Characterization of Mouse Interferon-Induced Transmembrane Protein-1 Expression in Mouse Testis

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ABSTRACT

Interferon induced transmembrane protein-1 (Ifitm-1) has been reported to have an important role in primordial germ cell formation, and it has expressed in female reproductive organ. In the present study, Ifitm-1 gene expression was identified in testes and all part of epididymis using western immunoblot and immunohistochemistry. Interestingly, Ifitm-1 expression was observed on the head of spermatozoa. To investigate the role of Ifitm-1 gene expression in behavior of spermatozoa after acrosome reaction, fresh sperm was incubated with calcium ionophore to induce acrosome reaction, whereas the expression of Ifitm-1 was not altered after the acrosome reaction. Then to identify the effect of Ifitm-1 in sperm motility and other seminal parameters, different concentration of Ifitm-1 antibody was incubated with spermatozoa, and seminal parameters were assessed using computer-assisted semen analysis (CASA). Interestingly, motility, progressive, and VAP were increased in the sperm with Ifitm-1 antibody treated compared to rabbit serum, however other parameters such as straightness were not changed. In order to identify the functional significance of Ifitm-1 in fertilization, capacitated spermatozoa were pre-incubated with anti- Ifitm-1 antibody and subsequently examined the ability to adhere to mouse oocytes. However, any defection or alteration in sperm-egg fusion was not found, Ifitm-1 antibody treated or non-treated spermatozoa showed a normal penetration. Although the precise role of Ifitm-1 in sperm motility and following fertilization need to be elucidated, this study suggests that the activation of Ifitm-1 on the sperm may enhance the motility of spermatozoa in mice.

(Key words : Ifitm-1, Testis, Fertilization, Sperm motility, Gene expression)

INTRODUCTION

Interferon-induced transmembrane protein-1(Ifitm-1) known as 9~27 or Leu13 was identified as a 16 kDa interferon inducible protein in endothelial cells, and this is a first member of the interferon-induced transmembrane protein families (Yang *et al*., 2007). Ifitm-1 has been suggested to have the functions in a variety of contexts, including immune cell regulation, cancerogenesis, spermatogenesis, and germ cell development. In human leukocyte cell lines, for example, Ifitm-1 is thought to mediate antiproliferative activities and cell-cell adhesion process (Bradbury *et al*., 1992; Evans *et al*., 1993). Ifitm-1 was found to induce purified T cells to aggregate when added to cultures in nanogram concentrations (Pumarola *et al*., 1986). In addition, Ifitm-1 contribute to the cellular adhesion properties of leukemic B cells and monoclonal antibody to the Ifitm-1 induced aggregation and inhibits proliferation of leukemic B cell (Evans *et al*., 1990). Increased expression of Ifitm-1 related to interferon (IFN) inhibited of cell growth in a subset of cell lineages and this gene is an important factor for anti-proliferative action of IFN-γ, and arrests cell proliferation in a p53-dependent manner (Yang *et al*., 2007). According to a recent report, Ifitm-1 co-localized with caveolin-1 (CAV-1) of the plasma membrane, and interaction between Ifitm-1 and CAV-1 induced inhibition of CAV-1 on extracellular signal-regulated kinase activation (Xu Y *et al*., 2009). As expression of Ifitm-1 is frequently found in a variety of cell- and tissue-associated diseases, Ifitm-1 can be served as a bio-

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markers of cis-platinum activity in esophageal squamous cell carcinoma (Fumoto *et al*., 2008), and human-Ifitm-1 expression profiles can beused to classify types of chronic myeloid leukemia (Akyerli *et al*., 2005).

Regarding to Ifitm-1 functions in reproduction, Ifitm-1 has been reported to involve in primordial germ cell (PGC) development (Tanaka *et al*., 2002). When the formation of PGCs is completed, the localization of PGCs in different germ layers is influenced by the expression of Ifitm-1. PGC precursor in the mesoderm expressed Ifitm-1, however the PGC were relocated to the endoderm, Iftim-1 expression was stopped (Tanaka and Mastui, 2002; Tanaka *et al*., 2002). In addition, Ifitm-1 has a role in repulsive interaction between the mesoderm and PGC precursors (Tanaka *et al*., 2005). In addition, recent gene expression study in uterus revealed that increased Ifitm-1 expression was observed in estrus stage, but decreased in diestrus stage (Park *et al*., 2011a). This study also reported that Ifitm-1 expression in uterus is highly correlated to follicle stimulating hormone (FSH) (Park *et al*., 2011). However, the characterization and function of Ifitm-1 in testis have not been widely studied. In this study, Ifitm-1 gene expression in mice testis and other male reproductive organ were identified, and putative functions of this protein in semen parameters were also observed.

