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Impact of Hyperosmotic Glycerol on Tight Junction In Vitro

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Abstract The tight junction regulates passage of molecules through the paracellular spaces. Occludin and claudins are the specific transmembrane proteins present at the tight junction and are believed to regulate the cell barrier functions. To examine the response of the tight junction to hyperosmotic solutions, I investigated the effects of hyperosmotic glycerol on function and protein expression of the tight junction in ECV304 cells. Cell cytotoxicity analysis showed that the high (10%) concentration of glycerol damaged 64.1% of the ECV304 cells ($p<0.001$), and this was confirmed morphologically. Treatment with 1%, 2% or 5% glycerol increased the paracellular permeability of fluorescein isothiocyanate (FITC) -labeled dextran by 4.7%, 18.7% and 29.4% ($p<0.05$), respectively. In addition, exposure to glycerol at any concentration strongly reduced the expression of occludin, whereas expression of claudin-1 was affected very slightly. These results suggest that hyperosmotic glycerol would certainly induce the paracellular pathway in vitro and modulate the expression of tight junctional molecules.

Introduction

The tight junction (TJ) is located at the most apical side of the cell border of epithelium and endothelium. Electron micrographs of ultrathin sections show that the TJ appears to be a set of discrete sites of apparent fusion involving the outer leaflets of the plasma membrane.[1] The freeze-fracture method also demonstrates continuous strands on the cell borders.[2] The TJ prevents various water-soluble molecules, electrolytes and proteins from passing freely through the paracellular space (barrier function).[3] It also separates the plasma membrane into the apical and basolateral parts to maintain cell polarity (fence function).[4]

Several proteins in the TJ have been identified, such as ZO-1[5], ZO-2[6], ZO-3[7], cinglin [8], 7H6 antigen [9], and symplekin.[10] These proteins are recruited and concentrated at the cytoplasmic surfaces close to TJ structures. Occludin was the first identified TJ-specific transmembrane protein.[11] Occludin has four transmembrane domains connected with a long carboxy terminal and forms a TJ strand itself.[12] The cytoplasmic domain is directly associated with ZO-1, ZO-2, and ZO-3 molecules.[13] Most recently, two novel TJ proteins, claudin-1 and -2, were cloned by the same method used to isolate occludin.[14] Claudins form the claudin family, with subtypes that have specific expression in the main organs

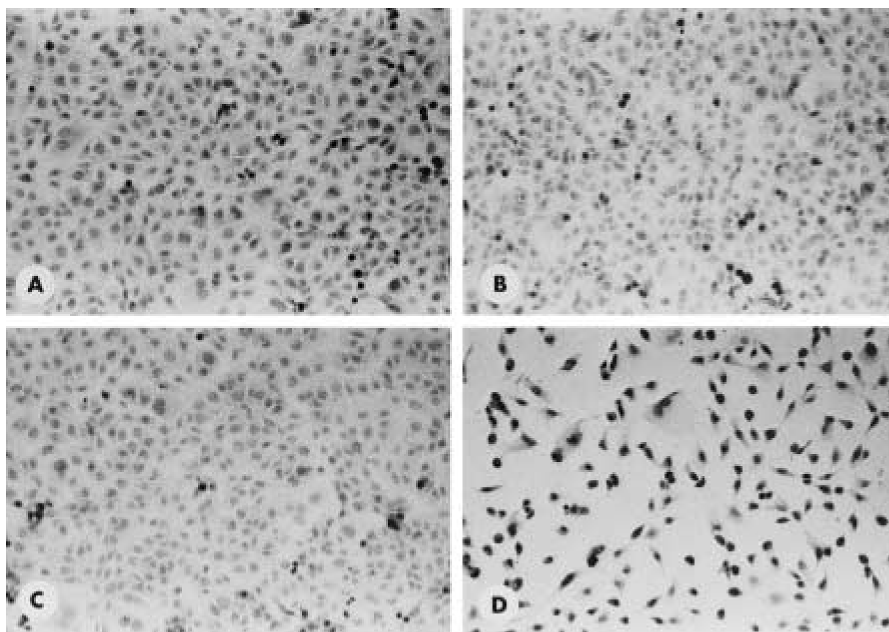

Fig. 1 Morphological examination of ECV304 cells in the presence of glycerol. ECV304 cells develop and form a monocellular layer in the basal medium (A). Cells were exposed to 1%, 5% or 10% glycerol in the flask for 1 h. Cells in 1% (B) and 5% (C) glycerol showed no apparent change of cell architecture, whereas those in 10% (D) glycerol demonstrated marked cell damage with extensive shrinkage. HE stain, x 100.

