NOTE TO USERS

MODULATION OF INTESTINAL EPITHELIAL PHYSIOLOGY AND SIGNAL TRANSDUCTION BY TRANSFORMING GROWTH FACTOR-BETA

By

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

MODULATION OF INTESTINAL EPITHELIAL FUNCTION BY TGFβ

DOCTOR OF PHILOSOPHY (2004) McMaster University

(Medical Sciences) Hamilton, Ontario

TITLE: Modulation of Intestinal Epithelial Physiology and Signal Transduction by Transforming Growth Factor-beta

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SUPERVISOR: Dr. D.M. McKay

NUMBER OF PAGES: xiv, 212

ABSTRACT

Intestinal epithelia contribute to gut homeostasis by acting as a selectively permeable barrier and establishing a driving force for water movement through vectorial ion transport. These processes are affected by cytokines. As TGFβ levels can be increased in gut inflammation, a situation where epithelial function is often altered, **the aim of this thesis was to define the effects of TGFβ on intestinal epithelial barrier and ion transport function.**

The first study characterized the kinetics and signal transduction pathway behind the novel observation that intestinal epithelial monolayers treated with TGFβ display reduced stimulated secretory responses. The mechanisms involved in this process were further delineated in a second study where it was determined TGFβ treatment causes a down-regulation and altered sub-cellular localization of the main apical chloride channel (CFTR). The final study defined the mechanism behind TGFβ-induced epithelial barrier enhancement in terms of signal transduction pathways and regulation of proteins involved in maintaining a physiologically "tight" barrier. Furthermore, it was determined that TGFβ treatment protects against barrier damage caused by pathogenic bacteria, and the mechanisms behind this have been revealed.

The first two manuscripts present a substantial body of evidence on the kinetics and mechanisms of TGFβ-induced diminished epithelial ion transport. In addition to furthering understanding of cytokine regulation of epithelial function, these data are particularly relevant to the field of enteric disease where water balance is often perturbed. In the final manuscript, the protective role of TGFβ on epithelial barrier function is

illustrated by its ability to preserve barrier function from damage caused by the pathogenic bacteria, enterohemorrhagic *E. coli* (EHEC) O157:H7. Having determined mechanisms underlying epithelial barrier enhancement and protection by TGFβ, potential therapeutic targets have been revealed that might be strategic in treating individuals with conditions of increased barrier permeability, such as inflammatory bowel disease relapses and EHEC infection.

PREFACE

As per the guidelines set by the School of Graduate Studies, McMaster University, I am required to list the contributions of each author in any multiple-authored work contained within the following thesis. Below is a timeline for each manuscript and a description of the work conducted by each author.

TGFβ Effects on Epithelial Ion Transport and Barrier: Reduced Cl^- Secretion Blocked by a p38 MAPK Inhibitor.

- 05/1999 – 07/2002
- K. Howe: responsible for the design and execution of all experiments, and wrote the manuscript
- J. Gauldie: provided adenoviral constructs
- D.M. McKay: supervision and advice

TGFβ Down-regulation of the CFTR: A Means to Limit Epithelial Chloride Secretion.

- 03/2001-03/2004
- K.L. Howe: responsible for the design and execution of all experiments, and wrote the manuscript
- A. Wang: assisted with RT-PCR experiments and agarose gels
- M.M. Hunter: assisted with RNA extraction and RT-PCR experiments; designed CFTR primers

- B.A. Stanton: provided MDCK epithelia stably transfected with GFP-tagged CFTR
- D.M. McKay: supervision and advice

TGFß Enhances Intestinal Epithelial Barrier and Protects Against EHEC O157:H7 Infection by Differentially Regulating Claudins.

- The TER data was collected throughout the thesis, beginning in 1999. However, the bulk of the work was conducted from 05/2002-05/2004.
- K. Howe: responsible for the design and execution of all experiments, and wrote the manuscript
- C. Reardon: performed ERK MAPK and SMAD signaling experiments
- A. Nazli: microbiology consultant, provided HB101 *E. coli* TER data, performed IL-8 ELISA
- D.M. McKay: supervision and advice

ACKNOWLEDGEMENTS

Science is a collaborative medium, not only in the greater academic community, but also right inside the laboratory. While certain individuals have already been mentioned, I am greatly indebted to Jun Lu for her guidance and to my fellow laboratory members for experimental assistance, constructive criticism, and useful discussions. As is typical of scientific research, I found moments of pure joy and absolute frustration. Without the invaluable support of my adopted family here at McMaster, the past five years would not have been such an incredible experience. There are numerous people with whom I have shared many laughs, a few tears, and lots of hugs. To all of you, I thank you for these memories and your friendship – you will remain in my heart forever.

