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Regulation of intestinal epithelial function: a link between opportunities for macromolecular drug delivery and inflammatory bowel disease

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Abstract

The intestinal epithelium performs a multitude of tasks related to digestion and homeostasis. As a consequence of ingestion, this tissue must also participate in activities associated with protecting the body from potential pathogenic agents and toxic materials. To efficiently perform tasks associated with digestion and these protective functions, the intestinal epithelium has established several anatomical, biochemical and physiological barriers to impede unregulated uptake of materials. In order to perform functions of digestion and homeostasis, the intestinal epithelium uses mechanisms that allow dynamic modulation of regulated uptake pathways that can respond rapidly to changes in diet, health and challenges from pathogenic agents and macromolecules. This review focuses on specific, recent advances made in understanding cellular pathways and mechanisms that regulate dynamic processes of these barriers and examines the feasibility of drug delivery strategies focusing on macromolecular therapeutics potentially useful in the treatment of inflammatory bowel disease (IBD).

Keywords: Intestinal epithelium; Intestinal barrier; Crohn's disease; Ulcerative colitis; Inflammatory bowel disease; Antibody delivery; Peptide delivery

Contents

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1. Introduction

Inflammatory bowel disease (IBD) currently affects approximately one million Americans and more than one million Europeans. Traditional therapies have focused on small molecule anti-inflammatory and immunosuppressive agents [1]. With the recent advent of biotechnology, new therapeutic opportunities involving peptides and proteins have been and are being explored for treatment of individuals with IBD. Optimal delivery (i.e. to the correct location and for the proper duration) will likely be critical for the effective and safe administration of some of these new therapeutic agents. In this review we initially describe the various barriers of the gut that limit the potential for delivery of proteins and peptides drugs and then discuss how modifications that occur to these barriers in the unique case(s) of IBD might affect newly identified opportunities for the delivery of protein and peptide therapeutics designed to affect corrective changes to diseased intestinal tissue.

1.1. The intestinal epithelia

Absorption of nutrients and drugs occurs primarily in the small intestine and colon. The luminal surface of the small intestine is specialized to increase surface area available for absorption and is arranged into macroscopic irregular folds, villi and microvilli. Colonic tissue lacks villi. Associated with microvilli is a glycoprotein coating (glycocalyx) that contains numerous proteolytic activities and establishes a biochemical barrier at the luminal surface of the gut designed to degrade proteins and peptides (Fig. 1). Atop the glycocalyx is a layer of mucus derived from glands and goblet cells and composed of mucin glycoproteins, which can provide a physical barrier

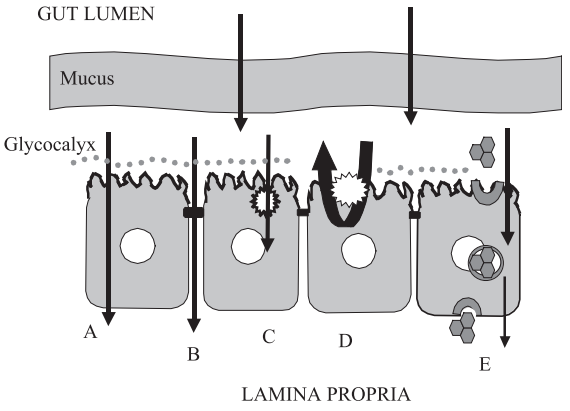

Fig. 1. The physical barrier of the intestinal epithelium to the passage of nutrients and drug molecules from the gut lumen to the basal lamina propria consist of a layer of mucus, the glycocalyx, and the cell membrane barrier; paracellular spaces are sealed by tight junctional proteins. Small drug molecules may cross the cell membrane barrier by different mechanisms: (A) passive transcellular diffusion, (B) passive paracellular diffusion, (C) active transport, or (D) be extruded back into the lumen by efflux proteins. Macromolecules are usually transported via transcytosis (E).

to the movement of proteins and peptides [2]. The primary barrier formed by epithelial cells themselves and their associated intercellular tight junction (TJ) structures establish a physical barrier to peptide and protein transport. The epithelial cell monolayer interacts at its basal surface with a variety of proteins (e.g. collagen and elastin) and polysaccharides that are organized into an extracellular matrix termed the basement membrane. Extracellular matrix components are synthesized and secreted by both the epithelial cells and cells within the lamina propria such as myofibroblasts. This combination of cellular and extracellular barriers maintains regulated vectoral transport of solutes, including ions, macromolecules, and even water.

Besides this critical activity in homeostasis, these barriers also act to repel pathogenic microorganisms. An important anatomical feature of the intestinal epithelium related to its responsiveness to pathogen challenge is its close association with loosely organized immune cells known as the gut-associated lymphoid tissue (GALT). Cells of the GALT are capable of responding to the presence of pathogens or toxic agents. Moreover, in response to signals secreted from underlying lymphocytes, epithelial cells can differentiate into M cells (Fig. 2). M cells sample antigens and other macromolecules in the gut lumen and transport them to the underlying tissues to be presented to the immune system [3]. Although these specialized cells constitute only a small fraction of the intestinal epithelial surface, intense efforts have gone into examining the potential for protein and peptide drug delivery at these sites (reviewed in Ref. [4]).

1.2. Transport routes across the intestinal epithelium

Cellular membranes represent a significant physical barrier of the intestinal epithelium that selectively inhibits the passage of nutrients and drug molecules.