of the body.[15]

In the central nervous system (CNS), the TJ of endothelium plays an important role in maintaining the homeostasis of neural function. This barrier function constructs the blood-brain barrier (BBB), which regulates the paracellular transport of molecules from the blood space to the brain parenchyma. [16] The BBB also restricts entry of many therapeutic drugs into the brain. Many trials have examined transient opening of the BBB to enhance the therapeutic effect. Hyperosmotic solutions (such as mannitol, glycerol, arabinose, lactamide and urea) are commonly used, in experiments and in clinical treatment.[17-21] The aims of this research are to determine how hyperosmotic solutions open the BBB in situ and to clarify the molecular mechanism of the hyperosmotic effect on the TJ, especially any direct influence on TJ molecules, with a view to modulating the function of the TJ artificially for clinical purposes. Although mannitol is commonly used, glycerol has some advantages, such as high osmolarity and low viscosity .[22] In this study, I focused on glycerol to examine directly its hyperosmotic influence on TJ molecules.

In this study, I investigated the abilities of glycerol to open the TJ barrier in vitro and to influence the expression of TJ molecules using the human cell line ECV 304. The results indicated that glycerol probably modulates the expression of TJ molecules, and thus may contribute to enhance paracellular permeability.

MATERIALS AND METHODS

Cells. ECV304 (ATCC/CRL-1998, kindly provided by Dr. Yasufumi Satoh, Tohoku University) and MDCK (ATCC/CCL-34) cells were used. The cells were fed and maintained with Dulbecco's Modified Eagle Medium (D-MEM) (Sigma) containing 10% fetal bovine serum (FBS), 2 mM glutamate and 1% penicillin/streptomycin at 37℃ under 5% CO_2 in humidified air.

Hyperosmotic solutions. Glycerol (1.09 M) (Glyceol®, Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) and D-mannitol (1.10 M) (Mannitol®, Nikkenn Co., Ltd., Toky o, Japan) were used as hyperosmotic solutions.

Morphology. ECV304 cells were cultured in a 12-well plate until the cells grew to confluence for three days. The fresh media containing glycerol were exposed for 1 h. After being washed twice with Dulbecco's phosphate-buffered saline (D-PBS) (Sigma), these cells were stained with hematoxylin-eosin and observed by light microscopy.

Cell cytotoxicity. Five thousand cells were cultured in each well of a 96-well plate for 24 h. After spent media had been aspirated, 100 μl of fresh medium containing glycerol was added to each well and incubated for 1 h. The medium was aspirated, then fresh medium containing 10% indicator solution (alamarBlue®, GIBCO) was added and incubated for another three hours. The plate was then scanned using a spectrophotometer (570 nm) (BioRad). The cell cytotoxicity (%) was calculated as the percentage of the untreated control, and is presented as mean±SD of seven independent measurements. Treatment with 0.5% Triton X-100 was done to obtain data for complete cell killing.

Permeability assay. To analyze the paracellular permeability through ECV304 cells, the two-chamber system was used. An insert with a collagen-coated microporous poly-tetrafluoroethylene (PTFE) membrane of 3.0 μm pore size and 6.5 mm diameter (Transwell-COL®, Costar) was used in the reflux measurement of fluorescence-labeled dextran (FD-4, MW3825, Sigma). The insert was gently placed into the recipient cluster well plate after first adding medium to its well. A total of 1 x 10^5 ECV304 cells/cm^2 formed a completely confluent monolayer after 24 h (Fig. 1). Following the exposure to glycerol, the permeability through the monolayer cells was determined by adding 1 μM FD-4 into the insert chamber. The transwell filters were incubated in the dark and samples (600 μl) were taken from the outer chamber just after 60 min. The fluorescence of samples was analyzed using a spectrofluorometer (Ex, 488 nm; Em,