I would also like to extend my sincere gratitude to my committee members, Drs. Steve Collins, Carl Richards, and former member, P.K. Rangachari. Your supervision and suggestions were critical in developing my understanding of the scientific process. I recognize that your participation on my supervisory committee has continued despite increasingly demanding schedules. On behalf of all the students whose lives you enrich, I thank you for your generous gift of time. It is greatly appreciated.

To my supervisor, Dr. Derek McKay, you have provided me with so many opportunities, both in my personal and professional lives. The growth I have experienced over the past five years is a testament to your exceptional supervisory abilities. You have given me a supportive learning environment, one where I was able to make mistakes and express my thoughts freely. I particularly appreciated the time and patience you put into explaining each suggestion throughout the many stages of my training. Through

entrusting me to supervise an undergraduate thesis project, I had the privilege to impart some of your teachings. Last but not least, your love of spinning a 'good yarn', encouraging laughter in the laboratory, and dancing a 'wee jig' when the data looked good, brought a smile to my face almost every day – you truly are one in a million!

To my friends, who have been with me throughout this entire process, you have each played a unique and significant role in my life. In particular, I thank the kindred spirits I have found in Shannon Taylor, Kersti Kents, and Meaghan Hunter. In addition, thank you to my Phoenix Jennifer Rayworth for the strength you transmit, Vincenza Di Leo for our continued exploration of human nature, and Heather Cameron for journeying with me throughout this phase of our lives.

I would like to thank my sister Laura for many fond memories over the years, but most of all, for teaching me about generosity of spirit. To my parents, I thank you for instilling in me the desire to learn about the wonders of our universe. As a result, for me learning has always been fun, and for this I feel extremely fortunate.

Finally, to Peter Ceponis, who started off as a goofy pal in 3N49 and has now become one of the most important people in my life. Thank you for your encouragement, never-ending support, and most of all, for illuminating my life.

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Chapter 1

Regulation of Intestinal Epithelial Function

The purpose of this introduction is to provide the reader with sufficient context underlying the aims of this thesis research, focusing on the primary functions of intestinal epithelia and discussing a range of factors that affect these functions. Specifically, the background and rationale for investigating the regulation of epithelial physiology and signal transduction by transforming growth factor-beta (TGFβ) will be provided.

IMPACT OF GASTROINTESTINAL DISEASE ON SOCIETY

The burden of gastrointestinal (GI) illness poses substantial economic costs and quality of life issues (Bodger, 2002). The health care system is challenged with meeting the chronic care demands typical of many GI illnesses, while many patients suffer the physical and emotional consequences of living a lifetime with pain, embarrassment, and frequent absence from employment. For example, Crohn's disease and ulcerative colitis, collectively referred to as inflammatory bowel disease (IBD), are chronic inflammatory conditions for which there is sub-optimal therapy and no cure, thus presenting a challenge to both health care workers and patients as they strive to manage the disease and the constant disruption to their lives. Similarly, outbreaks involving enteric pathogens are frequent around the world and cause high levels of morbidity and mortality. One such example includes enterohemorrhagic *Escherichia coli* (EHEC) O157:H7, which is a pathogen acquired from fecally contaminated food or water in developed nations. Outbreaks with EHEC, such as the one in Walkerton, Ontario in May 2000, caused seven deaths and made over 2000 people ill, demonstrating that such an infection poses a

significant threat to the health of our communities in the short-term. Furthermore, studies have shown that infection with acute gastroenteritis can lead to complications such as post-infectious bowel syndrome (Spiller, 2003), and point to the long-term impact of GI infections.

While much progress has been made in the last decade regarding the diagnosis and treatment of GI diseases, there is still a significant need for more effective therapies. Indeed, the recent success story of anti-TNFα therapy in Crohn's disease proves the bench-to-bedside approach works. Thus, research aimed at understanding the functions of the GI tract and the factors that influence its processes during health and disease may identify new therapeutic targets for the design of effective treatments for patients suffering from GI illnesses.

