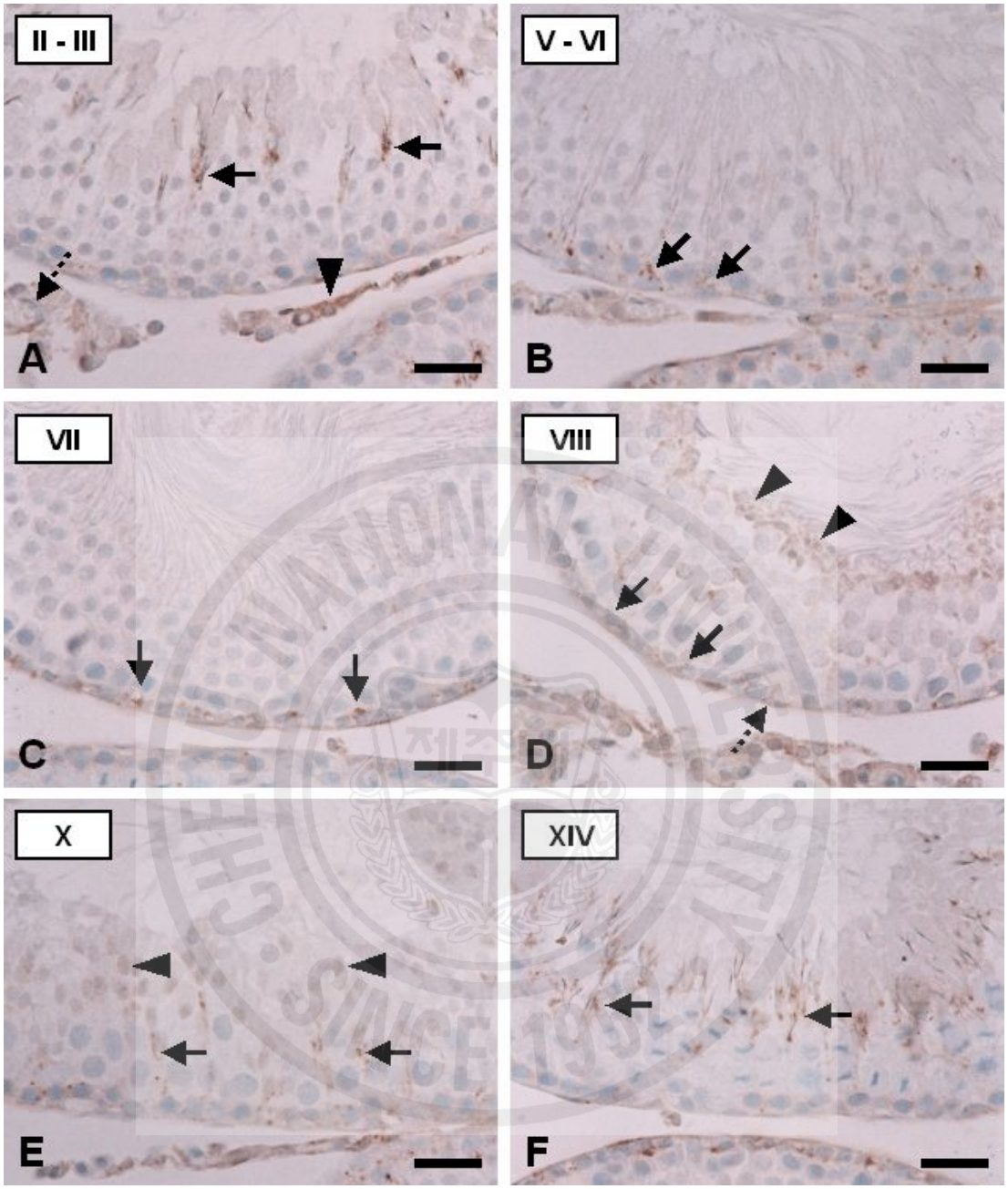

Figure. 3. Immunolabeling of flotilin-1 in the seminiferous tubules of the adult rat testis. A: a tubule at stage II/III of the cycle of the seminiferous tubule. Flotillin-1 was immunolocalised in the adluminal compartment of Sertoli cells (arrows). Some vessels (dotted arrow) and Leydig cells (arrowhead) also

labeled positively for flotillin-1. B: a tubule at stages V/VI of the cycle of the seminiferous tubule. Flotillin-1 was immunolocalised in the perinuclear and supranuclear regions of Sertoli cells (arrows). C: a tubule at stage VII of the cycle of the seminiferous tubule. Flotillin-1 was immunolocalised in the basal area of Sertoli cells. D: a stage VIII tubule, flotillin-1 immunolabeling was observed in the basal compartment of Sertoli cells (arrows), residual bodies (arrowheads), and peritubular myoid cells (dotted arrow). E: a stage of X tubule, flotillin-1 immunoreactivity was detected in the apical region of Sertoli cells (arrows) and elongating spermatids (arrowheads). F: a stage of the XIV tubule, flotillin-1 was immunolocalised in the adluminal compartment of Sertoli cells (arrows). Counterstained with hematoxylin. Scale bars = 30 μm.

