Claudins, dietary milk proteins, and intestinal barrier regulation

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The family of claudin proteins plays an important role in regulating the intestinal barrier by modulating the permeability of tight junctions. The impact of dietary protein on claudin biology has not been studied extensively. Whey proteins have been reported to improve intestinal barrier function, but their mechanism of action is not clear. Recent studies, however, have demonstrated increased intestinal claudin expression in response to milk protein components. Reviewed here are new findings suggesting that whey-protein-derived transforming growth factor β transcriptionally upregulates claudin-4 expression via a Smad-4-dependent pathway. These and other data, including limited clinical studies, are summarized below and, in the aggregate, suggest a therapeutic role for whey protein in diseases of intestinal barrier dysfunction, perhaps, in part, by regulating claudin expression.

INTRODUCTION

Epithelial monolayers form selective barriers to protect organ systems from the external environment. A critical example is the gut epithelium, which has evolved complex mechanisms not only to allow nutrient absorption but also to defend against entry of infectious agents and foreign antigens into the body. The impact of nutrients on gut epithelial function and, in particular, the gut epithelial barrier, is poorly understood. Molecular mechanisms of nutrient actions in this tissue are also unclear, but recent work has provided new insights into this area.

Absorption of nutrients, including minerals, can occur through both transcellular and paracellular mechanisms. Transcellular mechanisms usually involve specific cell-surface transporters or acceptor molecules that allow for selective uptake of molecules that are transported across the cell; this transport is often achieved with the assistance of carrier proteins that are either utilized by the intestinal epithelia or are exported through the basolateral membrane to the systemic circulation. In contrast, paracellular transport involves the movement of molecules, usually across a concentration gradient, between cells. The selectivity of paracellular transport relies on the specific composition of the proteins that form intercellular junctions. This review focuses on paracellular transport and the role of tight junctions in that process. Within an epithelial monolayer, cell-to-cell contacts, known as tight junctions, regulate the flux of water, ions, and proteins across tissue barriers by allowing for the movement of molecules between cells.1 Tight junctions are composed of membrane-spanning proteins, whose components traverse the paracellular space, and scaffolding proteins, which link membrane proteins directly to the actin cytoskeletal network. As shown in Figure 1,2 membrane-spanning proteins, including claudin, occludin, junction adhesion molecule, and scaffolding proteins, including the zona occludens, collectively regulate the permeability of tight junctions.3 Claudins are essential for barrier function by virtue of their critical role in regulating selectively permeable ion channels in tight junctions.4

Recently, the influence of nutrition on the biology of tight junctions and, specifically, on the regulation of activity and level of expression has become an area of investigative interest. Although the effects of nutrients
and of dietary protein in particular on claudin expression and intestinal barrier regulation have not been studied extensively, they are likely to be important, given the critical role of claudins in regulating the gut epithelial barrier. This review focuses on recent studies that report an effect of whey protein on claudin-4 expression and the impact this has on intestinal barrier function.5

DESCRIPTION AND STRUCTURE OF THE CLAUDIN FAMILY

The family of claudin proteins currently consists of 24 members. Claudins were first identified by Furuse et al.6 and derive their name from the Latin word “claudere,” which means “to close.”5 Claudins have a molecular mass ranging from 20 kDa to 27 kDa and are composed of four transmembrane domains, two extracellular loops, a short cytosolic N-terminus, and a longer cytosolic C-terminus that includes a PDZ-binding domain, which is present in nearly all isoforms (Figure 2).4 Extracellular loop 1 (EL1) consists of approximately 52 amino acids, including a conserved cysteine-containing motif,1,7 and is predominantly responsible for the charge-selective permeability of paracellular transport. Extracellular loop 2 (EL2), approximately 16 to 33 amino acids long, is presumed to fold into a helix motif and, along with EL1, is critical for claudin-claudin interaction.4 The length of the C-terminus varies between claudin isoforms, from 21 to 63 amino acids, and contributes to isoform-dependent paracellular selectivity.3 The C-terminus PDZ-binding motif facilitates direct interaction between claudins and scaffolding proteins. Not all claudins contain a PDZ-binding domain, claudin-12 being an example.8 The N-terminus consists of seven amino acids and has no known function.1

CLAUDIN FUNCTION

As noted above, intestinal absorption of nutrients can occur via transcellular or paracellular transport. In contrast to transcellular transport, which usually requires energy expenditure, paracellular transport is passive and depends on an electrochemical gradient. The tight junctions, including the claudin proteins, facilitate paracellular transport by forming ion-selective pores.9