GASTROINTESINTAL STRUCTURE AND FUNCTION IN HEALTH AND DISEASE

The GI tract, or alimentary canal, is a long tube-like structure originating at the oral cavity and terminating at the rectum. The structural organization of the canal is similar throughout its length, with four basic layers or tunics that from the lumen outward, are called the mucosa, submucosa, muscularis externa, and serosa (Ross, Romrell, and Kaye, 1995). First, the intestinal mucosa is composed of a single layer of epithelial cells resting on a basement membrane, which then overlie a sheet of connective tissue called the lamina propria. Here, immune cells are found interspersed between epithelia, within lymphoid aggregates (Peyer's patches or follicles), or spread diffusely

throughout. In addition, blood vessels, lymphatic capillaries (small intestine), and a small layer of circular and longitudinal muscle called the muscularis mucosae are present, the latter residing at the boundary of the next layer. The submucosa consists of dense connective tissue containing blood and lymphatic vessels, glands (esophagus and duodenum), and the submucosal nerve plexus which is a component of the enteric nervous system. In the muscularis externa, two concentric layers of smooth muscle overlay one another with the innermost sheet oriented in a circular fashion and the outermost sheet arranged longitudinally. Between the circular and longitudinal layers of muscle lies the myenteric plexus, a nerve plexus responsible for coordinating the rhythmic contractions (peristalsis) of the muscularis externa necessary for moving intestinal contents towards the anus. The final layer of the GI tract, the serosa, is composed of an epithelial membrane and its underlying connective tissue, which provide attachment to the peritoneal wall, contain adipose tissue, and serve as a conduit for large vessels and nerve trunks to reach the wall of the GI tract.

Functionally, the four layers of the digestive tract cooperate to serve the basic GI functions of selectively obtaining and processing nutrients necessary for survival, removing unwanted materials, and mounting effective local immune responses. The latter process limits extensive unnecessary systemic effects caused by contact with potentially noxious stimuli (McKay and Perdue, 1993). As a result of the many antigenic substances ingested, the vast numbers of bacteria (both resident microflora and pathogens from the environment), and the constant change in luminal composition, it is typical for the GI tract to be in a state of controlled inflammation. This situation reflects the

dynamic balance existing between factors that activate the host immune system and the defense responses put forth by the host, without which mucosal integrity and host well-being would not be maintained.

A key example where altered immunity contributes to disease pathogenesis is IBD. Both human studies with IBD patients and experimental animal models provide insight into the generation of chronic intestinal inflammation, and these have been reviewed extensively by Bouma and Strober (2003) and Strober, Fuss, and Blumberg (2002). While the contributions of multiple genetic and environmental factors are currently being investigated (e.g. NOD2/CARD15, smoking; Nayar and Rhodes, 2004), it has become increasingly clear that in the absence of a pathogenic agent, individuals with Crohn's disease mount inappropriate immune responses to luminal contents (e.g. their own microflora). The consequence of this altered mucosal immunity manifests in histological changes which functionally contribute to disturbed water and electrolyte imbalance (resulting in diarrhea), stricture formation and bowel obstruction, and a 'leaky' epithelium that allows passage of antigenic luminal materials typically excluded from the underlying mucosa (Meddings, 2000). Given that elevated levels of both pro- and anti-inflammatory cytokines are found in tissue from patients with Crohn's disease and ulcerative colitis (e.g. TNFα, IFNγ, IL-4, TGFβ, IL-10; reviewed in Sartor, 1995), there is a basic need to understand the effects of these mediators on the cells responsible for GI homeostasis. The effect of cytokines on enteric epithelia is particularly important since they function as key regulators of the intestinal barrier and water balance, both of which are perturbed in IBD.