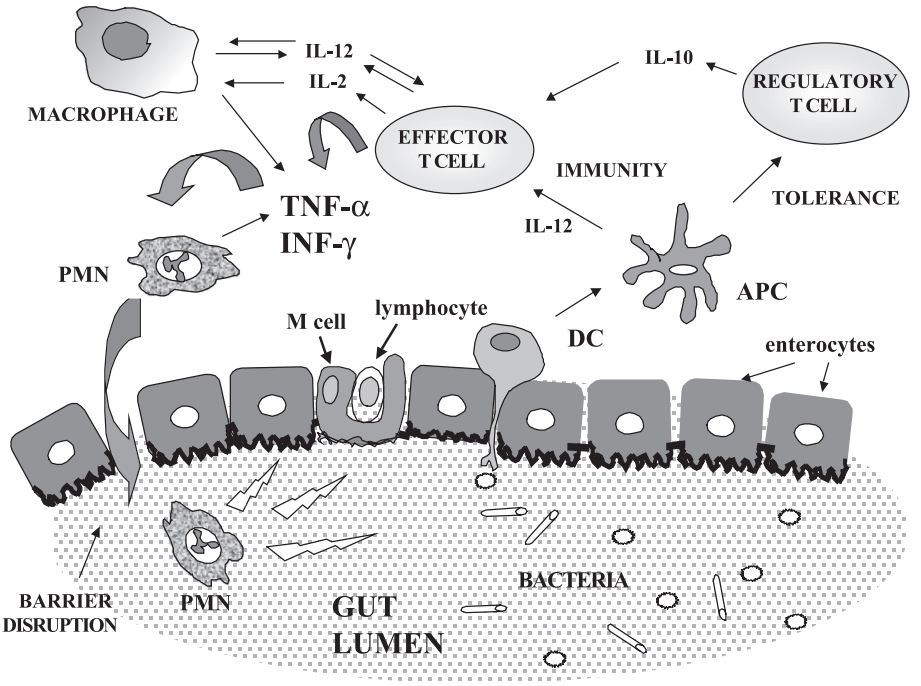

Fig. 2. Simplified diagram of interactions between intestinal microflora, intestinal epithelial cells and cells of the gut-associated immune system. Antigens sampled in the gut lumen by dendritic cells (DC) and M cells can trigger an inflammatory cascade mediated by antigen-presenting cells (APC) leading to the disruption of the epithelial cell barrier and the massive migration of polymorphonuclear cells (PMN) into the gut lumen. The endpoint is chronic epithelial injury.

These membranes are composed of a lipid bilayer; containing mostly cholesterol and phospholipids and are studded with a variety of proteins and protein complexes that act in the dynamic regulation of transport properties associated with this barrier. Drugs may cross intestinal epithelia by any combination of three main mechanisms: active transport, passive diffusion, and transcytosis. Typically, a molecule will preferentially utilize the route that best accommodates its physical and chemical properties.

1.2.1. Active transport

Highly specific and regulated transport mechanisms exist in human intestinal epithelia for the vectoral movement of nutrients, vitamins, ions, and water. Movement of compounds (either influx or efflux) through transporters is selective, saturable and energy-dependent. Several transport protein families, specific for the uptake of amino acids, sugar, nucleic acids, bile salts, etc., are strategically positioned along the intestinal tract to optimize digestion and nutrient absorption events [5]. Some transporters have the capacity to selectively move small peptides as part of nutrient uptake. Specialized uptake of small peptides, mainly di- and tri-peptides, from the intestinal lumen is carried out by the oligopeptide transporter, Pept-1 [6]. This mechanism appears necessary for absorption of protein digestion products in the proximal small intestine, but not in the colon where the transporter is not normally expressed.

Due to the sensitivity of peptides to peptidases present in the digestive tract, stable mimetics are often synthesized that emulate peptide-based therapeutic agents. Interestingly, Pept-1 can also transport a wide range of non-peptide molecules, such as β-lactam antibiotics [7] and thus may be important in the uptake of some peptide mimetics. Such compounds may also be a substrate for other transporters present in the intestinal epithelium. Such transporters could include those that move bulky charged organic molecules; examples are the organic cation/carnitine transporters (OCTNs) and the organic anion-transporting polypeptide (OATP) [8–10]. An entire class of membrane transporters also exists that have the capacity to direct efflux of charged lipophilic compounds across membranes. The most common of these proteins in the intestinal epithelium is the ATP-binding cassette family, which includes the MDR1 gene product P-glycoprotein, the multidrug resistance protein family (MRP1–9), and breast cancer resistance protein [11,12]. Thus, it is possible that one may observe a diminished effectiveness of a therapeutic peptide or peptide mimetic if they are recognized as substrates by these transporters (Fig. 1).

1.2.2. Passive diffusion

Two main routes of passive diffusion through epithelial cell monolayers are possible: the paracellular and transcellular (Fig. 1); the transcellular route is the more important route for lipophilic compounds. Passive diffusion through this route depends on the molecule's lipid solubility, degree of ionization and size. Small molecules (M.W.<500 Da) tend to penetrate membranes more rapidly than large ones and this involves a diffusion-driven process [13]. Molecules that are not capable of penetrating membrane barriers must rely on paracellular transport that is limited anatomically to the area between adjacent epithelial cells regulated by TJ structures [14]. This space is a small fraction of the total membrane surface area (about 0.01%). Compounds observed to diffuse passively via the paracellular route are typically hydrophilic in nature and transport via this route is size-dependent, with a cut-off value estimated to be about 250–400 Da [15–17], correlating well with calculated hydrodynamic radius values for these molecules [18].