MATERIALS AND METHODS

Immunohistochemistry

Reproductive organs were washed in PBS then the samples were frozen with optimal cutting temperature compound (Sakura Finetek, Tokyo, Japan) at −70℃ for 72 h. Frozen tissue slices with 3 μm thickness were mounted on a glass slides (Machine for tissue slice: Leica Microsystems, Mussloch, Germany). Frozen tissues were fixed in 4% formalin at 10 min, then the frozen tissues were treated with 0.1 % Triton X100 for permeabilization. Samples were blocked at room temperature for 1h in 10% BSA supplemented in PBS, then the slides were incubated in 1:200 anti-rabbit mouse Ifitm-1 polyclonal antibody at 4℃ for overnight. After three times wash with PBS, 1:500 dilutions of the corresponding rhodamin-conjugated anti-rabbit secondary antibody were applied. Sections were washed three times and a 4′-6-diamino-2-phenylindole (DAPI) staining was carried out for staining nuclei as described in previous report (Park *et al*., 2011b). For paraffin section of mouse reproductive organs, testis samples were fixed with 4 % paraformaldehyde for 72 hours in 4% paraformaldehyde and dehydrated for 1 hour in various percentages of ethanol from 70% to 100%. To make paraffin block, fixed testis samples were exposed in 100% xylene for 60 minutes, then exposed in paraffin solution for 3 hours at 72℃. Paraffin sections(3 μm) were imbedded on slide glasses.

Immunoblotting

Testes were lyzed in 1% triton X-100 buffer containing 25 mmol/l Hepes, 150 mmol/l NaCl, and 5 mmol/l $MgCl_2$, pH 7.5 at 4℃ for 1 hr. After centrifugation, the supernatants were collected, and the total protein was quantified using the DC protein assay kit (Bio- Rad, Chester, UK). Thirty micrograms of total protein were subjected to 14% polyacrylamide gel and performed electrophoresis. The membrane was incubated in TBS-T containing 1% bovine serum albumin for 1 hour at room temperature. After rinsing in TBS-T, the membrane was incubated with an anti-rabbit mouse Ifitm-1 antibody, then the samples were washed in TBS-T. The membrane was incubated with HRP-conjugated anti rabbit IgG antisera (Santa cruz, CA, USA). The protein bands were detected using enhanced chemiluminescence (Amersham, Buckighmshire, UK) and Hyper-film TM CELTM (Amersham, Buckighmshire, UK).

Analysis of Sperm Parameters

Sperms were collected from the cauda epididymis of sexually matured ICR mice (Chung Ang Biolab, Umsung, Korea). Epididymal cauda was carefully trimmed to remove adipose and other tissues, then rinsed in PBS and incubated in M2 media. After the incubation, the tissue was removed and the suspension was mixed gently by swirling. This suspension was then diluted 1:20 to 1:60 in M2 media to a concentration of $2\sim4\times10^5$ sperm/ml, equivalent to 50～120 sperm per microscope field for CASA. For the analysis of motility under non-capacitating conditions, sperm were diluted at the same ratio to M2 media. In addition, sperm were incubated in Ifitm-1 antibody (5, 10, 20, 40 μg/ml) and normal rabbit IgG contained M16 media for 2 hour, and the motility and other semen parameters were assessed using CASA.

Induction of Capacitation and PSA Staining

Collected sperm were incubated with 10 μM calcium ionophore A23187 in M16 media for 30 minutes to induce capacitation. Then sperm were fixed in formalin at 10 min. After 3times washing, sperm were treated with 0.1 % Triton × 100 at 3 minutes for permeable, and 10% FBS in PBS were treated for blocking. 1:200 Ifitm-1 antibody in 2 % BSA were incubated for 1 hr at room temperature, then *Pisum sativum* agglutinin(PSA)-Rhodamin and goat anti rabbit IgG-FITC were treated.

Sperm-Zona Pellucida Free Oocyte Binding Assay

ICR female mice (8 weeks old) were superovulated with 5 IU PMSG, and after 12 hours later 5 IU hCG was injected. After collecting the metaphase II stages of oocytes, control (none Ifitm-1 antibody treated) and 20 μg of Ifitm-1 antibody treated sperm were injected to zonapellucida free oocytes cultured media to induce *in vitro* fertilization (IVF). After sperm-egg fusion, zygotes were fixed in 4% formalin and permeabled in 0.1 % Triton ×100, then directly mounted in slide glass for DAPI staining.