THE INTESTINAL EPITHELIUM

Throughout the length of the GI tract, the intestinal epithelium forms a continuous single epithelial cell layer, although the epithelia are phenotypically distinct at different sites. For example, the base of intestinal crypts hosts a stem cell population from which all other epithelial cell types derive. The enterocyte predominates, appearing first as an immature pro-secretory-type cell located in the intestinal crypt and later, a mature pro-absorptive-type cell as it migrates towards the villus (small intestine) or surface epithelium (large intestine) (Goodlad, 1989). At the end of this differentiation and migration process the enterocyte is sloughed from the epithelial layer and replaced by its immediate successor, with the average lifespan of an enterocyte being 4-7 days. Interspersed between the enterocytes are mucus-producing goblet cells, enteroendocrine cells, and Paneth cells that produce anti-microbial compounds (e.g. defensins). Also situated between intestinal epithelial cells are immune cells specific to the gut, called intraepithelial lymphocytes. Furthermore, in regions overlying lymphoid follicles specialized epithelia called 'M' cells monitor the luminal environment through continuous antigen sampling, emphasizing the interplay between epithelia and immune cells (Jang *et al.*, 2004). Altogether, the intestinal epithelium serves three general functions: 1) to absorb nutrients and selectively transport electrolytes (and thus water) between the lumen of the gut and the body; 2) act as a regulated physical barrier; and 3), via production of a variety of messenger molecules, to serve as an endocrine or immune accessory cell.

Epithelial Ion Transport Function

Intestinal epithelia (i.e. enterocytes) secrete and absorb multiple ions, thus driving water movement to ease the passage of intestinal contents, facilitate diffusion of nutrients to digestive enzymes, and flush noxious substances through the gut lumen. The structural mechanism behind vectorial ion transport is polarization, giving an enterocyte an apical and basolateral side to permit asymmetric distribution of ion channels, transporters and absorptive modifications. Next, the functional mechanism underlying vectorial electrolyte transport is the establishment of an electrochemical gradient, or put another way, the tendency of an ion to move passively from one point to another as a result of concentration and electrical potential differences between the two points. This gradient is created by the basolateral Na^+/K^+/ATPase that continuously pumps 3 Na^+ out of the cell interior, consuming one molecule of ATP in the process, in an unequal exchange for 2 K^+ that enter from the external environment (Figure 1.1). Consequently, both a net negative charge is maintained inside the cell and a reduced intracellular Na^+ concentration. Together, these establish an electrochemical gradient where electrically, Na^+ is attracted to the net negative charge, and where chemically, Na^+ will move from areas of highest concentration to lowest, creating a condition in which there is an overall driving force for Na^+ to enter into the cell (Barrett, 1993). Thus, with the electrochemical gradient set up in this fashion, many cotransporters and exchangers exploit this driving force, using Na^+ entry to help transport other molecules or ions into the cell (e.g. Na^+/glucose, $Na^+/K^+/2Cl^-$, Na^+/H^+) (Greger, 2000).

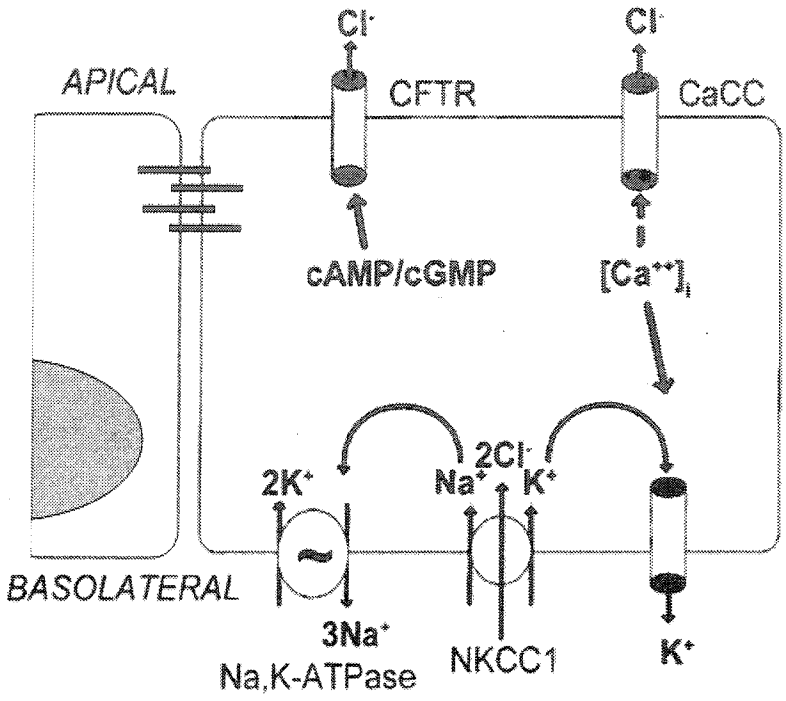

Figure 1.1. Model of chloride secretion in intestinal epithelial cells. The Na/K/ATPase continually pumps Na^+ out of the cell, Cl^- enters via the cotransporter, and K^+ passively exits following its electrochemical gradient. Agents that increase cAMP (or cGMP) open the CFTR (solid arrow), permitting Cl^- efflux. Dashed arrow indicates possible activation of Ca^{2+}-activated Cl^- channels (CaCC) by cAMP/cGMP. See text for details. Adapted from Barrett and Keely (2000).