1.2.3. Transcytosis

Uptake of macromolecules from the intestinal lumen is severely restricted by several mechanisms [2]. In general, macromolecular uptake occurs through a process known as receptor-mediated endocytosis. Although intestinal epithelial cells can absorb macromolecules from their apical surface through random pinocytosis events, organized uptake of macromolecules occurs through either clathrin-coated or caveolin-based vesicular structures that form through a coordinated clustering of plasma membrane components [19]. Spherical clathrin-coated vesicles (~120 nm in diameter) are established through complex organizations of the clathrin protein with epsin and AP-2 on the cytoplasmic surface leaflet of membrane bilayers. As these organizations bud to form nascent vesicles, their curvature is initiated and stabilized by amphiphysin and their pinching from the membrane is

facilitated by the actions of dynamin. Caveolae (little caves) are flask-shaped structures of ~60 nm in diameter that form through organizations of caveolin dimers that occur selectively in cholesterol and sphingomyelin-enriched membrane domains known as lipid rafts. Events stimulated through $G_{\alpha i}$-dependent activation of the Src tyrosine kinase trigger caveolae endocytosis. Both clathrin-coated pits and caveolae act to concentrate a variety of receptors that recognize macromolecular ligands. As macromolecules enter into cells through these vesicular structures, they are trafficked to various sites within the cell based upon a series of intracellular signals and surface structures related to their contents. Most endosomes are trafficked to sites where they fuse with vesicles containing a variety of hydrolytic enzymes as well as proton transport complexes that drive the internal pH of the newly formed degradation vesicle to acidic conditions. Thus, the ultimate fate of most macromolecules entering into the apical surface of intestinal epithelial cells through these endocytosis pathways involves delivery to lysosomes where they are destroyed.

Some macromolecules do avoid this fate of destruction and are released at the basal–lateral membrane (transcytosis) through vesicular fusion with that domain of the plasma membrane. Considering the destructive pathways as a default mechanism used by the intestinal epithelial cell to ensure that unwanted (even potentially toxic) macromolecules in the lumen of the intestine do no readily enter the body, the fact that some vesicles (and their contents) avoid this outcome suggests mechanisms of altered vesicular trafficking within the cell. There are only a few examples presently known that describe direct macromolecule transport from the apical to the basolateral surface of intestinal epithelial cells [20].

1.3. The tight junction (TJ)

Intestinal epithelial cell TJs establish the physical barrier to unrestricted movement of molecules through the paracellular route [14]. TJ complexes are composed of several types of proteins that are associated with and organized by a cytoskeletal structure at the apical neck of intestinal epithelial cells, termed the perijunctional actomyosin ring [21]. Transmembrane proteins present at the TJ include occludin, claudins, and the junctional adhesion molecule (JAM). Associated with these transmembrane proteins are a series of scaffold proteins such as the zonula occludens family members ZO-1, -2, and -3. ZO proteins are members of the membrane associated guanylate kinase (MAGUK) superfamily that also contain PSD-95, Discs Large, *Z*O-1 (PDZ) and SH3 domains. Much of the dynamic aspect of TJ function appears to involve contractile events involving myosin light chain kinase (MLCK) activation that results in constriction of the actomyosin perijunctional ring that interacts with claudin and occludin proteins (TJ elements that establish the paracellular barrier) through a series of scaffolding structures [18,22,23]. Importantly, a number of potential regulatory proteins have also been localized to the TJ and these appear to play active roles in the formation, stabilization and down-regulation of functional TJ structures. To date proteins such as the non-receptor tyrosine kinase c-yes, $G_{\alpha 12}$, the zeta isoform of protein kinase C, the regulatory p85 subunit of phosphotidylinositiol-3-kinase have been identified [24,25]. In summary, the TJ is composed of a complex of proteins involved in establishing a functional barrier that interact with cellular cytoskeletal elements in a manner that can be dynamically regulated through the actions of a variety of regulatory proteins.

2. Intestinal disorders and epithelial function

2.1. Inflammatory bowel disease

Inflammatory bowel disease (IBD) is divided into two conditions: Crohn's disease (CD) and ulcerative colitis (UC). Both diseases are characterized by relapsing and remitting episodes of active inflammation and chronic mucosal injury. Risk of disease correlates with genetic pre-disposition, although other host-related and environmental factors such as smoking and diet may also have causative roles. Aberrant immune responses to ingested antigens, commensal and pathogenic microorganisms have been recently suggested as major determinants of IBD (reviewed in Ref. [26]). Complexities in the etiology of CD and UC indicate that multiple, interconnected mechanisms are likely to be involved in regulating inflammatory events at the intestinal epithelium leading to the onset

and continuance of these diseases through an imbalance of pro- and anti-inflammatory agents [3,26,27]. It has been suggested that agents which stimulate IBD events act to shift this balance to a more pro-inflammatory outcome and that this occurs primarily through the actions of cells associated with the gut-associated lymphoid tissues (GALT). Intestinal epithelia have functionally dynamic TJ complexes capable of excluding macromolecules from the paracellular route. In the case of IBD, this balance point appears to be shifted to favour the actions of pro-inflammatory stimulators. This results in a continuously activated GALT and an intestinal epithelium exhibiting increased paracellular permeability [28].

2.2. Modifications of the intestinal epithelium

Both acute and chronic inflammatory events can modify functional aspects of the intestinal epithelium. In general, acute mechanisms of inflammation drive a series of events that, when continued over time, lead to morphological alterations of the tissue that in turn can affect actions and outcomes of inflammatory agents in that modified setting. Actions and outcomes of inflammatory agents can result from and be affected by environmental cues, responses to pathogens and even emotional stress [29,30]. Overall, it is easy to see that mechanisms initiating and perpetuating IBD-related events represent a complex and still poorly defined etiology. There are several aspects of biological responses related to IBD occurring at both the cellular and tissue level that can be of significance in identifying rational strategies to not only treat IBD but also to identify how to effectively deliver potential therapeutics in this unique biological setting.