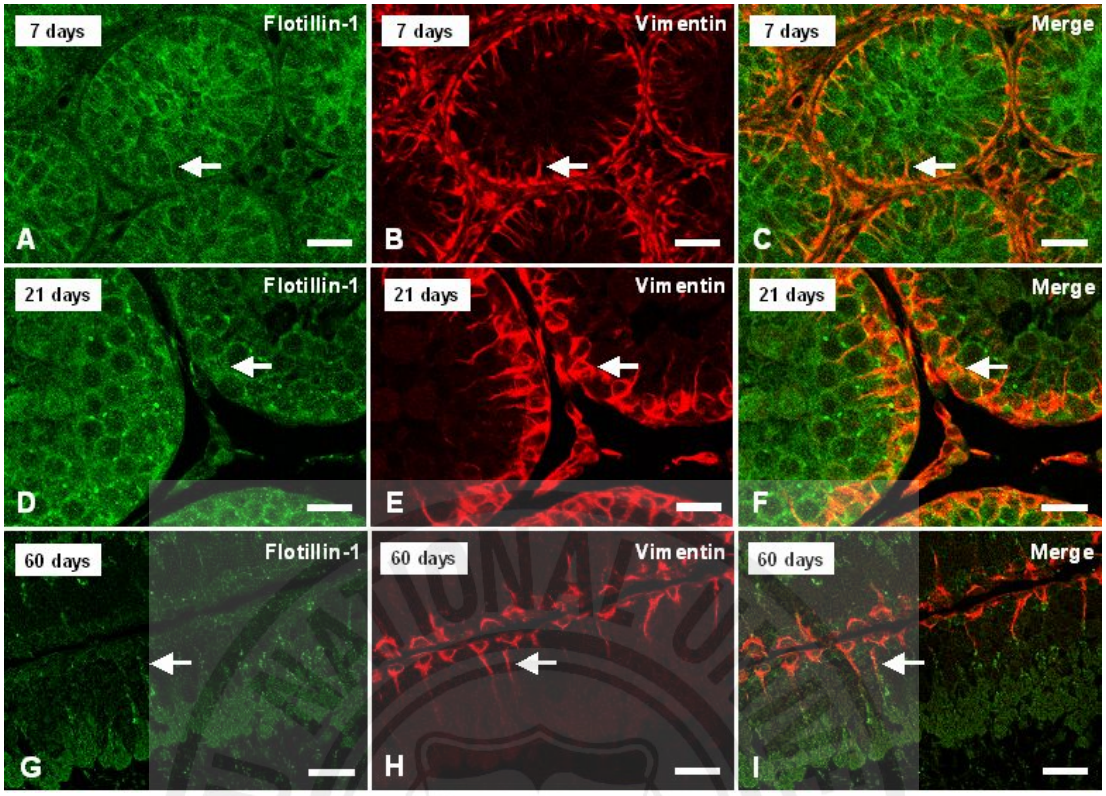

Figure. 4. Immunoflourescent co-localization of flotillin-1 (A, D, G) with vimentin (B, E, H) in the testis at postnatal days 7 (A-C), 21 (D-F), and 60 (G-I). The arrows indicate flotillin-1 immunoreactivity in the vimentin-positive Sertoli cells. C, F, and I are merged images. Scale bars: in A-F, 20 μm; in G-I, 40 μm.

CHAPTER II

Immunohistochemical Study of Flotillin-1 in the Rat Testis during Ischemia/Reperfusion Injury

II.1. Abstract

To investigate the involvement of flotillin-1 in acute experimental testicular torsion, we examined the expression and cellular localization of flotillin-1 and cathepsin D in the rat testis with ischemia/reperfusion (I/R) injury. Western blot analysis showed that the expression of flotillin-1 increased significantly 6 h after I/R and that the level remained elevated for 48 h. Immunohistochemically, flotillin-1 was constitutively localized in some Sertoli cells, peritubular myoid cells, and interstitial cells in the normal testis. After I/R injury, Sertoli cells in the damaged tubules were intensely immunostained for flotillin-1 at 24 and 48 h after I/R. Flotillin-1 was also detected in some inflammatory cells in the interstitial space around damaged tubules. Furthermore, flotillin-1 was colocalized with cathepsin D, a lysosomal marker, in normal testis (mainly in Sertoli cells), and the colocalization was greater in Sertoli cells and macrophages in I/R injured testes. Therefore, we postulate that flotillin-1 immunoreactivity is increased in some Sertoli and inflammatory cells (especially in ED1-positive activated macrophages) in testicular torsion and that flotillin-1 in the injured testis associates with lysosomes in Sertoli cells and macrophages, activating subsequent signals in inflammatory macrophages and Sertoli cells after I/R.

Keywords: Cathepsin D; Flotillin-1; Macrophage; Sertoli cell; Testis; Testicular torsion

II.2. Introduction

Flotillins are enriched in the detergent-insoluble, glycolipid-enriched membrane domains [1] that are involved in signal transduction, vesicular trafficking, and protein tyrosine nitration in cells [3]. There are two isoforms of flotillin, flotillin-1 and flotillin-2 [1]. Flotillin-1, a structural protein in lipid rafts, is found in cells that express caveolin as well as in those that lack caveolin [1]. At a certain stage of activation, flotillin-1 is segregated from the lipid raft and is involved in the stimulation of intracellular signal pathways. In addition, flotillin-1 is often found in lysosomes, suggesting that it is directly or indirectly associated with the formation of cathepsin D (a lysosomal marker)-positive phagolysosomes [9, 13].

The cathepsins constitute a group of proteases in the endosomal/lysosomal proteolytic system [18]. Cathepsins often leak into the cytoplasm following the endocytosis of oxidizable substrates that destabilize lysosomal membranes through lipid peroxidation [18]. Cathepsin D is a major component of lysosomes [21]. Recently, it was reported that both flotillin-1 and cathepsin D are distributed in various testicular cell types [2, 6]. Little is known about the expression of lipid raft proteins, including flotillin-1, in testicular torsion.