Claudins can be classified as either pore forming or pore sealing. Pore-forming claudins increase paracellular permeability through the formation of channels, whereas pore-sealing claudins reduce paracellular permeability. A wide variety of incompletely understood factors determine whether claudins enhance or reduce paracellular permeability. These include claudin-claudin interactions, which can occur head-to-head between the same claudins on adjacent cells (homotypic interactions) or, much less frequently, between different claudins (heterotypic interactions). Claudins can also interact side-to-side in homomeric or heteromeric interactions4 (see also the citations in Overgaard et al.4). Additionally, claudins may interact with other components of the tight junction, including...
ocludins and zona occludens 1, and can undergo post-
translational modification such as palmitoylation and
serine-threonine phosphorylation. These complex inter-
actions in the microenvironment of the tight junction, as
well as the tissue-specific expression of certain claudins,
deride their varied effects on permeability, which are for
the most part poorly understood.

Based in part on their molecular size, solutes are
thought to cross tight junctions. Solutes less than 4 ang-
stroms (e.g., sodium, magnesium) follow a pathway that
carries electrical charge during transport. Increased
claudin-2 expression in cultured epithelia increases per-
meability for solutes less than 4 angstroms. The pathway
for solutes greater than 4 angstroms (e.g., mannitol,
inulin) carries no electrical charge and is speculated to
represent temporary breaks in otherwise continuous tight
junctions.

Charge-selective regulation of claudin proteins is
determined by the structural elements in EL1. The nega-
tively charged residues in EL1 strongly influence cation
d pore formation. For example, the replacement of basic
residues with acidic residues in the EL1 domain of
claudin-4 increased cation permeability. EL2 collabora-
tion with EL1 seems to be required for proper pore
formation.

**CLAUDINS AND INTESTINAL BARRIER REGULATION**

Several claudins have been reported to have functional
roles in the intestine. Claudin-1, -3, -4, -5, and -8 function
as pore-sealing proteins, whereas claudin-2 forms charge-
selective pores, for example, facilitating the paracellular
transport of calcium. Claudin-12 has also been reported
to increase paracellular calcium transport in entero-
cytes. Claudin-7 appears to reduce tight-junction per-
meability, as indicated by studies in knockout mice.

The role of claudin-15 in regulating intestinal barrier
function is unclear, but this claudin seems to have distinct
biological actions in enterocytes, where it increases cell
proliferation.

Paracellular permeability can inappropriately
increase with disease and age, as well as during periods of
stress. Understanding the mechanisms by which claudin
proteins regulate the intestinal barrier will provide
insight into the pathophysiology of intestinal barrier mal-
function as well as identify targets for therapeutic inter-
vention. Consistent with this idea, recent studies indicate
that manipulation of claudin function in experimental
models affects the intestinal barrier. Ewaschuk et al. determined that claudin-2 expression is suppressed in
T84 cell monolayers (an entocyte cell model) following
4 hours of treatment with metabolites secreted by the
commensal organism *Bifidobacterium infantis*. Thus, one
mechanism by which microorganisms could affect in-
testinal permeability is by altering the expression of clau-
dins. Suzuki and Hara discovered that the most
common flavonoid present in nature, quercetin, decreases
paracellular flux across Caco-2 cells and increases
claudin-4 protein expression. Yeh et al. determined that
chitosan, a widely used food additive, increases paracellu-
lar flux in Caco-2 cells. During the postexposure period,
when tight-junction function is reestablished, the inves-
tigators found that claudin-4 transcription increased.
Taken together, the above findings suggest that
claudin activity and expression can be dynamically altered by the diet and by changes in the intestinal
microbiota.