Chloride Secretion

Although bicarbonate and potassium secretion also occurs along the length of the intestine, fluid secretion is driven primarily by chloride (Barrett and Keely, 2000). Using the electrochemical gradient established by the Na^+/K^+/ATPase pump, chloride enters via the electroneutral $Na^+/K^+/2Cl^-$ cotransporter (NKCC) located on the basolateral membrane, accumulates above its electrochemical equilibrium, and upon receipt of an appropriate stimulus, exits from the apical membrane through specific chloride channels during normal physiologic processes (Barrett and Keely, 2000). Subsequently, there are multiple mechanisms for Cl^- ion secretion. For example, in cyclic adenosine monophosphate (cAMP)–driven Cl^- secretion, the activation of adenylate cyclase (e.g. by vasoactive intestinal peptide) converts ATP to cAMP, which in turn, activates protein kinase A (PKA). PKA phosphorylates the regulatory domain of the main apical chloride channel, the cystic fibrosis transmembrane conductance regulator (CFTR), inducing a conformational change to the open configuration (Akabas, 2000). However, while phosphorylation is necessary to open the CFTR, it is not sufficient as channel gating of the CFTR additionally requires ATP binding and hydrolysis. Once the CFTR opens Cl^- is driven to leave the cell due to the negatively charged cell interior. Water movement occurs secondarily, following osmotic forces and hydrostatic pressure differences. While the flow of water is predominantly paracellular, lesser volumes of water may move via the transcellular route given the recent identification of aquaporin water channels in epithelia located throughout the GI tract (Ma and Verkman, 1999).

In addition to cAMP-driven chloride secretion, there is also a calcium-mediated pathway. The accepted working hypothesis is that intracellular calcium concentration rises, due to either increased permeability of the cell to extracellular Ca^+ or release from intracellular stores, and this causes the basolateral Ca^+-activated K^+ channels to open (Barrett and Keely, 2000). Efflux of the positively-charged potassium ion renders the cell interior even more negative and causes Cl^- to exit via apical CFTR, limited only by the number of channels found in the open configuration at any given point in time. It should be noted, however, that recent evidence from a human intestinal epithelial cell line has indicated that Ca^+-activated Cl^- channels also contribute to chloride secretion (Mohammad-Panah *et al.*, 2001). While their exact contribution to chloride secretion is yet to be determined, these channels clearly provide an alternate pathway for Cl^- efflux. Thus, further studies should delineate alternate chloride channel expression and determine their physiological role during intestinal epithelial chloride secretion.

Epithelial Barrier Function

The intestinal epithelium has an extensive surface area of >300 m^2, over which it must provide an effective barrier between the external environment (gut lumen) and host tissues. As a first line of defense, a mucous gel layer (secreted by goblet cells) rests above the epithelium of the small and large intestine and limits epithelial contact with potentially pathogenic microorganisms (Deplancke and Gaskins, 2001). A paradox exists in the gastrointestinal system, however, since nutrient absorption must occur alongside exclusion of noxious dietary and luminal substances (e.g. bacteria). Therefore,

establishing a barrier that can regulate passage of foreign materials is another essential property of gut epithelia. Selective regulation can take place via transcellular (through the cell) or paracellular (between cells) pathways. Transcellular transport is accomplished by endocytic pathways that route vesicles carrying large molecules from one side of the polarized epithelial cell to the other. This is a significant area of research that is often considered in hypersensitivity/food allergy (Yu and Perdue, 2000), and an in-depth discussion is beyond the scope of this thesis.