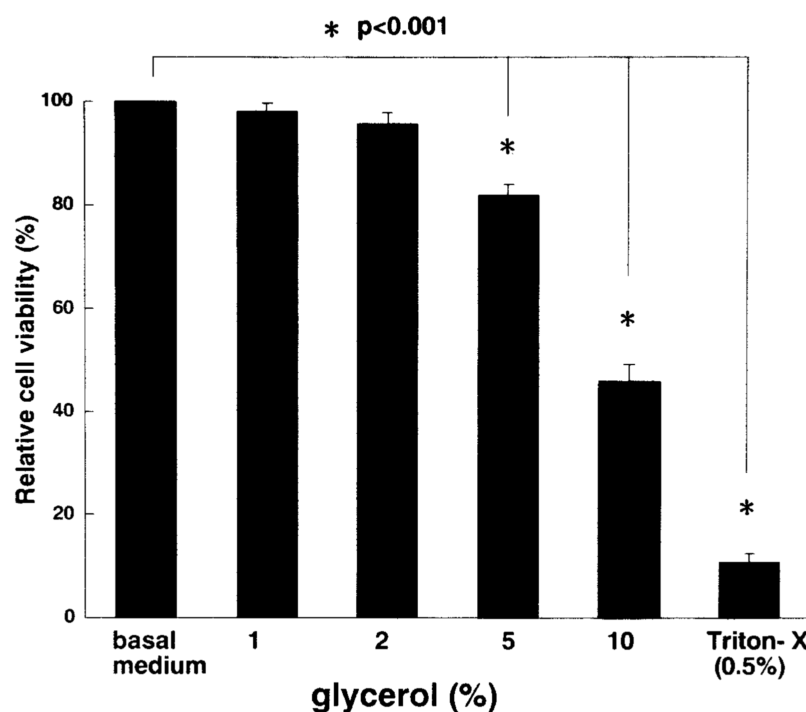

Fig. 2 Cell cytotoxicity against glycerol of ECV304 cells. The relative viability rate in 5% glycerol decreased slightly ($p<0.001$), whereas that in 10% changed strongly ($p<0.001$) compared with viability in the basal medium. In contrast, the viability of cells exposed to 1% and 2% glycerol did not change significantly. Treatment with 0.5% Triton X-100 was done to indicate the effect of complete cell killing. Cell viability is shown as mean±SD of seven independent measurements.

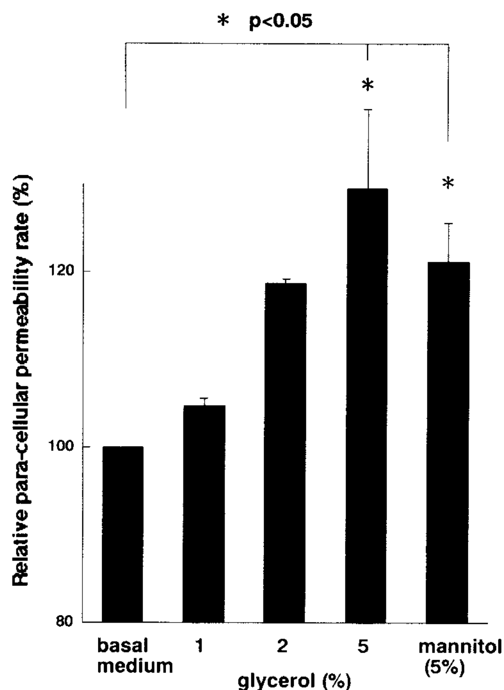

Fig. 3 The paracellular permeability induced by glycerol. Treatment with 5% glycerol increased the permeability significantly ($p<0.05$) compared with that of cells grown in the basal medium. Low concentrations (1% and 2%) of glycerol also induced the permeability moderately. Glycerol induced the paracellular permeability dose-dependently. The transcellular permeability with 5% mannitol also increased significantly ($p<0.05$). The relative transcellular permeabilities are shown as mean±SD of triplicate experiments.

510 nm) (HITACHI) to determine the amount of fluorescent marker passing through the monolayer. The amounts of transfused tracer were calculated relatively from a standard curve measured with known amounts of fluorescence. The mean relative permeability rate (%) compared with control medium is presented as mean±SD of three independent measure ments. A p value <0.05 was considered to indicate a statistically significant difference.

Electrophoresis and immunoblotting. ECV304 cells were prepared with 0.5% trypsin-EDTA (Sigma). Proteins were extracted by homogenizing cells on ice in the lysis buffer containing 20 mM Hepes (pH 7.5), 1% Triton X-100 and 0.25 mM saccharin. Protein concentrations were measured by the Bradford assay (BioRad). The concentration of whole extracts was normalized with sample buffer containing 0.5% sodium dodecyl sulfate (SDS). After incubation at 100℃ for 3 min, equal amounts of protein (total 100 μg) were electrophoretically resolved by SDS-polyacrylamide gel electrophoresis (PAGE). The gel was equilibrated in transfer buffer containing 48 mM Tris, 39 mM glycine, 0.03% SDS and 20% methanol. Proteins were electrophoretically transferred to the nitrocellulose filter (Hybond-P+®, Amersham) followed by Ponceau-S staining. Filters were blocked in 5% skim milk in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl containing 0.05% Tween-20 (TBS) at room temperature for 30 min. Then the filters were incubated with rabbit polyclonal anti-occludin antibody (Zymed), anti-claudin-1 (Zymed) or anti-actin (Santa Cruz) at room temperature for 1 h. The filters were washed three times with TBS containing 0.05% Tween-20, and then the primary antibodies were reacted with horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz) at room temperature for 1 h. After two

washes with TBS, immunoreactive bands were detected by ECL detection solution (Amersham).