**MILK PROTEIN COMPONENTS AND INTESTINAL
CLAUDIN EXPRESSION**

Recent work by Hering et al. adds to the heretofore
limited understanding of how nutrition influences
claudin biology. The investigators focused on whey, the
so-called “fast-digested milk protein,” specifically whey
protein concentrate 1 (WPC1). Consistent with earlier
studies in both humans and rodents, these investiga-
tors found that WPC1 protected against an interferon
gamma (IFNγ)-induced tight-junction barrier distur-
bance. They explored the molecular mechanisms by
which whey protein, and WPC1 in particular, exert this
effect. Milk, whey protein, and WPC1 all contain high
levels of transforming growth factor β1 (TGFβ1), which
has been reported to improve intestinal tight-junction
function. To determine if TGFβ1 mediates the effects of
WPC1 on intestinal tight junctions and to clarify the
molecular mechanism by which TGFβ1 acts, the authors
first showed that WPC1 and TGFβ1 both increased trans-
epithelial resistance. To explore the downstream signaling
cascade that mediates this effect, the authors next inves-
tigated known TGFβ1 signaling intermediaries. It had
been previously reported that milk and TGFβ1 activate a
canonical TGFβ reporter construct. However, it had
also been suggested that Smad-independent pathways
mediated the effects of TGFβ on epithelial tight junc-
tions. As noted, claudin-4 has been previously reported
to regulate tight-junction function. Interestingly, these
investigators found that WPC1 and TGFβ1 increased
expression of claudin-4 but did not affect the expression
of claudin-1, -2, -5, -7, or occludin. They therefore focused
on claudin-4 and used a claudin-4 promoter construct to
evaluate cell-signaling pathways entrained by WPC1 and
TGFβ1. As compared with nonfat dried milk, which has
less TGFβ1, WPC1 activated the claudin-4 promoter
more effectively. TGFβ1 was also able to induce this
construct. This activation was significantly enhanced by over-
expression of Smad-4, a transcription factor known to be
a target of TGFβ1. Deletional analysis, as well as muta-
tion of the Smad-4 binding site of the claudin-4 promoter, established that Smad-4 binding was required for the ability of both TGFβ1 and WPC1 to activate the promoter. Thus, HT-29/B6 cells treated with TGFβ1 (60 ng/L) for 48 hours showed an increase in the activity of the claudin-4 promoter. Overexpression of Smad-4 augmented this effect, while mutation of the Smad-4 binding site in the claudin-4 promoter abolished the ability of TGFβ1 to induce claudin-4 promoter activity. To determine if TGFβ1 mediated the effects of WPC1 on claudin-4 transcription, cells were transfected with a claudin-4 promoter and treated with WPC1 in the presence of neutralizing antibodies to TGFβ1, 2, and 3. These neutralizing antibodies blocked the stimulatory effect of WPC1 on the promoter.

Hering et al.5 concluded that WPC1 mediates its action on intestinal barrier function in part by inducing claudin-4 expression via a TGFβ/Smad-4 signaling cascade. These studies provide a plausible molecular mechanism by which milk and whey protein exert their salutary effect on intestinal epithelial integrity. While these models of overexpression support a role for TGFβ1 in Smad-4-mediated regulation of tight junctions, complementary studies involving knockdown of endogenous Smad-4 would provide key additional support for this model.

The potential clinical relevance of this proposed molecular pathway is supported by in vivo evidence that TGFβ has beneficial effects on the intestinal barrier. In a mouse model, oral administration of 500 μL of cow milk containing TGFβ1 (3,000 ng/L) daily for 2 weeks before induction of colitis and endotoxemia ameliorated both tissue damage and mortality.18 In the neonate, TGFβ1 has been found to be important for gut homeostasis and regulation of inflammation.22 Hering et al.5 suggest that TGFβ1 in concentrations as little as 60 ng/L can be beneficial to mucosal restitution. The WPC1 used by Hering et al.5 contained 30–60 ng/g of TGFβ1. Although the dilutional effect of gastric and intestinal secretions on ingested WPC1 is not known, it seems reasonable to assume that the ingestion of several grams of WPC1 would result in luminal TGFβ1 concentrations in the 60 ng/L range. It is plausible that ingestion of WPC1 could induce biologically significant effects on intestinal barrier protection. Thus, TGFβ1-rich WPC1 may have clinical efficacy in gastrointestinal disorders characterized by barrier dysfunction. This is especially appealing in the neonate, in whom the dilution factor between the mouth and duodenum is less than in the adult.

In addition to whey protein, casein, the most abundant protein in milk, may influence barrier function and claudins. Visser et al.23 reported that a high-casein diet improved intestinal barrier function in diabetes-prone BioBreeding (DP-BB) rats. At weaning, DP-BB rats were placed on a high-casein (HC) diet (containing 200 g/kg casein) or a standard plant-based diet for 65 days. Rats on the HC diet had improved intestinal barrier function as assessed by measuring the ratio of lactulose to mannitol in the urine after oral administration of lactulose and mannitol.23 Serum zonulin levels, which correlate strongly with intestinal permeability, were lower in the animals on the HC diet.23 The HC animals also had increased transepithelial resistance as compared with animals on the standard diet. Quantitative polymerase chain reaction was performed using RNA isolated from the ileum of the HC animals to investigate the effect of the diet on expression of tight-junction proteins. Rats on the HC diet had increased expression of pore-sealing claudin-1 and a reduced expression of pore-forming claudin-2.23 Interestingly, Hering et al.5 found that casein-based diets contain little TGFβ1. These data suggest that the HC diet reduces intestinal permeability in this experimental rat model by a TGFβ1-independent mechanism.