Statiscal Analysis

All data were expressed as means and standard deviation. One-way ANOVA for Ifitm-1 mRNA expression at different time courses and Scheffe post hoc test was used to determine the significance. Also signinicances in semen parameters were also analyzed in same manner. Significance for all analyses was at $p<0.05$.

RESULT AND DISCUSSION

Ifitm-1 Expressed in Spermatozoa in Adult Testes

To identify the expression of Ifitm-1 protein in testes and epididymis, total protein extractions from the whole testes, caput, corpus, cauda epididymis were analyzed. 18 kilo Dalton of Ifitm-1 protein was identified in all tested organs by western immunoblot (Fig. 1A). Immunohistochemistry data showed that Ifitm-1 protein was observed mostly in rumen of seminiferous tubule area, whereas very few cells around basal membrane were identified (Fig. 1B). In the epididymis, relatively strong Ifitm-1 signal was identified on the cells in inside of the epididymal rumen (Fig. 1B). As most of cells in the rumen of epididymis are spermatozoa, therefore Ifitm-1 positive cells in epididymis can be regarded as the spermatozoa. To determine whether the spermatozoa expressed Ifitm-1, immumocytochemistry was performed, and the data clearly showed that the Ifitm-1 was expressed on the head and mitochondrium of spermatozoa (Fig. 1C). Male germ cells have many genetic and physiological changes during the spermatogenesis. This complex process was regulated by the thousands of genes and proteins, which influence to transcriptional mechanism of genes involved in spermatogenesis (Hecht, 1998; Eddy *et al.*, 1998). Although, the function of Ifitm-1 gene in sperm has not been identified, expression of Ifitm-1 in gonadal primordial germ cells (PGCs) has been observed, and its expression was reported to relate to PGC migration (Tanaka and Mastui, 2002). In addition, Ifitm-1 expression was increased in germ cell during germ cell development at the stages from A1 to A4 spermatogonia (Lacham-Kaplan, 2004). However, Ifitm-1 expression has not been

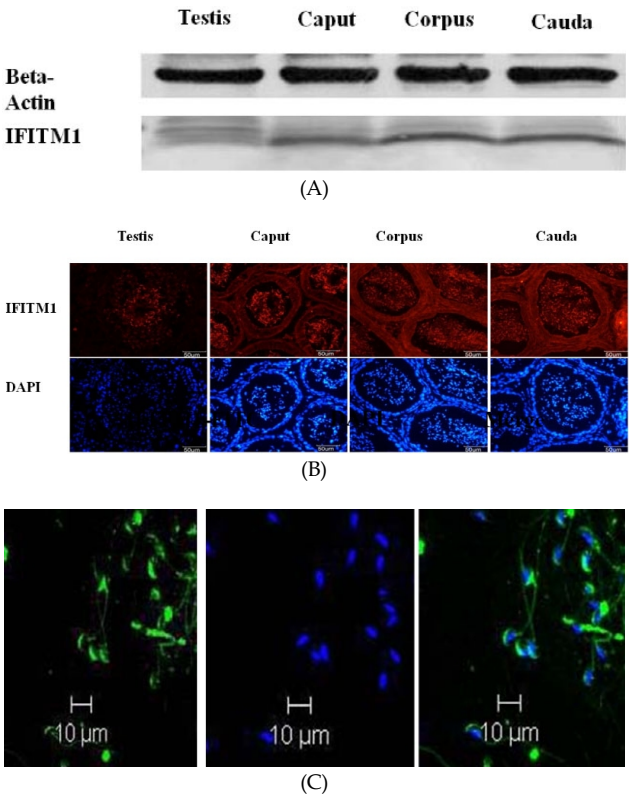

Fig. 1. Ifitm-1 expression in mice whole testis and epididymis. (A) western immunoblot using total protein extract from whole testis, caput, corpus and cauda epididymis. Quantification of protein loading was compared to level of beta-actin protein. (B) Immunohistochemistry of whole testis and each part of epididymis. DAPI staining was performed to identify the location of cells. The scale bar represents 50 μm. (C) Immunocytochemistry of spermatozoa. The scale bar represents 10 μm. Anti-rabbit mouse Ifitm-1 antibody was used in all three experiments.

reported in developing male germ cells during the spermatogenesis. In the present study, Ifitm-1 expression on the head and mitochondrium of spermatozoa has reported for the first time, and elucidating the roles of this protein in sperm behavior and fertilization should be conducted.