Testicular torsion has been implicated in testicular injury and infertility. The main pathology of testicular torsion is ischemia and reperfusion (I/R) injury of the testis [7]. I/R injury causes the infiltration of inflammatory cells into the interstitial space surrounding damaged tubules during the delayed phase after reperfusion. This suggests that, during the peak and delayed phases of reperfusion, increased numbers of hematogenous macrophages

stimulate the generation of nitric oxide (NO) and oxidative stress, which results in induced germ cell death [14, 15, 17]. Therefore, the inflammatory response contributes to testicular I/R injury and is a potential cause of infertility.

A recent study reported that many lipid raft structural proteins are present in testis cells [6], and their expression might change during the inflammatory process. Furthermore, in some neurological disorders, flotillin-1 is abundant within the phagosomes of phagocytes, which suggests that flotillin-1 is involved in the phagocytic process [9, 13]. However, little is known of the functional role and temporal profile of flotillin-1 in testicular inflammation.

This study examined the expression and localization of flotillin-1 in acute experimental testicular torsion to investigate the role of flotillin-1 in testicular inflammation with I/R injury.

II.3. Materials and methods

Surgical procedure

Sprague–Dawley rats were purchased from Daehan Biolink (Chungbuk, Korea) and were bred in our animal facility. Twenty 8- to 9-week-old male rats were used in the experiments. Male adult rats (250–300 g) were anesthetized with chloral hydrate (Fluka, Buchs, Switzerland; 375 mg/kg body weight, intraperitoneal injection). All surgical procedures were performed as described previously [14, 16]. The left scrotum was incised, and the left spermatic cord was rotated 720° clockwise to minimize individual variation in blood flow. After 1.5 h, the torsion (i.e., ischemia) was relieved (i.e., reperfusion), and the testis was returned to the scrotum. Testes were collected at 6, 24, and 48 h after reperfusion. The testes were removed immediately after death, and then fixed routinely in 10% buffered formalin for paraffin embedding or stored at −70 °C for later biochemical analysis. All experiments were conducted in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

Antibodies

Rabbit polyclonal anti-flotillin-1 (clone H104) and goat anti-cathepsin D (IgG, clone R-20, catalog no. sc-6487), which is a lysosomal marker (Girardot et al., 2003), were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal anti-beta-actin was obtained from Sigma (St. Louis, MO, USA). ED1 (mouse monoclonal anti-rat macrophages) was obtained from Serotec (London, UK). ED1 recognizes a lysosomal membrane-related antigen on both monocytes and macrophages [4].

Western blot analysis

Western blot analysis was performed according to a previously described protocol [13]. In brief, testis tissue was homogenized in immunoprecipitation buffer [20 nM HEPES (pH 7.2), 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 150 mM NaCl, 10 μm/ml leupeptin, 10 μm/ml aprotinin, 1 mM phenylmethylsulfonylfluoride] with 20 strokes in a homogenizer. The homogenate was transferred into microtubes and centrifuged at 14,000 rpm for 20 min, after which the supernatant was harvested.

For the immunoblot assay, supernatant samples containing 20 μg of protein each were loaded into individual lanes of 10% SDS-polyacrylamide gels, electrophoresed, and immunoblotted onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA). The residual binding sites on the membrane were blocked by incubation with 5% non-fat milk in Tris-buffered saline (TBS; 10 mM Tris–HCl, pH 7.4, and 150 mM NaCl) for 1 h. Subsequently, the membrane was incubated for 2 h with rabbit polyclonal anti-flotillin-1 antibody (1:3000 dilution). The membranes were washed three times in TBS containing 0.1% Tween 20 before being incubated with horseradish peroxidase-conjugated anti-mouse IgG (Vector, Burlingame, CA, USA) for 1 h. Immunoreactive bands were developed using a chemiluminescent substrate (WEST-one™ kit; iNtRON Biotech, Kyungki, Korea).

After imaging, the membranes were stripped and reprobed using anti-beta-actin antibody (Sigma). The optical density (per mm2) of each band was measured with a scanning laser densitometer (GS-700, Bio-Rad, Hercules, CA, USA), and these values are presented as means ± SEM. The ratios of

the density of each flotillin band relative to that of the beta-actin band were compared using Molecular Analyst software (Bio-Rad).

The data were analyzed using one-way analysis of variance (ANOVA) followed by the Student-Newman–Keuls post hoc test for multiple comparisons. In all cases, $p < 0.05$ was considered statistically significant.

Immunohistochemistry

Immunohistochemical and double immunofluorescence labeling were performed as described elsewhere [13]. In brief, 5-μm sections of paraffin-embedded testis were deparaffinized, treated with citrate buffer (0.01 M, pH 6.0) in a microwave for 3 min, and then treated with 0.3% hydrogen peroxide in methyl alcohol for 20 min, to block endogenous peroxidase activity. After three washes with PBS, the sections were incubated with 10% normal goat serum or rabbit serum and then incubated for 1 h at room temperature with primary antibodies consisting of either rabbit anti-flotillin-1 or goat anti-cathepsin D antibodies. The immunoreactions were visualized using avidin-biotin peroxidase complexes (Vector Elite ABC kit; Vector, Burlingame, CA, USA), and the peroxidase reaction was developed using a diaminobenzidine substrate kit (Vector).