In general, one might consider modifications in TJ function as a critical acute initiator of events that lead to chronic outcomes associated with IBD. These chronic events are characterized by marked disruption of the intestinal mucosa architecture, involving modification of the mucus layer, destruction of the extracellular matrix, alteration of expressed proteolytic activities and modified transport functions. These alterations may have significant impact on specific aspects related to the delivery of therapeutic proteins and peptides. In the case of some agents there may be enhanced transport capacity, and in other cases these modifications that occur under chronic conditions of IBD may result in serious limitations to protein and peptide drug delivery. These points will be discussed in greater detail in later sections.

2.2.1. Intestinal permeability

Several studies have demonstrated IBD pathogenesis to be characterised by increased intestinal permeability, primarily using methods that measure the enhanced transport of poorly transported solutes from the intestinal lumen into the urine [13]. Not only can this enhanced transport property be readily demonstrated in individuals suffering from IBD [31], but non-affected, genetically at-risk individuals can also demonstrate this characteristic as a potential prelude to acquiring IBD symptoms [32]. Thus, it appears that an increased intestinal permeability is an early stage event of eventual IBD symptoms. There are several potential mechanisms whereby this increased permeability characteristic may be initiated and these have been the focus of several strategies for clinical intervention.

Increased intestinal permeability involves either modification of TJ function or frank lesions in the intestinal mucosa. We will focus on mechanisms where the TJ is modified in its function rather than where it is absent due to tissue trauma. Such modifications could be the result of external agents acting directly to destroy or damage critical TJ components. In the case of IBD, increased levels of proteinases such as trypsin, thrombin and tryptase have been suggested to correlate with disease. It is also possible that these enzymes activate proteinase-activated receptors (PARs) to initiate apoptotic signalling pathways within enterocytes leading to disruption of tight junctional ZO-1 [33]. Thus, dramatic changes in the proteolytic environment could initiate alterations in intestinal permeability.

Alternately, or possibly in conjunction with changes in proteolytic activities, other soluble factors such as pro-inflammatory cytokines may act to alter intestinal permeability. Two proteins stand out as hallmark agents of inflammation—tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ). Although each of these cytokines can act individually to enhance paracellular permeability of the intestinal mucosa [34,35], the combined actions of TNF-α and IFN-γ are clearly synergistic [36]. These agents down-regulate the expression of occludin [37] and

up-regulate myosin II regulatory light chain phosphorylation [38]. Besides these soluble factors, interactions of pathogenic (or even commensal) organisms with intestinal epithelial cells may act to alter permeability properties [39]. The common thread between these agents is their ability to modify TJ protein components and it may be that a mixture of such agents acts to establish an environment capable of sustained TJ dysfunction.

The ability of these factors to modulate TJ function underscores the central critical role of this structure in normal barrier function of the intestinal epithelium. TJ structures have mechanisms to allow for dynamic regulation of this barrier since white blood cells must occasionally pass through this structure and the replacement of senescent cells with nascent ones must involve the transient opening and closing of these structures. Thus, it is quite probable that many mechanisms involved in the altered permeability properties of this barrier associated with IBD are extreme outcomes of processes that normally occur on a regular, but controlled basis. Therefore, one might consider IBD as an instance where uncontrolled modulation of TJ function has occurred. Based upon this analysis, therapies that focus on restoring normal TJ function may be useful in IBD.

2.2.2. Modification of the mucus layer

The mucus layer protecting the small and large intestine is largely comprised of members of the mucin glycoprotein family secreted by epithelial Goblet cells. Mucins are either bound to the apical membrane of epithelial cells, extending into the glycocalyx, or are secreted into the lumen, where they form a gel-like structure serving as a barrier to protect the epithelium from mechanical stress and microorganisms. Because of its critical protective function, defects in mucin expression or organization have been proposed to contribute to the etiology of IBD [40,41]. In UC the mucus layer is abnormally thin, while in CD it is thicker than what is commonly observed in healthy individuals [42]. Reduction in the mucus layer observed in UC is consistent with the observed depletion of Goblet cells associated with this condition. It is unclear how an increased mucus layer occurs in CD since affected individuals do not demonstrate an increased number of goblet cell. Alternately, pro-inflammatory or other stimulatory agents may accelerate the expression or release of mucins to result in this increased mucus layer thickness.

Biochemical abnormalities have been identified in mucins produced by both CD and UC patients. These changes include variations in protein chain length and degree of glycosylation, which may impact the viscosity and binding properties of mucus barrier [41], potentially hampering its protective function. For example, Goblet cells in the small and large intestine synthesize and secrete not only mucins, but also TFF3 peptide, a member of the trefoil factor family found in mucus-secreting cells in the stomach and intestine. TFF3 has a protective effect on the epithelium, possibly by organising the mucin layer which protects the mucosa from damage and promoting cell migration to the area of injury [43,44]. Thus, biochemical modifications to mucin could alter its ability to interact with other protective factors, such as TFF3, and could lead to increased sensitivity to environmental insults. Changes to mucin could also affect permeability properties for protein and peptide-based therapeutics following oral administration. For example, it has been shown that intestinal mucus can bind IgG via a unique Fc binding protein secreted by Goblet cells, which has probably evolved as a mechanism to block IgG-antigen complex molecules on the mucosal surface, where they can be degraded and washed away [45]. Alteration in mucin expression level or composition could also alter the transport properties of antibody-based therapies by changing antibody binding properties. In some instances this may act to increase antibody permeability and in other cases it could impede antibody accessibility to the epithelial cell surface.

