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Original Research

Equine Tight Junctions: Tissue-Specific Localization and Expression of Junction Adhesion Molecule-A, Zona Occludens-1, and Occludin

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ABSTRACT

Tight junctions regulate physiological homeostasis by controlling paracellular transportation and forming physical barriers. *Equus ferus caballus,* the domestic horse, is an economically important animal and studies on equine cell-to-cell junctions are insufficient. In fact, common equine diseases such as colic, skin carcinoma, and West Nile disease are related to tight junction degradation. In this study, expression and distribution of tight junction proteins including junction adhesion molecule-A, zona occludens-1, and occludin were examined to understand the physiological characteristics of equine tight junctions. Protein expression of these three factors was quantified by Western blotting using proteins extracted from liver, kidney, lung, duodenum, heart, testis, ovary, and uterus. Localization of the tight junction proteins was observed in those tissues via immunohistochemistry. Expression of the proteins was primarily detected in the epithelium and endothelium. Expression pattern in equine tissues for the three tight junction proteins tested is different from that reported in mice or humans. Results from this study can help elucidate the organization of tight junctions in equine tissues as well as mechanisms underlying tight junction-related disease in horse.

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1. Introduction

Cells are connected to one another and communicate with each other. Intercellular connections include tight junctions (TJs), gap junctions, desmosomes, and adherens junctions [1]. TJs are reported to have various functions including sealing the intercellular space of the epithelium and forming various types of physiological barriers such as the blood-brain barrier or blood-testis barrier. These barriers regulate physiological characteristics like permeability, material transport, and maintenance of osmotic balance [2] and processes such as spermatogenesis [3]. Furthermore, these structures are subcellular organelles that hold cells together by joining the cytoskeletons of adjacent cells [4]. In studies using knockout mice models, imperfect formation of tight junction barriers resulted in dehydration, brain edema, malabsorption, hypernatriuria, and gastritis [5]. Additionally, loss in cohesion of the TJ structure can facilitate tumor cell invasion and then ultimately lead to the metastasis of cancer cells [6].

Equus ferus caballus, the domestic horse, is an economically important animal. Few studies on equine cell-to-cell junctions have been performed. Like mice and humans, tight junction-related disorders occur in horses. For example, West Nile disease is a zoonosis that affects horses

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and humans. In mice injected with West Nile virus, the expression of junction adhesion molecule-A (JAM-A), zona occludens-1 (ZO-1), and occludin (OCLN) is significantly reduced [7]. Colonic obstruction causes profound and sometimes fatal abdominal disease in horses and is characterized by severe structural changes to intestinal tight junctions [8].

The present study evaluated three protein components of tight junctions: JAM-A, ZO-1, and OCLN. JAM-A and OCLN are typical transmembrane proteins, whereas ZO-1 is a cytoplasmic plaque protein. JAM-A, a member of the immunoglobulin superfamily, is found in intracellular junctions of the endothelium and epithelium of various organs [9]; it is also expressed in platelets, antigen-presenting cells, and lymphocytes [10]. JAM-A binds to other junctional proteins including as ZO-1 and OCLN and regulates homeostasis by forming a physical barrier [11].

ZO-1 is essential for the formation of tight and adherens junctions [12] as a scaffolding protein [13]. ZO-1 controls paracellular permeability by coupling with perijunctional actin and myosin [14] and participates in the regulation of cell growth by interacting with nuclear proteins [15]. OCLN contains four transmembrane domains, two extracellular loops of similar size, and a long carboxyl terminal region [16]. For assembling tight junctions, the COOH terminal of OCLN has a specific binding site for ZO-1 [17]. It would appear that OCLN is not essential for tight junction formation as studies using OCLN-knockout mice and mouse embryonic stem cells indicate that tight junctions may be formed in its absence [18,19]. However, OCLN-deficient mice showed deafness through defective positioning of tricellulin and progressive apoptosis of cochlear hair cells [20].

We measured protein levels of JAM-A, ZO-1, and OCLN using Western blotting methods, and confirmed the distribution of these factors by tissue immunohistochemistry in equine liver, kidney, lung, duodenum, heart, testis, ovary, and uterus.