Double immunofluorescence

To examine the cell phenotype of flotillin-1 expression, double immunofluorescence was applied using cell type-specific markers, including ED1 for rat macrophages. First, paraffin sections were reacted sequentially with primary rabbit anti-flotillin-1, secondary biotinylated anti-rabbit IgG (Vector, Burlingame, CA, USA), and tetramethyl rhodamine isothiocyanate

(TRITC)-labeled streptavidin (Zymed, San Francisco, CA, USA). Then, the slides were incubated with second primary reagents, including ED1, and this was followed with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (1:50 dilution; Sigma).

To observe the colocalization of cathepsin D with ED1 or anti-flotillin-1 in testis lesions, sections immunostained with one of the primary reagents, e.g., ED1 or rabbit anti-flotillin-1, were reacted with biotinylated anti-mouse IgG or anti-rabbit IgG (Vector, Burlingame, CA), followed by TRITC-labeled streptavidin (Zymed, San Francisco, CA, USA), and then with the second primary antisera (anti-cathepsin D, ×800 dilution), followed by FITC-labeled rabbit anti-goat IgG (Sigma).

To reduce or eliminate lipofuscin autofluorescence, the sections were washed in PBS (three times for 1 h) at room temperature, dipped briefly in distilled H_2O, treated with 10 mM $CuSO_4$ in ammonium acetate buffer (50 mM CH_3COONH_4, pH 5.0) for 20 min, dipped briefly in distilled H2O again, and then returned to PBS. The double immunofluorescence-stained specimens were examined with an FV500 laser confocal microscope (Olympus, Tokyo, Japan).

II.4. Results

Flotillin-1 protein is significantly increased in testicular torsion

Western blot analysis was used to examine flotillin-1 expression in the testis with testicular torsion (I/R). Flotillin-1 was expressed constitutively in the testes from normal control rats, but its expression was increased significantly in the testes with testicular torsion (Fig. 1). As seen in Fig. 1, flotillin-1 expression was significantly increased at 6 h (0.27 ± 0.02 [mean ± SEM], n = 3, $p < 0.05$) and was further enhanced at 24 (0.35 ± 0.02, n = 3) and 48 h (0.33 ± 0.05, n = 3) after testis I/R, compared with the normal control (0.11 ± 0.01, n = 3).

Increased flotillin-1 immunoreactivity is localized in Sertoli and spermatogenic cells and macrophages in the testis after I/R

In the normal rat testis, weak flotillin-1 immunoreactivity was found in some Sertoli cells, spermatogonia, peritubular myoid cells, and interstitial cells in the testis (Fig. 2A). In the Sertoli cells, flotillin-1 was mainly located in the cytoplasm.

At 24 h after testis I/R, when a significant increase of flotillin-1 was seen in the Western blot analysis, some Sertoli and spermatogenic cells in the damaged seminiferous tubules showed increased flotillin-1 expression compared with the normal controls. Some multinucleated germ cells were detected and were positive for flotillin-1 (Fig. 2B, inset). Furthermore, flotillin-1 was immunostained in the ED1-positive monocyte-like macrophages in the interstitial space surrounding the damaged seminiferous tubules (Fig. 2C–E). The immunoreactivity of flotillin-1 in testis I/R at 48 h was similar to that at

24 h after I/R (data not shown).

Cathepsin D immunoreactivity is increased in the testis after I/R

In normal control rat testis, immunoreactivity for cathepsin D was found in some Sertoli cells, spermatogonia, and interstitial cells (Fig. 3A). At 24 h after testis I/R, cathepsin D immunoreactivity was increased in some Sertoli and spermatogenic cells in the damaged seminiferous tubules compared with the normal control (Fig. 3B).

Flotillin-1 expression is colocalized with cathepsin D in the normal testis and testicular I/R

To examine the relationship between flotillin-1 and cathepsin D, double labeling was performed (Fig. 4A–F). In the normal control, cathepsin D was colocalized in flotillin-1-positive cells, including Sertoli cells, spermatogonium, and interstitial cells (Fig. 4A–C). At 24 h after testis I/R injury, cathepsin D immunoreactivity was colocalized in both flotillin-1-positive cells (Fig. 4D–F) and ED1-positive macrophages (Fig. 4G–I). This indicates that flotillin-1 is closely associated with cathepsin D in Sertoli cells and macrophages in testicular torsion.

II.5. Discussion

This study demonstrated that the expression of flotillin-1 protein was increased significantly in rat testis after I/R. In addition, flotillin-1 immunoreactivity was localized in various testicular cell types in normal controls, whereas flotillin-1 reactivity increased in Sertoli and spermatogenic cells and in monocyte-like macrophages after I/R. These results suggest that flotillin-1 responds to the progression of testicular I/R.

Flotillin-1 is associated with lipid rafts, which are important sites for signal transduction and vesicular trafficking [1]. Flotillins have been included in an expanding list of proteins that are colocalized at caveolae, including protein kinase C alpha, Ras, Rap Src-like kinase, and glycosylphosphatidylinositol-linked receptors [10, 20]. In pathological lesions of other organs, flotillin-1 may promote the trafficking of various molecules and subsequently activate signal transduction [9, 13]. In addition, it has been suggested that their interaction has a role in the vesicular transport of materials between Sertoli and spermatogenic cells [6]. In the present study, after I/R, flotillin-1 expression increased strongly in the membranes of spermatogenic cells (particularly spermatogonia and primary spermatocytes) and Sertoli cells (Fig. 4), suggesting that there is cross-talk between Sertoli and spermatogenic cells, which activates subsequent signals in both spermatogenic and Sertoli cells.