2.2.3. Proteolytic activities and the extracellular matrix

Extracellular matrix breakdown is an important process for the normal function of the intestine. The movement of cells responding to immune stimuli and the normal events of tissue remodelling require the actions of a variety of proteolytic enzymes secreted by intestinal epithelial cells and by resident non-epithelial cells such as macrophages, fibroblast and neutrophils. Some of the best studied of these enzymes are the families of matrix metalloproteinases (MMPs), a family of peptidases that regulate tissue turnover by

degrading extracellular matrix proteins, and serine proteases [46,47]. Some of these proteolytic activities have been considered a major component of IBD-related events [46,48,49]. For example, elevated non-specific proteolysis activity (e.g. elastase, trypsin, chymotrypsin) has been found in the gut lumen of patients with ulcerative colitis and the extent of these abnormalities correlate with disease severity [50–52]. Activation of intracellular proteases by proteolytic cascades is also a necessary mechanism for inflammatory responses resulting in the conversion of cytokines from inactive to active forms. Trypsin, thrombin and tryptase have also been shown to activate proteinase-activated receptors (PARs) at the apical membrane of enterocytes. This activation not only acts in increased intestinal permeability but it also incites epithelial cell apoptosis [33].

It is thought that many of these proteases, particularly trypsin, are responsible for the proteolytic activation of MMPs and that this activation is further facilitated by the increased permeability properties of TJ structures associated with IBD [46]. It is generally accepted that destruction of the extracellular matrix is a common feature of both CD and UC [53]. MMPs are themselves regulated by endogenous protease inhibitors collectively known as tissue inhibitors of metalloproteinases (TIMPs). It is thought that the imbalance between MMPs and TIMPs may have some role in the initiation of inflammation [47]. It is also important to realize that MMPs can also degrade various non-matrix proteins, including human IgG [54]. Since collagen, also present in the extracellular matrix, has been shown to reduce the diffusion coefficient of IgG [55], it is also possible that modifications to the collagen characteristics of the extracellular matrix may act to further limit antibody-based drug transport following absorption across the epithelial cell barrier. Thus, modified expression of proteolytic enzymes, by their ability to remodel extracellular matrix composition, may affect the oral uptake of protein and peptide therapeutics in the IBD patient by either imposing a greater enzymatic barrier or by reducing the potential binding sites that can limit transport through the extracellular matrix.

2.2.4. Transporters

A susceptibility locus for development of IBD has been identified on chromosome 7q, which lies intriguingly near to the MDR1 gene [56]. This finding prompted the speculation that the gene product of MDR1, P-glycoprotein (P-gp), which is normally expressed at high levels on the apical membrane of columnar epithelial cells of the intestine, may be linked with the development of IBD. Genetically engineered mice lacking intestinal epithelial cell P-gp developed an intestinal inflammatory condition histologically similar to IBD [57]. Since this condition could be reversed by treatment with antibiotics, it has been speculated that P-gp may be important in maintaining homeostasis in the gut through modulation of intestinal responses to bacteria, although the mechanism for this action is not known. On the other hand, the effect of IBD on the expression of P-gp is unclear, elevated P-gp expression in T-lymphocytes and intestinal epithelial cells of patients with IBD who failed glucocorticoid therapy has been reported [58]. Expression and functionality of P-gp in the large intestine of a mice model of colitis however was reduced before severe symptoms appeared [59].

Another intestinal epithelial cell transporter that may not only be important in normal intestinal epithelial cell function but also affect the development of IBD is Pept-1. Studies have shown that Pept-1 transports bacterial-derived pro-inflammatory *n*-formyl peptides produced in the gut lumen, to facilitate movement of neutrophils across the epithelial monolayer through the induced expression of accessory immune molecules [60]. Furthermore, unlike normal colon tissue that is devoid of Pept-1, expression of this transporter is induced in the colon of patients with IBD [61], possibly in response to the local overproduction of bacterial proinflammatory peptides. It may be important to note that some peptide drugs and possibly peptide mimetics may enter enterocytes via Pept-1 and that the expression of this transporter can be regulated by certain pharmacological agents, such as cyclosporine (reviewed in Ref. [62]). Overall, a better understanding of the role of some intestinal epithelial cell surface transporters would be valuable, as their expression may not only correlate and affect the severity of disease, but modulation of their expression might also affect uptake of some potential IBD biotherapeutics.

3. Strategies for treating inflammatory bowel disease

Existing animal models and information obtained from patients with IBD point toward an altered T-cell inflammatory response to ingested antigens, commensal and pathogenic microorganisms as initiators of disease flares. Such bouts of inflammation can occur as a result of excessive effector T-cell function and overproduction of pro-inflammatory cytokines [63]. Some of these cytokines, such as TNF-α, IFN-γ and interleukin (IL)-12, stimulate T_H1-type responses while others, such as IL-4, IL-5, IL-13 stimulate T_H2-type outcomes (Fig. 2). It is widely accepted that CD is a T_H1-mediated inflammatory disease [64], while T_H2 subset dysfunction leads to UC [65]. Alternatively, inflammation can develop as a result of deficient regulatory T-cell function, known as T_H3 cells, which in normal conditions establishes mucosal homeostasis (also known as "oral tolerance") necessary to self-limit the everyday challenge of dietary antigens [65]. This mechanism is mediated by production of suppressive cytokines, such as transforming growth factor beta (TGF-β), which specifically inhibits its release of TNF-α and other proinflammatory mediators [66].