Ifitm-1 Expression Was not Altered by Acrosome Reaction

Acrosome reaction (AR) was induced to identify the changes of Ifitm-1 expression using calcium ionophore, and the AR was assessed by Pisum astivum agglutinin (PSA) staining. When spermatozoa undergoes AR, the head of spermatozoa are turned to red by PSA staining. As shown in Fig. 2, spermatozoa treated with calcium ionophore stained red in PSA, but control spermatozoa did not stained with PSA. However, Iftim-1 expression was not altered in both AR positive and negative (Fig. 2). This data suggests that Ifitm-1 expression and localization are not related to acrosome reac-

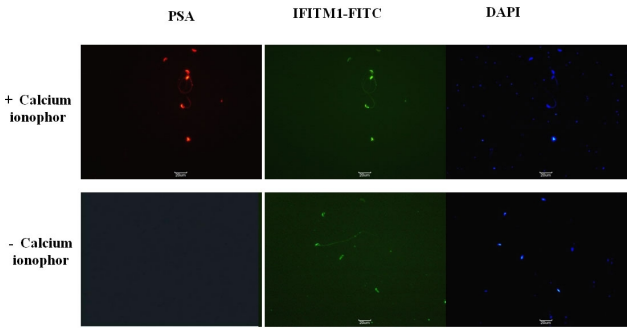

Fig. 2. Immunocytochemistry of spermatozoa. Spermatozoa were treated with calcium ionophore to induce acrosome reaction. Activated spermatozoa by calcium ionophore stained in red with *Pisum astivum* agglutinin (PSA), and green color represents the Ifitm-1 positive cells. Cellular localization was detected with DAPI staining.

tion. Generally, spermatozoa in the testes and epididymis are not able to recognize and move to oocytes, they must have these abilities such as AR (Aida *et al*., 2000). Spermatozoa with AR can move and recognize to oocytes, and can be reacted to signals from oocytes (Edda *et al*., 2000). It is not clear that specific gene expression is changed after the AR, however certain functions of spermatozoa are able to be altered such as motility. In this regard, although the protein expression level was not changed following AR, it cannot be ruled out that Ifitm-1 may contribute functional changes of spermatozoa.

Effects of Ifitm-1 on Sperm Motility and Fertility

Vigorous sperm motility, including the transition from progressive to hyperactivated motility that occurs in the female reproductive tract, is required for normal fertilization in mammals. In this study, spermatozoa from the caudal epididymis were incubated for 30 minutes in M16 media containing 3 mg/ml BSA at 37℃. Then either with Ifitm-1 antibodies at the concentrations of 5, 10, 20, and 40 μg/ml or 20 μg/ml of normal rabbit serum was treated for 0.5, 1, 1.5, and 2 hour to observe the effect of Ifitm-1 protein action on semen parameters. After incubation of sperm with antibody incubation, semen parameters including motility, progressive, velocity average path (VAP), velocity curvilinear (VCL), velocity straight line (VSL), and straightness (STR) were assessed using CASA. Spermatozoa treated 10 and 40 μg/ml for 0.5 and 1 hour, respectively, and 5 μg/ml for 1.5 hour showed a significant increase in motility compared to normal rabbit serum treatment (Fig. 3A). The significantly increased number of progressive spermatozoa was observed in 10 and 20 μg/ml for 0.5 h, 5 to 15 μg/ml for 1 h, and 10 μg/ml for 1.5 h of antibody treatment (Fig. 3B). In VAP, spermatozoa with 10 and 15 μg/ml for 0.5 hour, 5 to 15 μg/ml for 1 hour, and 5 μg/ml for 1.5 hour Ifitm-1 antibody treatment group showed an increase (Fig. 3C). For the VSL and VCL, only 1 hour incubation with 15, and 5 to 10 μg/ml of Ifitm-1 antibody showed the significances, respectively (Fig. 4D and E), However the overall straightness has not been altered (Fig. 4F). This data suggest that Ifitm-1 on the spermatozoa may be activated by antibody, then promoted semen parameters including motility, progressive, and