There is a general agreement that flotillin-1 is associated with the formation of phagosomes in macrophage cell lines [3, 8, 19]. Several authors have described phagosomes in Sertoli cells as well as in macrophages [11, 12]. Here, flotilin-1 was colocalized with cathepsin D, a lysosomal marker,

indicating that flotillin-1 accumulates in lysosomes in macrophages and Sertoli cells in the testis after I/R. This suggests that flotillin-1, a lipid raft protein, is either actively or passively involved in the formation or activation of phagolysosomes, resulting in the clearance of cellular debris in macrophages and Sertoli cells in the process of testicular I/R injury.

In conclusion, this study suggests that the lipid raft protein flotillin-1 is involved in the degenerative process after testicular torsion, possibly via the activation of signal transduction enzymes and the formation of phagolysosomes. The precise role of flotillin-1 after testicular injury remains to be elucidated.

II.6. References

1. **Bickel PE, Scherer PE, Schnitzer JE, Oh P, Lisanti MP, Lodish HF**. Flotillin and epidermal surface antigen define a new family of caveolae-associated integral membrane proteins. J Biol Chem 1997, 272, 13793-13802.

2. **Burdan F, Szumilo J, Dudka J, Dabrowski A, Maciejewski R, Wojtowicz Z**. The activity and immunoexpression of cathepsin D in rat male reproductive organs. Folia Morphol (Warsz) 2006, 65, 111-115.

3. **Dermine JF, Duclos S, Garin J, St-Louis F, Rea S, Parton RG, Desjardins M**. Flotillin-1-enriched lipid raft domains accumulate on maturing phagosomes. J Biol Chem 2001, 276, 18507-18512.

4. **Dijkstra CD, Dopp EA, van den Berg TK, Damoiseaux JG**. Monoclonal antibodies against rat macrophages. J Immunol Methods 1994, 174, 21-23.

5. **Dremina ES, Sharov VS, Schoneich C**. Protein tyrosine nitration in rat brain is associated with raft proteins, flotillin-1 and alpha-tubulin: effect of biological aging. J Neurochem 2005, 93, 1262-1271.

6. **Evans 4th WE, Coyer RL, Sandusky MF, Van Fleet MJ, Moore JG, Nyquist SE**. Characterization of membrane rafts isolated from rat Sertoli cell cultures: caveolin and flotillin-1 content. J Androl 2003, 24,

812-821.

7. **Filho DW, Torres MA, Bordin AL, Crezcynski-Pasa TB, Boveris A**. Spermatic cord torsion, reactive oxygen and nitrogen species and ischemia-reperfusion injury. Mol Aspects Med 2004, 25, 199-210.

8. **Garin J, Diez R, Kieffer S, Dermine JF, Duclos S, Gagnon E, Sadoul R, Rondeau C, Desjardins M**. The phagosome proteome: insight into phagosome functions. J Cell Biol 2001, 152, 165-180.

9. **Girardot N, Allinquant B, Langui D, Laquerrière A, Dubois B, Hauw JJ, Duyckaerts C**. Accumulation of flotillin-1 in tangle-bearing neurones of Alzheimer's disease. Neuropathol Appl Neurobiol 2003, 29, 451-461.

10. **Harris J, Werling D, Hope JC, Taylor G, Howardc CJ**. Caveolae and caveolin in immune cells: distribution and functions. Trends Immunol 2002, 23, 158-164.

11. **Igdoura SA, Morales CR**. Role of sulfated glycoprotein-1 (SGP-1) in the disposal of residual bodies by Sertoli cells of the rat. Mol Reprod Dev 1995, 40, 91-102.

12. **Jabado N, Canonne-Hergaux F, Gruenheid S, Picard V, Gros P**. Iron transporter Nramp2/DMT-1 is associated with the membrane of phagosomes in macrophages and Sertoli cells. Blood 2002, 100, 2617-2622.

13. **Kim H, Ahn M, Moon C, Matsumoto Y, Koh CS, Shin T**. Immunohistochemical study of flotillin-1 in the spinal cord of Lewis rats with experimental autoimmune encephalomyelitis. Brain Res 2006, 1114, 204-211.

14. **Moon C, Shin T**. Involvement of macrophages in germ cell death in the rat testis with acute experimental testicular torsion. Korean J Vet Res 2004, 44, 329-334.

15. **Moon C, Ahn M, Kim S, Yasuzumi F, Shin T**. Increased expression of both constitutive and inducible forms of nitric oxide synthase in the delayed phase of acute experimental testicular torsion. J Vet Med Sci 2005, 67, 453-456.

16. **Moon C, Yasuzumi F, Okura N, Kim H, Ahn M, Shin T**. Enhanced expression of tyrosine kinase receptor a in germ cells of rat testis with acute experimental testicular torsion. Urol Int 2005, 74, 79-85.

17. **Moon C, Jeong CW, Kim H, Ahn M, Kim S, Shin T**. Expression of CD44 adhesion molecule in rat testis with ischemia/reperfusion injury. J Vet Med Sci 2006, 68, 761-764.

18. **Nakanishi H**. Neuronal and microglial cathepsins in aging and age-related diseases. Ageing Res Rev 2003, 2, 367-381.

19. **Ng Yan Hing JD, Desjardins M, Descoteaux A**. Proteomic analysis reveals a role for protein kinase C-alpha in phagosome maturation. Biochem Biophys Res Comm 2004, 319, 810-816.

20. **Razani B, Woodman SE, Lisanti MP**. Caveolae: from cell biology to animal physiology. Pharmacol Rev 2002, 54, 431-467.

21. **Steinfeld R, Reinhardt K, Schreiber K, Hillebrand M, Kraetzner R, Bruck W, Saftig P, Gartner J**. Cathepsin D deficiency is associated with a human neurodegenerative disorder, Am J Hum Genet 2006, 78, 988-998.