We have depicted a number of cellular and soluble drivers of IBD in a simplified model (Fig. 2). Dendritic cells (DC) are the primary cell for antigen sampling from the gut lumen. CDs also act as antigen-presenting cells (APC) to local populations of T- and B-lymphocytes. Cytokines present in the surrounding milieu at the time of antigen presentation determine both immune outcome (activation versus suppression) and immune disposition (T_H1/T_H2 balance) with IL-12 and IL-2 stimulating T lymphocyte growth and expansion. TNF-α, released from macrophage can amplify inflammation by activating other cells to release additional cytokines and mediators such as eicosanoids and nitric oxide. IL-10 secreted by regulatory T cells inhibits IL-12 synthesis and proinflammatory cytokine release. Inflammatory events also recruit neutrophils into the intestinal lumen, via a paracellular route, where they can cause tissue necrosis (Fig. 2).

Normal responses to a variety of environmental challenges appear to have been lost in the case of IBD. Thus, strategies to treat IBD attempt to rectify or mute resulting immune imbalances. Lack of success for some of these approaches has demonstrated that there is still much to understand concerning the underlying mechanisms of IBD. For example, introduction of a suppressive cytokine, such as IL-10, failed to show efficacy in the clinic [67]. Several proteins and peptides, however, have now been identified as potential therapeutic agents; the issue of how best to deliver these agents and what issue to consider in these deliveries is an important current topic for discussion [68,69].

3.1. Antibody-based therapies

Therapeutic antibodies are, at the moment, the most successful anti-inflammatory agents. They can inhibit the action of specific cytokines or other soluble mediators by directly binding to these factors, blocking their ability to function in target cell stimulation. Alternately, an antibody could be targeted toward a specific cell-surface receptor and in this way bind to and possibly stimulate the clearance of a discrete lymphocyte population involved in disease initiation or propagation. There are a variety of potential soluble or cell-surface antibody targets for the treatment of IBD (Fig. 2). The challenge is to select targets that provide an adequate efficacy to safety profile. Because of such concerns, focused delivery of potential antibody therapeutics may be useful to increase this efficacy to safety ratio.

Due to its central role in pro-inflammatory responses, such as that observed in IBD, TNF-α has been a primary therapeutic target for clinical intervention. The biological actions of TNF-α are extremely varied and include the ability to reversibly disrupt the TJ permeability barrier [35]. Therefore, the actions of any therapy designed to impede TNF-α at cell surface receptors may not only block immune cell activation but re-establish proper intestinal epithelium barrier properties. A reduction in intestinal paracellular permeability properties may reduce the amount or extent of environmental stimulus absorbed from the intestinal lumen, reducing the presence of agents that drive IBD symptoms.

Infliximab (Remicade) was the first monoclonal antibody to enter the market for the treatment of Crohn's disease. It is a chimeric protein composed of a human IgG framework containing a mouse-derived

variable region that selectively binds human TNF-α. Adalimumab (Humira) is a new phage-display-derived anti-TNF-α monoclonal antibody currently prescribed for rheumatoid arthritis but potentially useful for Crohn's disease as well [69,70]. Another antibody-based anti-TNF-α therapy currently used in the clinic is the TNF-α receptor-IgG chimera etanercept (Enbrel). Other co-stimulatory signalling events necessary for T cell activation have also been targeted, such as the OX40/OX40L interaction [71]. OX40 is a TNF receptor superfamily member expressed on T cells that binds to OX40L (ligand) expressed on activated B cells and dendritic cells. An anti-OX40L Mab effectively prevented onset and progression of experimental colitis in a murine model. A complicating safety factor associated with any anti-TNF-α therapeutic strategy relates to the critical role played by this cytokine in responding to infective events—leading to potential life-threatening infections in patients on this antibody therapy. Serious adverse events, including malignancy and demyelination, have also been reported [72].

3.2. Delivery opportunities for antibody-based therapies

From a pharmaceutical perspective, one limitation for widespread use of antibodies and antibody-based therapies is that these and other protein macromolecules are not suitable for oral delivery due to (1) poor stability in the gastric and intestinal environment and (2) limited uptake across the intestinal epithelium. Antibody-based therapies for IBD are therefore currently administered by repeated intravenous infusions, or self-administered by subcutaneous injections. As a result of this delivery strategy, these agents must be given in very large quantities, sometimes with a total dose of nearly a gram of protein per patient per treatment. In many cases, this amount of protein is required to achieve sufficient systemic levels to reduce the levels of an agent (i.e. TNF-α) that has been released into the circulation as well as into the intestinal epithelia and its surrounding tissue. Once sufficient serum levels of an antibody-based treatment have been achieved, that agent will typically remain in the blood at appreciable levels for several weeks due to mechanisms of slow elimination and re-uptake mechanisms for antibodies. On a positive note, this reduces the need for frequent dosing. As a concern, the therapeutic agent cannot be readily cleared from the body if some untoward outcome occurs. Because of these issues, it would be potentially advantageous to deliver antibodies for the treatment of IBD from the intestinal lumen if this was possible.

Pharmaceutical companies have prepared antibody-based therapeutics using the IgG class of immunoglobulins. This class represents the major class of antibodies present in serum. At the mucosal surface of the gut, secretory IgA (sIgA) typically dominate, having achieved that location through a complex receptor-mediated transport pathway that initiates at the basolateral plasma membrane and finishes at the apical membrane with the release of a dimeric antibody structure decorated with a J-chain protein and fragment of the IgA receptor/transport complex (reviewed in Ref. [73]). A small amount of IgG can also be found at the apical surface of mucosal epithelia [74]. Thus, systemic delivery of antibody-based therapies utilizing IgG components will have only minimal access to the apical surface of epithelial cells; a location where some critical events associated with IBD may be occurring (Fig. 3). IgA-based therapeutics may be better candidates to reach this site following a systemic administration. But even then, these antibodies would go to every mucosal surface in the body and not necessarily just those implicated in IBD. Thus, a large dose of an IgA-based therapy might also be required to achieve local, sufficient concentrations at affected sites of the gut.