The paracellular pathway, on the other hand, is equipped with a tight seal that limits the passive diffusion of solutes and allows cells to maintain polarity, displaying apical and basolateral domains (Tsukita and Furuse, 1999). This seal, formed by tight junctions (TJ), is located in the apical domain and has been confirmed by a variety of complementary techniques. For example, electron microscopy (EM) identifies the TJ as a series of membrane fusion sites or 'kissing points', which have been further revealed by freeze-fracture EM to appear as networks of continuous anastomosing strands with complementary grooves and ridges between adjacent cells (Staehelin, 1974). Surpassing the initial 'static' concept that lateral association of tight junction strands between adjacent cells created regions of absolute occlusion, physiological studies showed wide ranges in solute permeability between various epithelia that correlated with the level of TJ protein expression (Anderson and Van Itallie, 1995).

Molecular composition of tight junctions

The discovery of the ~65 kDa protein occludin in 1993 by Furuse *et al.* (1993) stands as the first time an integral membrane protein was localized to the tight junction. This finding was re-affirmed by induction of cell-cell adhesion in fibroblasts by overexpressing occludin (Van Itallie and Anderson, 1997). Additionally, expression level of occludin correlates with the number of TJ strands, demonstrating the structural contribution of occludin in tight junction formation (Saitou *et al.*, 1997). A functional role for occludin was established after observing enhanced barrier function in cultured epithelial cells overexpressing occludin (McCarthy *et al.*, 1996, Balda *et al.*, 1996). Thus, occludin is clearly an important TJ protein. However, the observation that occludin-deficient embryonic stem cells still differentiated into polarized epithelial cells expressing tight junctions pointed to a functional role for other proteins in the TJ (Saitou *et al.*, 1998). Also, due to the notion that a single protein was unlikely to account for the range in permeability characteristics between epithelia, and that only short and poorly developed tight junction strands were formed in fibroblasts overexpressing occludin, research sought additional tight junction proteins (Tsukita and Fursuse, 1999).

In 1998, Furuse *et al.* (1998) characterized a structural role for claudin-1 and claudin-2 in TJ formation through their reconstitution of TJ strands in fibroblasts and recruitment of occludin to the TJ. The identification of the claudin gene family revealed a series of ~22 kDa transmembrane proteins within the tight junction that, similar to occludin, have two extracellular loops with amino- and carboxy-terminal ends retained within the cytoplasm. Unlike occludin, however, there are over 20 identified claudin

proteins that display varied tissue distribution. Consequently, differential claudin expression likely helps explain varied epithelial permeability observed among different tissues (Mitic, Van Itallie, and Anderson, 2000). Experimental evidence also demonstrates the physiological significance of claudins, with overexpression of claudin-1 improving two markers of epithelial barrier function (Inai, Kobayashi, and Shibita, 1999). Indeed, claudins can form homotypic and heterotypic associations with each other to regulate the TJ barrier (Heiskala, Peterson, and Yang, 2001). Claudin dysregulation can also cause disease, as mutations in claudin-16 (also known as paracellin-1) alter Mg^{2+} transport resulting in kidney disease (Simon *et al.*, 1999). Moreover, claudin-3 acts as a receptor for enterotoxin produced by *Clostridium perfringens*, further emphasizing the importance of TJs in disease pathogenesis (Fujita *et al.*, 2000). Thus, while heterogeneous interactions between claudin-1, -3, and -5 increase the barrier function of human airways epithelia (Coyne *et al.*, 2003), the contribution of specific claudin-claudin interactions to TJ barrier function needed to be addressed in a tissue-specific manner.

Perijunctional actin-myosin ring

Near the apical pole of the intestinal epithelial cell there is a perijunctional ring of actin and myosin (PAMR), the contraction or relaxation of which causes alterations in tight junction structure and paracellular permeability (Madara, Moore, and Carlson, 1987). Specifically, contraction of the PAMR decreases epithelial barrier function. Therefore, while transmembrane TJ proteins are necessary for occluding the paracellular pathway, tight apposition of adjacent epithelial cells can be maintained when the PAMR

is in a 'relaxed' state. The linkage between TJ proteins and the PAMR was revealed by the discovery of the tight junction-associated protein, zonula occludens (ZO)-1 (Figure 1.2). This protein mediates interactions with the actin cytoskeleton whereby the carboxy-terminus of ZO-1 binds F-actin, and specific localization to the tight junction whereby the amino-terminus of ZO-1 binds occludin and ZO-2 (Fanning *et al.*, 1998). Furthermore, ZO-1 and the related proteins ZO-2 and ZO-3 were shown to bind claudin-1 and claudin-2 at the tight junction in transfected fibroblasts (Itoh *et al.*, 1999). *In vitro* binding assays further determined that ZO-2, ZO-3, and occludin also directly bind to F-actin (Wittchen, Haskins, and Stevenson, 1999). While their exact contributions remain to be determined, there are additional proteins that may play a role in the regulation of intestinal epithelial barrier function given their localization to the TJ, and these include cingulin, 7H6, symplekin, and junctional adhesion molecules (Mitic, Van Itallie, and Anderson, 2000). Thus, factors that induce PAMR contraction and/or disrupt any component of the tight junction complex have the potential to cause changes in epithelial barrier function.