Statistical analysis. The statistical significance was analyzed using Student's t-test, and p-values are presented.

RESULTS

Morphological observation in glycerol. The tolerance of ECV304 cells to glycerol was observed morphologically. In the presence of 1% (109 mM), 2% (218 mM) and 5% (545 mM) glycerol, the cells were not different from those in the control medium (Fig. 1). In contrast, the cells showed severe shrinkage and most of them became detached when exposed to 10% (1.09 M) glycerol, which suggested extensive cell destruction. The morphological observations indicated that 5% glycerol might be the maximum that ECV304 cells can tolerate in vitro for 1 hour.

Cytotoxic effect of glycerol. To confirm the morphological results, the cytotoxicity of glycerol on ECV304 cells was examined using a quantitative cytotoxicity assay. The cell viability rates upon exposure to 5% or 10% glycerol for 1 h decreased to 81.9±2.1% ($p<0.001$) and 45.9±3.1% ($p<0.001$) of the viability in control media, respectively (Fig. 2). In contrast, exposure to 1% and 2% glycerol did not significantly affect cell viability (1%, 97.9±1.7%; 2%, 95.6±2.0%). These data indicate that 10% glycerol would severely injure more than half the population of ECV304 cells in vitro. In consequence, it was suggested that ECV 304 cells should be exposed to less than 5% (545 mM) glycerol in vitro.

Enhanced paracellular permeability with glycerol. To examine the barrier function of ECV304 cells, the paracellular permeability of FITC-labeled dextran was measured (Fig. 3). Treatment with 5% glycerol increased permeability by 29.4±9.0% ($p<0.05$) compared with that of cells in the basal media. In addition, the lower concentrations of 1% and 2% glycerol enhanced permeability by 4.7±0.9% and 18.7±0.6%, respectively. Treatment with 5% (275 mM) mannitol also increased permeability by 2 1.1±4.4% ($p<0.05$). These results indicate that glycerol enhances the paracellular permeability of molecules in vitro.

Protein expression of tight junction molecules. The expression of occludin and claudin-1 was analyzed by Western blotting analysis. ECV304 cells express occludin constitutively, whereas the expression pattern of claudin-1 has not yet been determined. The results showed that ECV304 cells expressed claudin-1 as well as occludin. Exposure to glycerol suppressed the expression of occludin completely,

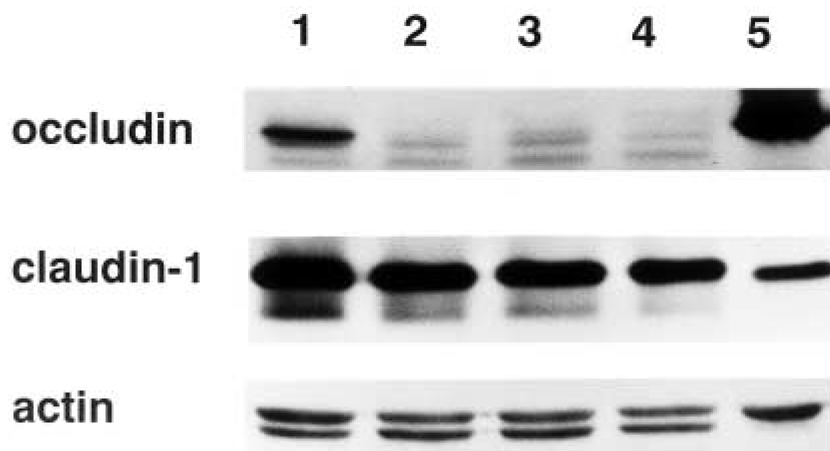

Fig. 4 Immunoblotting analysis of occludin, claudin-1 and actin molecules on ECV 304 cells. The expression of occludin in the presence of glycerol was suppressed completely with any concentration (1, basal medium; 2, 1% glycerol; 3, 2% glycerol; 4, 5% glycerol; 5, MDCK cells in basal medium). In contrast, the expression of claudin-1 was affected very slightly. Actin was not affected at any concentration.

even at a glycerol concentration of 1%, whereas expression of claudin-1 was affected very slightly (Fig. 4). Moreover, there was no apparent change in expression of actin. Surprisingly, glycerol-induced cell shrinkage did not affect actin filaments, which are assumed to participate in junctional regulation by linking to occludin or claudins. These data demonstrate that glycerol suppresses the expression of occludin, but not that of claudin-1 or actin. Thus, the two TJ molecules react differently when exposed to hyperosmotic glycerol.