Is apical application of an IgG antibody-based therapy a viable therapeutic option for IBD? As discussed above, conditions of the mucus layer and glycocalyx composition may either complicate or enhance the stability and access of antibodies in the lumen to the apical surface intestinal epithelial cells. Entrapment of protein macromolecules in these sites can act to facilitate the actions of proteolytic activities. Once at the intestinal cell surface, IgG transport across the intestinal epithelium does not occur passively to any appreciable extent. Fortunately, however, there is a transcytosis pathway present in intestinal cells that may provide for efficient receptor-mediated transcytosis of IgG-based therapeutics [75]. This transepithelial IgG transport occurs through selective

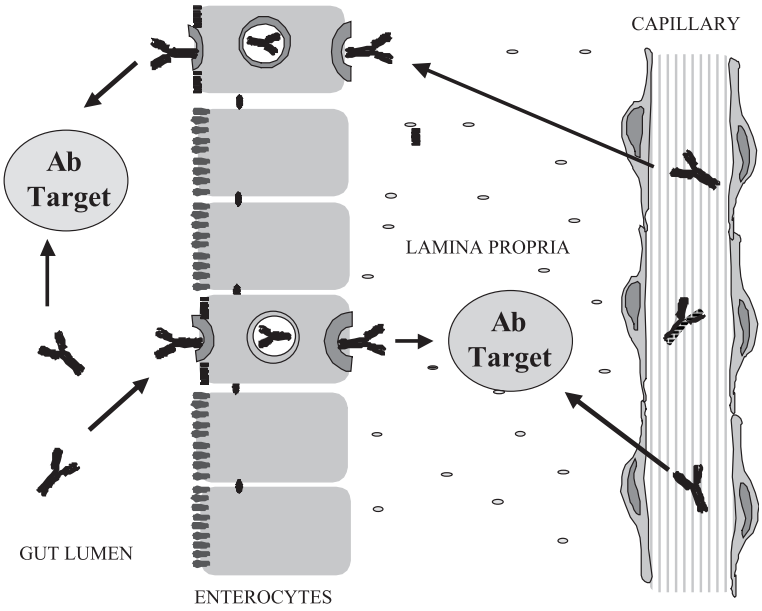

Fig. 3. Targets for antibody-based therapy for IBD exert their pro-inflammatory functions in the lamina propria or, possibly, in the gut lumen. Current antibody-based therapies for IBD are administered by injection (or infusion) and must reach their targets via the systemic circulation. The oral route, however, could bring the antibody molecule closer to the target.

interactions between the Fc region of the antibody with the MHC-I-related transmembrane protein FcRn [76].

FcRn was initially identified as part of a receptor mechanism to supply the immature fetal/newborn immune system with maternal antibodies present in colostrum. FcRn was demonstrated to mediate apical (AP) to basolateral (BL) transport of IgG through intestinal epithelial cells. In newborn rodents, intestinal absorption of maternal IgG from breast milk into the systemic circulation is well documented. FcRn is highly expressed in rodent's gut immediately after birth but is down-regulated afterwards to the point of being almost completely lost at the time of weaning [77]. In humans, maternal IgGs are mainly transmitted before birth to the fetus across the placenta. As in rodents, FcRn mediates AP to BL transcytosis of IgG [78]. Differently from rodents though, FcRn is expressed in the human adult and it has been found in a variety of tissues, including kidney and intestine, in the latter being localised mostly on the apical membrane of epithelial cells [79]. In a polarized human intestinal cell line, T84, FcRn can mediate bi-directional IgG transport, i.e AP to BL and BL to AP [74].

A slightly acidic apical pH is a key factor in IgG transport: FcRn–IgG interaction is strong at acidic pH but substantially weaker at neutral pH [80]. Thus, binding of IgG to FcRn is strong at the apical plasma membrane of the intestinal epithelial cells where a slightly acidic surface pH is established through the actions of sodium-mediated proton exchange. Once associated with FcRn and internalized into intestinal epithelial cells IgG transcytosis occurs in the AP to BL direction with privileged intracellular trafficking that evades the lysosomal pathway—the fate of most materials brought into intestinal epithelial cells from their apical surface. Ultimate delivery of the IgG–FcRn complex at the basolateral serosal surface results in exposure to a neutral pH environment and promotes antibody release. Thus, receptors for IgG transport are present and functional on the apical surface of the adult intestinal epithelium and could be exploited for the oral delivery of IgG-based antibody therapeutics, and possibly other macromolecules as well.

Degradation by enzymes through the digestive tract is a major challenge to oral delivery of antibody-based therapeutics [81,82]. As discussed earlier, in patients with IBD the situation may be even more complicated by enhanced proteolytic activities which

are a hallmark of this disease. This is further complicated by the fact that there may be great patient-to-patient and site-to-site variability of epithelial dysfunction due to disease. In general, protein stability in the gut can be improved by conjugation to non-degradable polymers such as polyethylene glycol (termed pegylation) [83]. Studies have shown pegylation to stabilize antibodies and antibody fragments from proteolytic destruction [84]. Other strategies to stabilize IgG proteins within the gut involve incorporation into materials or devices that release their contents at discrete sites and within close approximation of the apical surface of the intestinal epithelium. Additionally, several strategies for engineering metabolically stable antibodies have been investigated [85,86]. So far, however, no chemical modification, coupling, or other pharmaceutical strategy has been developed with complete success.