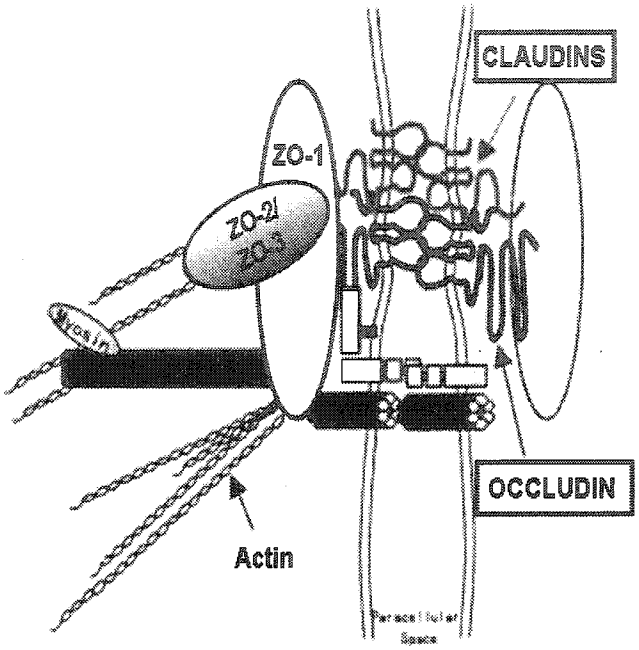

Figure 1.2. Schematic model of claudin, occludin, and ZO protein interactions at the tight junction (TJ). Notice the connection between the TJ architecture and the actin cytoskeleton. Adapted from Mitic, Van Itallie, and Anderson (2000).

METHODS FOR STUDYING EPITHELIAL BARRIER AND ION TRANSPORT

Cell culture models provide researchers with a tool for examining epithelial function under controlled experimental conditions *in vitro*. T84, HT-29, and Caco-2 are human intestinal epithelial cell lines employed routinely in the study of epithelial ion secretion and barrier function (McKay, Philpott, and Perdue, 1997). T84 cells, employed throughout the studies in this thesis, are human colonic crypt-like cells that form confluent polarized monolayers (Figure 1.3) (Dharmsathaphorn *et al.*, 1984). These monolayers form tight junctions, demonstrate high electrical resistance (see below), and are capable of vectorial ion transport. In addition, T84 cells express receptors for neurotransmitters and cytokines found *in vivo*, and are often used to delineate pathogenesis of bacterial enteropathogens, making them an appropriate experimental model for studying epithelial responses to physiological stimuli.

Dating back to 1951 (Ussing and Zehran), and employed by laboratories world-wide as the gold standard method, Ussing chambers permit the measurement of net active ion secretion across intestinal tissue segments or epithelial cell culture monolayers in response to physiological stimuli. In this procedure an epithelial monolayer is first grown on a removable insert such as a Transwell®, and then is mounted in the Ussing chamber, bathed in physiological buffers at 37°C, and clamped at zero voltage. The amount of current injected to maintain a zero voltage is referred to as short circuit current (Isc), and reflects net active ion secretion. Although baseline Isc values may vary between monolayer preparations, net active ion transport (ΔIsc) across the monolayer is routinely increased in response to a range of mediators that elevate cAMP (e.g. forskolin) and Ca^{2+}

(e.g. carbachol), making these secretagogues useful in assessing epithelial secretory responses. Epithelial monolayers can thus be treated with various factors (e.g. biological mediators, bacterial products, or cytokines) and the impact of these factors on epithelial Isc responses to stimuli compared against untreated controls.