While the anti-inflammatory effects of TGFβ within the intestine have long been known24,25 and are mediated, in part, by modulation of the relative proportions of pro- and anti-inflammatory CD4+T cell subsets, these data support an alternative mechanism. The studies of Hering et al.5 and Visser et al.23 are consistent with the idea that milk protein components improve intestinal barrier function, in part, by altering the expression and functions of claudins via both TGFβ1-dependent and TGFβ1-independent pathways. In support of this hypothesis, previous reports demonstrate that a variety of intestinal diseases characterized by altered permeability are accompanied by changes in claudin expression.26,27

ALTERED CLAUDIN EXPRESSION IN INTESTINAL DISEASE

As many as 1.4 million Americans suffer from inflammatory bowel disease.28 Altered intestinal barrier function is a common feature of the major inflammatory bowel diseases, Crohn’s disease and ulcerative colitis, as well as diseases such as collagenous colitis. Increased antigen uptake into the circulation can initiate and intensify epithelial inflammation, while water uptake from the circulation to the intestinal lumen can result in diarrhea. This can lead to an auto-intensifying cycle of disease in which increased permeability results in increased antigen uptake, which further worsens the inflammation and diarrhea.

Zeissig et al.27 recently reported changes in claudin expression in colonic biopsies from patients with Crohn’s disease. Expression of pore-sealing claudin-5 and claudin-8 was downregulated, while expression of pore-forming claudin-2 was strongly upregulated. Claudin-5 and claudin-8 were also found to redistribute away from
the tight junction, as determined by immunohistochemistry. In addition to the changes in the molecular composition of the tight junction, the rate of epithelial cell apoptosis was also found to be upregulated.

Alterations in tight-junction structure and function have also been observed in ulcerative colitis. Expression of pore-forming claudin-2 is upregulated, accompanied by an increase in the rate of epithelial cell apoptosis, resulting in epithelial lesions. In collagenous colitis, intestinal barrier regulation is significantly altered, with downregulation of pore-sealing claudin-4 and upregulation of pore-forming claudin-2 observed in some patients.26 Interestingly, and in contrast to the findings in Crohn’s disease and ulcerative colitis, rates of epithelial cell apoptosis were not increased in collagenous colitis.

Since downregulation of claudin expression has been reported to occur frequently in inflammatory bowel disease and since TGFβ upregulates expression of at least claudin-4, it could be postulated that TGFβ might serve as a potential therapeutic agent in inflammatory bowel disease, in part by helping to reestablish intestinal barrier function via its effect on claudins. Consistent with that notion, Fell et al.29,30 have reported that a polymeric diet enriched in TGFβ induced healing in pediatric patients with Crohn’s disease.

CONCLUSION

Tight junctions, including the claudin proteins, are a crucial component of the intestinal barrier responsible for integrity and permeability. The varied effects of claudins on permeability are, for the most part, poorly understood. It has been suggested that claudin function, and thereby tight-junction permeability, can be dynamically altered by the diet and changes in the intestinal microbiota.15–17 Specifically, Hering et al.5 and Visser et al.23 suggest milk protein components improve intestinal barrier function, in part, by altering the expression and functions of claudins. These studies5,23 are limited by the fact that animal models exclusively were studied, leaving uncertain the relevance of these findings in humans. The reliance on overexpression studies to establish the role of Smad-4 in the TGFβ1/claudin-4 pathway by Hering et al.5 requires that additional studies be performed before this signaling cascade can be accepted as broadly applicable in vitro and in vivo.

Nonetheless, the studies summarized above5,18–23 add to the current understanding of how nutrition influences barrier regulation. Several mechanisms contribute to intestinal barrier dysfunction and disease. Investigation of tight-junction protein regulation of intestinal disease may provide new targets for therapeutic intervention. Further research is necessary to confirm the mechanisms by which milk protein components improve intestinal integrity, as well as whether milk protein components should be considered as a therapy for diseases characterized by intestinal barrier compromise. Clinical trials using whey-protein isolates in humans with intestinal barrier dysfunction would be of considerable interest. In addition to noninvasive assessment of intestinal barrier function, intestinal mucosal biopsies to assess claudin expression would be of interest. If well-designed and properly controlled, such studies would significantly aid in determining whether claudins deserve attention as potential therapeutic targets, and, if so, which ones should be pursued.

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