It is interesting to note that antibodies are absorbed by neonates after delivery to the gut in colostrum and milk. In humans, sIgA is the major immunoglobulin found in breast milk, followed by secretory IgM and IgG. Neutralization of some viruses and other pathogens is in part carried out by a mechanism that requires antibody transcytosis to the basolateral side of the epithelial barrier and back, with neutralization of pathogens occurring in endosomal vescicles [87]. Recently, IgGs delivered in bovine colostrum has been shown to survive transit through the human GI tract [88]. Others also have shown bovine colostrum as a safe and effective delivery vector for antibodies [89] and this approach may serve as a model to study a possible alternative strategy for administering antibodies to the intestinal mucosa.

It is possible that some of the numerous ingredients contained in colostrum protect antibodies from degradation or may modify their uptake or action(s). The presence of peptide growth factors has been linked to improvements in the mucosal integrity and repair in a variety of gastrointestinal conditions [90–93]. Interestingly, some peptide growth factors involved in intestinal epithelial repair have been shown to resist degradation and have improved activity when co-administered with colostrum as compared to other media [91]. This is not surprising, considering that colostrum has evolved in nature to specifically deliver immediate passive immunity (i.e. IgG) and growth factors that may be critical in the final maturation steps of the GI tract of the newborn. Many other properties are less understood; for example colostrum has been shown to prevent gut damage induced by NSAID and it has been suggested that it may be of value for the treatment of other ulcerative conditions of the gut [94]. An additional potential benefit of colostrum to inflammatory gut conditions is the presence of high concentrations of TGF-β. As discussed earlier, TGF-β is a regulatory component of the immune system, with suppressive effects on effector T-cells [65]. Although the potential application of colostrum to protect and possibly facilitate IgG uptake by the intestinal epithelium appears promising, it is currently unclear what issues might develop from chronic exposure to colostrum in adults due to its diverse spectrum of biologically active agents.

3.3. Peptide-based therapies

When considering potential therapeutic opportunities for the treatment of a disease or condition, it is often beneficial to target therapies that address not only the clinical consequences of the disease but its root cause(s). In the case of IBD, the observed intestinal inflammation and hyper-permeable properties of the intestinal epithelium appear to have intertwined early roles in the initiation of this disease. When considering the cellular events involved, a central role for TJ dysfunction is clear. MLCK activation results in a decline in TJ barrier properties in vitro, suggesting that this kinase is increased in its activity in intestinal epithelial cells in IBD [95–97]. Recently, we have shown that impaired TJ functionality can be restored in vitro by inhibition of MLCK using a short peptide (PIK) that emulates a specific sequence of the regulatory domain of this protein [38].

The ability of the PIK peptide to reverse disease-related permeability is likely due to two factors: its affinity for the non-muscle MLCK catalytic domain and its ability to pass efficiently across the plasma membrane of human intestinal epithelial cells. After administration, PIK is preferentially localized at the inner surface of the plasma membrane along with TJ elements. PIK is a nonapeptide rich in positively charged lysine and arginine residues, with a sequence highly analogous to a similar short peptide from HIV-1 TAT protein transduction domain. HIV-1 TAT is one

of the best-studied oligopeptides having such membrane translocation properties [98]. Many other short peptide (frequently arginine-rich) sequences that can penetrate cell membranes have been described and are collectively termed membrane translocation signals, or protein transduction domains (PTDs) [99,100]. Several reports have confirmed the usefulness of these PTDs for the delivery of macromolecules into cells (reviewed in Ref. [101]). Thus, peptide-based therapeutics for the treatment of IBD could be readily absorbed following oral administration and PIK specifically has the ability to reduce the permeability properties of the TJ barrier.

3.4. Delivery opportunities for peptide-based therapies

The mechanism of cell penetration of the arginine-rich PTDs is not clear. Receptor-mediated endocytosis is not a likely mechanism since there is no decrease in uptake at 4 °C as compared to 37 °C suggesting the lack of an energy-dependent transport process [102]. Furthermore, D-amino acid peptides cross cellular membranes as efficiently as the natural L-counterparts [98]. Since receptors normally recognize only L-form molecules it seems unlikely that a receptor would be involved. The fact that D-amino acid PTDs sequences cross membranes equally well to their L-amino acid counterparts, the opportunity to prepare an enzymatically stable form of PIK can likely be achieved using this strategy. Thus, membrane-permeant, D-amino acid-based peptides that interfere with cellular activation events leading to increased paracellular permeability may be able to correct at least some aspects of IBD following an oral administration.

4. Conclusions

Less than a decade ago there would have been little to write for a review discussing the potential for treatment of IBD using orally administered protein and peptide drugs. Two critical events have changed that. The first is the identification of a potential trans-epithelial uptake mechanism for IgG-based proteins. In the case of IgG-based therapies, the extracellular (soluble and cell-surface associated) targets already identified through clinical studies are being extended by further work to define cytokines and pro-inflammatory cascades that are activated in IBD to provide even more potential targets for the future. Second, there is a better definition of critical TJ components and interactions that are involved in regulating functional properties of this barrier to paracellular permeability. Identification of these components and methods to manipulate their aberrant activity can lead to novel methods of treating IBD. Since many of the events associated with TJ structure and function rely on highly specific protein–protein interactions that can be emulated by synthetic peptides, opportunities for the identification and delivery of orally delivered peptide-based therapeutics is also promising.

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