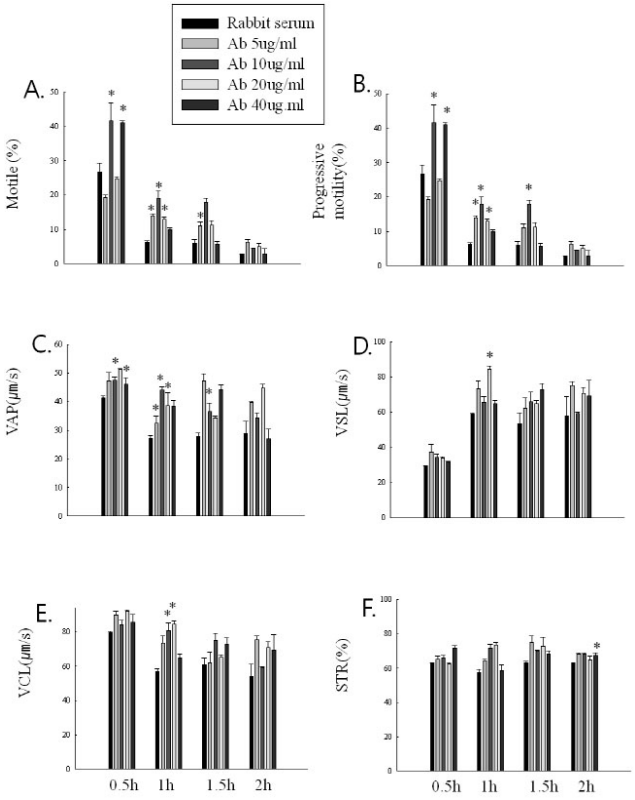

Fig. 3. Assessment of semen parameters using computer-assisted sperm analysis (CASA). (A) motility, (B) progressive, (C) velocity average path (VAP), (D) velocity straight line (VSL), (E) velocity curvilinear (VCL), and (F) straightness (STR) were analyzed after the sperm were incubated with normal 20 μg/ml of rabbit serum or 5, 10, 15 and 20 μg/ml of anti-Ifitm-1 antisera. * $p<0.05$

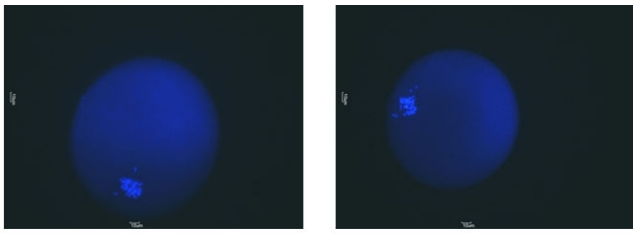

Fig. 4. Sperm-oocyte binding assay. Spermatozoa treated with 20 μg/ml of anti-Ifitm-1 antisera were incubated with zonapellucida free oocytes to induce *in vitro* fertilization. DAPI staining was performed to identify the number of spermatozoa in oocyte. The experiment was replicated 5 times using a minimum 10 oocytes per treatment.

VAP. It has been reported that Ifitm-1 can be regulated by wnt/β-catenin signaling and bone morphogenic protein (BMP) 4 during embryogenesis (Saitou *et al*., 2003), because Ifitm-1 is a downstream molecule of wnt/β-catenin signaling pathway (Lickert *et al*., 2005). In mouse, continuous activation of wnt/β-catenin signaling pathway caused a weakness of seminiferous tubule formation results in sterility (Alexandre *et al*., 2008). Generally, activated spermatozoa by capacitation and acrosome reaction are fate to death. In this regard, Ifitm-1 activation in spermatozoa may enhance the migration ability to oocytes, however spermatozoa itself goes to death. In support of this hypothesis, spermatozoa exposed to anti-Ifitm-1 antibody for over 1 hour with high dose showed less than 10 % of motile sperm, suggesting that hyperactivation of spermatozoa reduced its viability but increase the motility.

The penetration of the zonapellucida is a crucial step during fertilization. Only capacitated sperm are enabled to recognize the oocyte and respond to the oocyte signals in an appropriate manner. Close to time of ovulation sperm are released from the oviductal epithelium and swim to site of fertilization. Sperm-oocyte fusion is most important result in fertilization ability of sperm. To assess the possible role of Ifitm-1 in sperm-oocyte fusion, spermatozoa were incubated with Ifitm-1 antibody, and normal *in vitro* fertilization (IVF) was conducted with zonapellucida free oocytes. After IVF performed, embryo was stained with DAPI to identify the chromosome formation. As a result, both Ifitm-1 antibody treated and none-treated spermatozoa showed a normal fertilization, and they formed the full zygotic nucleus (Fig. 4). This data suggest that activation of Iftim- 1 protein is not involved in sperm-egg fusion.

In the present study, Ifitm-1 expression was identified on the head and mitochondrium of spermatozoa for the first time, and the protein expression has not been altered following acrosome reaction. However, activation of Ifitm-1 by anti-Ifitim-1 antibody increased the semen parameters for enhancing fertilization such as motility, progressive, VAP. This data may contribute to understand the mechanism of movement behavior of spermatozoa in female reproductive organ.

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