DISCUSSION

Hyperosmotic glycerol induces paracellular permeability in vitro. It is well known that hyperosmotic solutions help some substances transfer across endothelial cells.[16] Most studies in vivo confirm that this barrier opening is transient and reversible, which improves the delivery of therapeutic agents, especially in the central nervous system.[17,20] For instance, this enhancement of drug delivery is currently applied to the intraarterial treatment of malignant brain tumors with anticancer drugs.[20] Currently, mannitol is the most commonly used hyperosmotic solution for barrier opening, but glycerol is also a hyperosmotic solution. Glycerol reduces the intracerebral pressure significantly with less change to the water-electrolyte balance in the serum than with mannitol.[22,23] In addition, glycerol increases the regional cerebral blood flow and improves the neuronal cell metabolism.[24] Thus, glycerol is useful for the treatment of CNS crises. Among hyperosmotic solutions, glycerol has certain advantages, such as 1.75 times the osmolarity and 0.71 times the viscosity of mannitol.[22] Although mannitol is commonly used for BBB opening, we thought that glycerol might be better.

ECV304 cells contact each other with tight linear cell borders, which prevent transcellular leakage by forming TJs.[25,26] ECV304 cell line is now known to be equivalent to T24 bladder carcinoma.[27] However, I consider that ECV304 cell line is favorable to investigate alternations of TJ proteins because ECV304 cells stably express TJ proteins. I first examined the cytotoxic effects of glycerol on ECV304 cells. If ECV304 cells are damaged by the toxic effect of glycerol, the paracellular permeability should increase. The cell viability in 10% (1.09 M) glycerol decreases dramatically (Fig. 2), and these findings are consistent with the morphological examinations (Fig. 1). Therefore, we suggest that 5% (545 mM) glycerol might be the maximum concentration that ECV304 cells can tolerate in vitro. The tolerance is important for observing the barrier function of cells, because any toxic effect would damage the cell permeability.

A number of experiments using hyperosmotic solutions have been performed in vivo. A positron emission tomography (PET) study performed in baboons using the potassium analog rubidium-82 showed that 1.4 M mannitol opens the barrier.[21] The mean influx constant of the mannitol-perfused hemisphere increased rapidly by 100%. In a rat model, the permeability of 14C-sucrose was reported to increase by 723% after infusion of 1.6 M arabinose into the carotid artery.[19] Our data indicate that treatment with glycerol increases the paracellular permeability in vitro (Fig. 3). However, it is difficult to compare results in vitro with those in vivo in general. The difference in the rate of increased permeability between this study and previous ones in vivo may have resulted from differences in tracer sensitivity.

Glycerol modulates expression of TJ molecules. It has been proposed that hyperosmotic barrier opening may be caused by cell shrinkage, leading to opening of tight junctions between endothelial cells.[19] However, a direct effect of hyperosmotic solutions on the TJ itself has not yet been confirmed. Occludin was the first transmembrane TJ protein to be identified.[11] Recently, two other proteins were identified as novel membrane proteins: claudin-1 and -2.[14,15] These two proteins have structural similarity at the amino sequence level, but no similarity to occludin. TJ strands in situ are believed to comprise various combinations of these proteins.[29,30]

It is known that occludin is incorporated in the claudin-based strands, but it is not clear how occludin and claudins are arranged to form TJ strands.[15]

In the present study, we showed that the expression of occludin decreased in the presence of glycerol, although that of claudin-1 was influenced very slightly. These findings suggest that hyperosmotic solutions directly influence cell junctional molecules, but not uniformly. Occludin responds to glycerol dramatically, whereas claudin-1 is significantly tolerant to it. Although both molecules are expressed at the TJ, their biological functions may be extremely different, at least during hyperosmotic exposure. In fact, claudin-1 shows no sequence similarity to occludin.[14] The claudin family is involved in the formation of TJ strands and, in addition, TJ strands can be reconstituted from gene products of claudin.[15] Moreover, occludin-deficient endoderm cells still develop the network of TJ strands.[28] It seems that glycerol may not influence TJ strands themselves, but may modulate expression of some molecules at the TJ. Although the combined function of occludin and claudins is still unclear, we have demonstrated that the molecular architecture and function of the TJ might be much more complex than expected, and that occludin and claudin-1 may have different molecular functions at the TJ in the presence of hyperosmotic solutions.

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