II.7. Figures

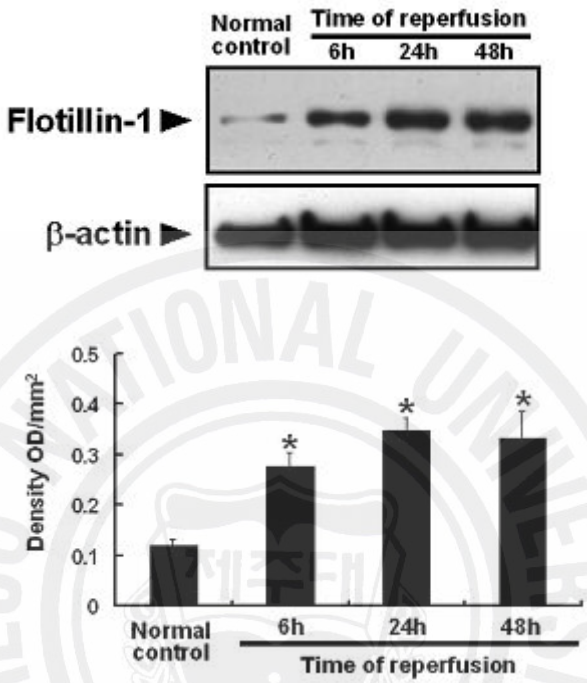

Figure 1. Western blot analysis of flotillin-1 in the testis at various times after ischemia/reperfusion (normal control, and 6, 24, and 48 h). (A) Representative photographs of Western blots with arrowheads indicating the expression of flotillin-1 (48 kDa) and beta-actin (45 kDa). (B) Semiquantitative analysis of flotillin-1 immunoreactivity in the testes normalized to the intensity of beta-actin expression in the same immunoblot. The flotillin-1 immunoreactivity is detected at low levels in the testes of normal controls and is significantly increased at 6, 24, and 48 h after reperfusion. The data (mean ± SEM) are from three experiments. *, $p < 0.05$ vs. normal control.

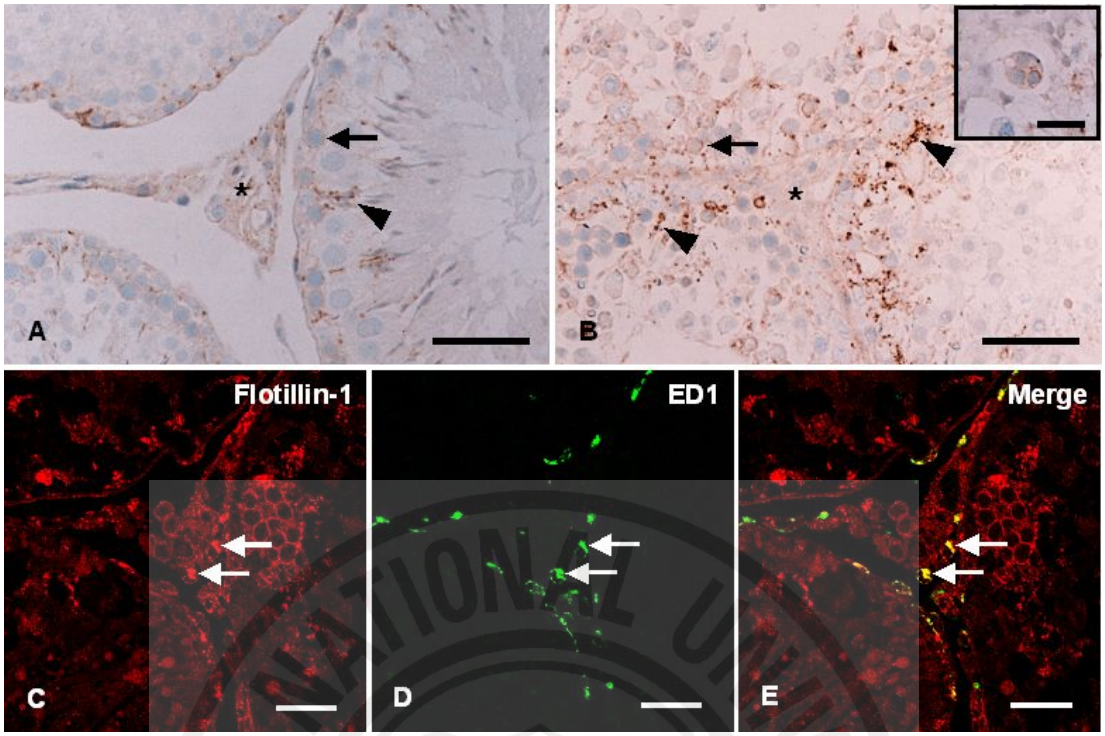

Figure 2. Increased flotillin-1 immunoreactivity is localized in Sertoli and spermatogenic cells and macrophages in testis after I/R. In the normal rat testis, weak flotillin-1 expression is detected in some spermatogonia (A, arrow), Sertoli cells (A, arrowhead), and interstitial cells (A, asterisk). At 24 h after relieving the torsion, flotillin-1 immunoreactivity is increased in spermatogonia (B, arrow), Sertoli (B, arrowheads) and interstitial (B, asterisk) cells. Furthermore, some flotillin-1-positive multinucleated germ cells are observed in the rat testis 24 h after reperfusion (B, inset). In the interstitial space around damaged tubules, flotillin-1 (C, red, arrows) expression is detected in some ED1-positive macrophages (D, green, arrows). The sections in A and B were immunoperoxidase-stained for flotillin-1 and counterstained with hematoxylin. Scale bars: in A–B, 50 μm; in C–E, 20 μm; in inset, 25 μm.