2. Materials and Methods

2.1. Ethics and Animal Experiments

Tissues were harvested from four horses immediately postmortem at the slaughterhouse and included liver, kidney, lung, duodenum, heart, testis, ovary, and uterus. Horse A was a 4-year-old stallion, horse B was a 6-year-old gelding, horse C was a 14-year-old mare, and horse D was a 5-year-old mare. Dissected tissues were washed out with cold sterile saline (0.9% NaCl) and transported to the laboratory on ice. Some tissues were fixed in 10% neutral-buffered formalin for immunohistochemistry, and extracted RNA and protein were stored at −70°C until use. The study was approved by the Committee on the Ethics of Animal Experiments of the Chungbuk National University (approval number CBNUA-257-1004-01; approval date 30 November 2010).

2.2. Western Blot Analysis

Proteins were extracted with cold Pro-prep (InTron) according to the manufacturer's instructions. Tissues were homogenized with a Dounce homogenizer at 4°C and centrifuged at 15,300 g for 20 minutes at 4°C. The supernatant containing soluble proteins was transferred to a new tube, after which the protein concentration was determined by BCA assay (Sigma-Aldrich) at 562 nm. Protein (50 μg) was loaded onto a 12.5% sodium dodecyl sulfate polyacrylamide gel for electrophoresis at 60V–90V for 2 hours and then transferred to a nitrocellulose membrane (millipore) at 200 mA for 2 hours. The blot was blocked with tris-buffered saline containing 0.5% Tween-20 (TBS-T) including 5% skim milk for 1 hour at room temperature (RT), and then, each blot was probed overnight at 4°C with the primary antibodies diluted in TBS-T including 1% bovine serum albumin: rabbit anti-human JAM-A IgG (Santa Cruz biotechnology, sc-25629, 1:1,000), rabbit anti-human ZO-1 IgG (Invitrogen, 61-7300, 1:1,000), rabbit anti-human OCLN IgG (Invitrogen, 71-1500, 1:1,000), mouse anti-human GAPDH IgG (Santa Cruz biotechnology, sc-137179, 1:1,000). After washing three times for 5 minutes each with TBS-T, blots were incubated with the horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz biotechnology, sc-2004, 1:4,000) or goat anti-mouse IgG (Bioss, bs-0296G-HRP, 1:4,000) antibody in TBS-T containing 2.5% skim milk for 2 hours at RT. After washing three times for 5 minutes each with TBS-T, the blots were exposed to enhanced chemiluminescence reagent (Santa Cruz Biotech) and then were developed by using the GenGnome5 imaging system (Syngene). The optical density of the target band was examined by NIH ImageJ software.

2.3. Immunohistochemistry

Fixed pieces of liver, kidney, lung, duodenum, heart, testis, ovary, and uterus were embedded in paraffin, cut into 4 μm sections, mounted on glass slides, deparaffinized using xylene, hydrated in descending graded ethanol solutions, and washed in tap water for 10 minutes. For JAM-A and ZO-1 antigen retrieval, tissues were boiled for 10 minutes in acidic citrate buffer (pH 6.00) and for 5 minutes in basic citrate buffer (pH 9.00), respectively. Tissues for OCLN detection were incubated in tris-EDTA buffer with proteinase K 20 μg/μL for 20 minutes at 36.5°C. Endogenous peroxidase activity of tissues was blocked in TBS-T with 3% hydrogen peroxide for 30 minutes and washed with distilled water. To prevent nonspecific reaction, tissue sections were incubated with 10% normal goat serum and then probed at RT overnight with primary antibody. Primary antibodies used for immunohistochemistry were the same as those used in Western blotting. After washing three times for 5 minutes each with TBS-T, the tissue sections were incubated with biotinylated anti-rabbit (Vector laboratories) or anti-mouse (Vector laboratories) antibodies for 1 hour at 37°C, washed with TBS-T, and exposed to the biotin/avidin interaction incorporated in the Vectastain ABC kit (Vector Laboratories) for 1 hour at 37°C. After washing three times for 5 minutes each with TBS-T, sections were exposed to diaminobenzidine (Sigma) and were counterstained with hematoxylin before mounting in Canada balsam.

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