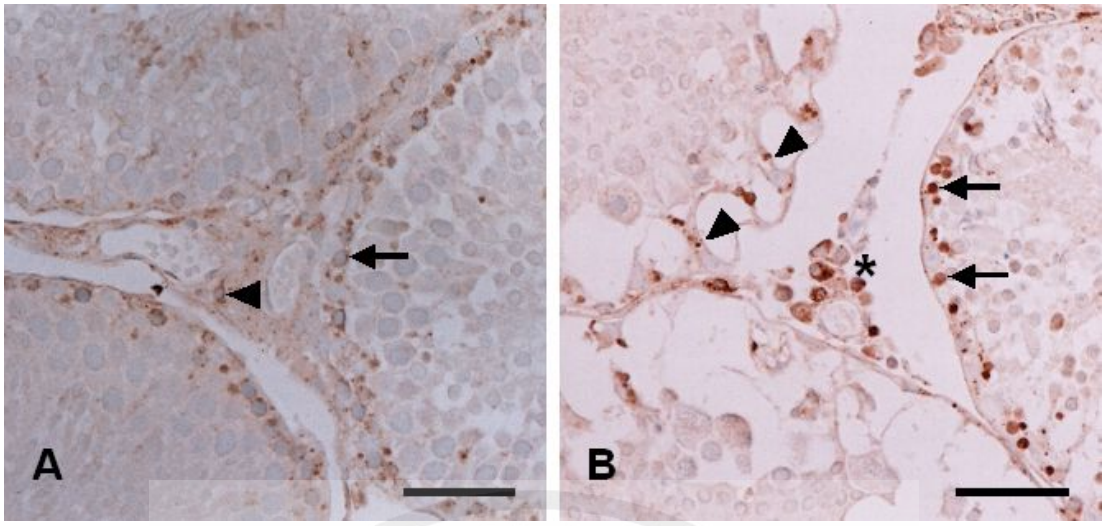

Figure 3. Cathepsin D immunoreactivity is increased in the testis after I/R. In normal control rat testis, cathepsin D immunoreactivity was found in some Sertoli cells (A, arrow), spermatogonia, and interstitial cells (A, arrowhead). At 24 h after testis I/R, cathepsin D immunoreactivity is increased in some Sertoli (arrowheads), spermatogenic (arrows), and interstitial (asterisk) cells in the damaged tubules. Counterstained with hematoxylin. Scale bars = 50 μm.

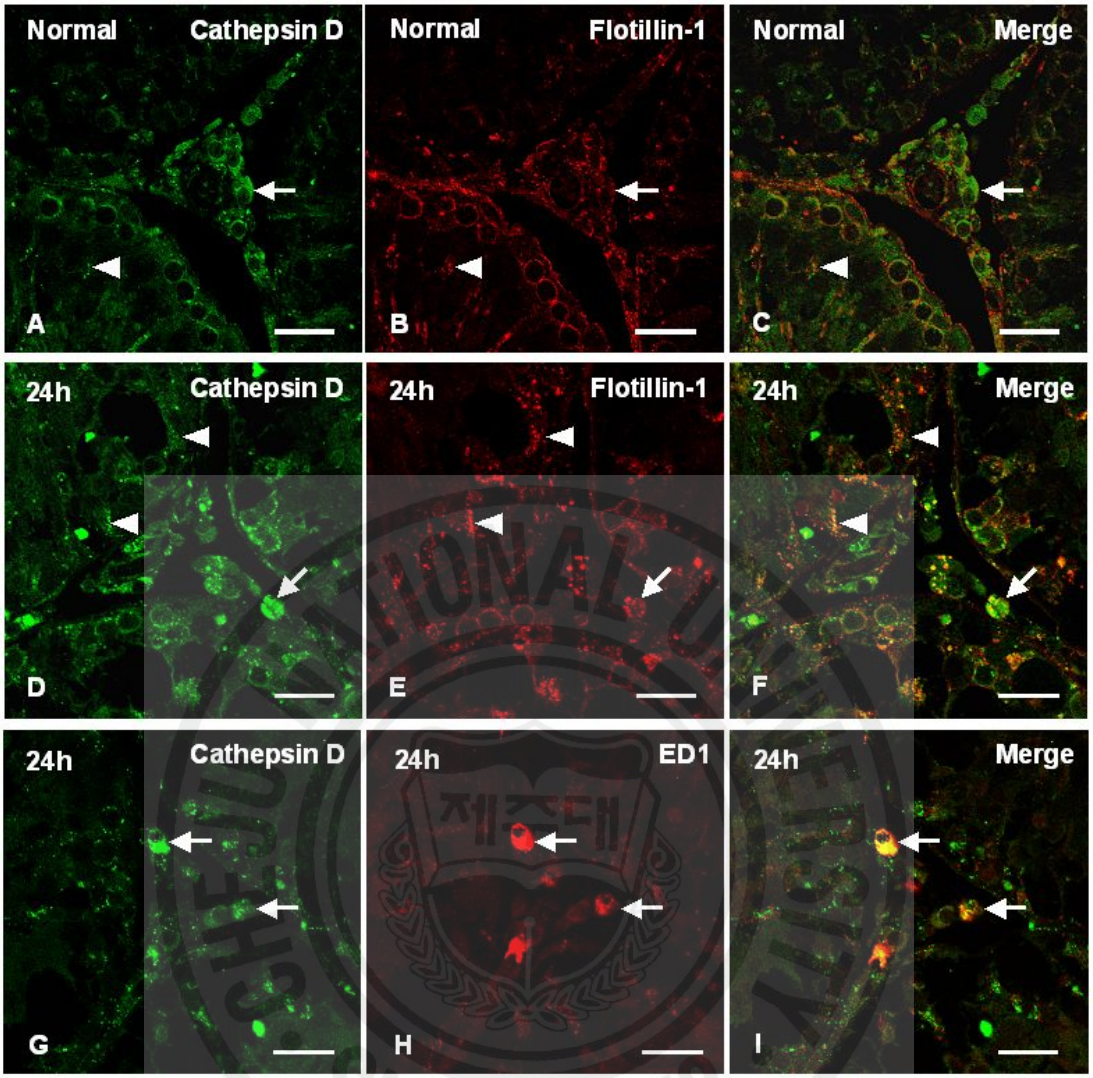

Figure 4. Immunofluorescence colocalization of cathepsin D (A, D, G) with flotillin-1 (B, E) and ED1 (H) in the testis of normal control rats (A–C) and rats at 24 h after relieving the torsion (D–I). In the normal controls, some cathepsin D-immunoreactivity (A, green, arrowhead) is colocalized in flotillin-1-positive Sertoli cells (B, red, arrowhead) in seminiferous tubules (C, merge, arrowhead) and some interstitial cells (A–C, arrow). At 24 h after relieving the torsion, many cathepsin D-positive Sertoli cells (D, green, arrowheads) are positive for flotllin-1 (E, red, arrowheads) in damaged tubules

(F, merge, arrowheads) and interstitial cells (D–F, arrow). In addition, cathepsin D (G, green, arrows) expression is detected in some ED1-positive macrophages (H, red, arrows) in the rat testis at 24 h after reperfusion (I, merge, arrows). Scale bars = 20 μm.

감사의 글

박사학위과정에 입학한 것이 엊그제 같은데, 흐르는 강물처럼 시간이 흘러 졸업을 앞두게 되었습니다. 지난날들을 돌이켜 보면서 감사한 분들께 말씀을 드리겠습니다.

먼저, 1998년 추운 겨울에 방황하고 있는 저를 따뜻하게 맞이해주신 후, 지금까지 학문적, 인격적 가르침을 주신 신태균 교수님께 감사드립니다. 진실로 제 20대 인생의 길에서 신태균 교수님을 만날 수 있었던 것은 저에게 있어서 감당할 수 없는 축복 이였습니다. 다시 한 번 더 감사드립니다. 그리고 제 학위 논문을 심사하시면서 많은 가르침과 조언을 아끼지 않으신 지영흔 교수님, 이용덕 교수님, 전남대학교 수의과대학 문창종 교수님, 일송생명과학연구소 진재광 박사님께 감사드립니다.

수의학과에 입학하여 지금까지 많은 가르침을 주신 김희석 교수님, 박전홍 교수님, 배종희 교수님, 이두식 교수님, 이경갑 교수님, 임윤규 교수님, 우호춘 교수님, 이영재 교수님, 정종태 교수님, 손원근 교수님, 김재훈 교수님, 황규계 교수님, 주홍구 교수님, 윤영민 교수님, 이주명 교수님, 강태영 교수님, 박현정 교수님, 한창훈 교수님, 그리고 고 이국경 교수님께 감사의 마음을 전합니다.

학위과정동안, 여러 신경외과적 기술을 접할 수 있게 도움을 주시고, 삶에 대해여 여러 조언을 주신 의과대학 신경외과 심기범 교수님께 감사드립니다. 그리고 실험 및 연구하는데 많은 부분을 지원해주고, 격려의 말씀을 해주신 의과대학 의학과 현진원 교수님께도 감사드립니다. 또한, 수의학회

에 참석할 때마다 언제나 따뜻하게 맞이해주시고 많은 조언을 해주신 전남대학교 수의과대학 김성호 교수님께도 감사드립니다.

제가 학위를 수료한 후 미국 연구소 생활을 접할 수 있게 초청해주신 보스턴대학교 의과대학 류훈 교수님과 이정희 교수님께도 감사드립니다. 그리고, 미국생활 하는데 여러 도움을 주신 김진호 박사님, 이효순 선생님, Swati, Edward, Kerry, Jane께 감사드립니다.

실험실 생활하는데 여러 격려의 말씀과 도움을 주신 박민수 원장님, 김승준 박사님, 김황룡 선배님, 강재윤 선배님, 김철 선배님, 김정훈 선배님, 강성진 사장님, 강종철 선배님, 안미정 박사님, 김도현 선배님, 허승담 선배님, 박달수 선배님, 이기현 선배님께 감사드립니다. 또한 지금 미국 켄터키주에서 연수중인 광협형과 일본 농공대학교에서 박사과정에 있는 경숙에게도 감사드립니다. 실험실에서 같이 실험하고 많은 일들을 함께 해준 김미아 실장님과 지영, 태기, 지성, 찬우, 정태, 진우, 주연, 지훈, 규영에게 감사드립니다.

마지막으로, 저를 지금까지 많은 사랑으로 키워주신 부모님과 제가 학위과정에 전념할 수 있게 도움을 주신 이모부, 이모님께 감사드립니다. 또한 며칠 없으면 결혼하시는 형님과 형수님, 그리고 멀리 일본에 계신 할머니와 친척 분들을 비롯하여 모든 가족분들께 작은 결실을 드립니다.

2007. 12. 25.

김희철