Epithelial paracellular permeability or barrier function can be assessed in one of three ways: 1) recording transepithelial electrical resistance (TER) of a monolayer mounted in the Ussing chamber; 2) measuring TER using a voltmeter and chopstick electrodes; and 3), determining the movement (flux) of small molecules restricted to the paracellular space (e.g. mannitol). First, the voltmeter and Ussing chamber both measure the passive flow of ions across the epithelial preparation. Using Ohm's law (Voltage=Current x Resistance), the resistance of the tissue to passive ion flow is calculated, such that higher resistance values correspond to a "tighter" barrier. Also, flux studies are often used as a complementary assessment of epithelial permeability. This involves measuring the amount of selected tracer molecules such as ^{51}Cr or fluorescent-labeled dextrans that cross the epithelium. Finally, it should be noted that the terminology used to describe epithelial barrier function can be confusing; thus, increased epithelial permeability is the same as decreased transepithelial electrical resistance, where both imply reduced barrier function.

Although the experimental models used to assess epithelial monolayer function are reductionist, in that the intestinal epithelium is studied outside of the complex arrangement of the gut, they have revealed that the intestinal epithelium is a dynamic

structure capable of responding to a vast array of stimuli, and thus contributed to our overall understanding of gut function.

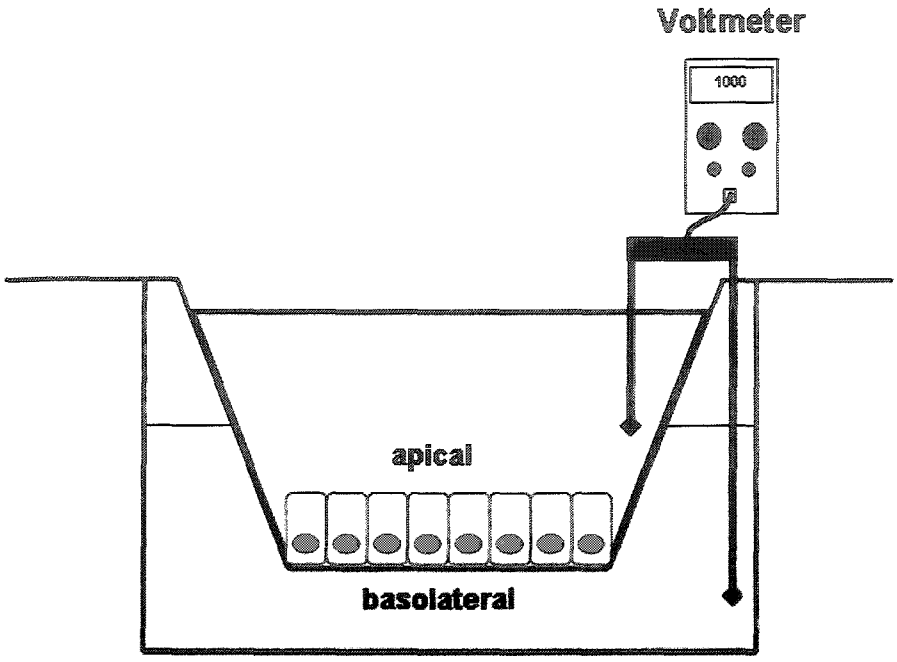

Figure 1.3. Schematic of a T84 intestinal epithelial monolayer grown on a filter support. The voltmeter and chopstick electrodes are depicted to the right of the image and are used for assessing transepithelial electrical resistance (TER). The apical and basolateral compartments of the transwell system are separated by a semipermeable membrane (0.4 μm pore size), upon which the monolayer is grown.

FACTORS AFFECTING EPITHELIAL FUNCTION

Agents that can alter epithelial function include biological mediators released from cells in the local environment, bacteria and their products, and also cytokines. The impact of these 'signals' on the epithelium can be beneficial or detrimental, and may even be both, depending on the context in which they are received.

Biological mediators

The enteric nervous system is intimately associated with the intestinal epithelium through a network of nerve fibres originating from the mucosal and submucosal plexuses. In conjunction with enteroendocrine cells, the neuroendocrine system releases several neurotransmitters and neuropeptides that can act on intestinal epithelia including vasoactive intestinal polypeptide (VIP), acetylcholine (ACh), neuropeptide Y (NPY), substance P (SP), and serotonin (5-HT) (Wapnir and Teichberg, 2002). In intestinal epithelial monolayers, VIP and ACh induce chloride secretion through receptor-mediated activation of the cAMP and Ca^{2+}-driven pathways respectively (Dharmsathaphorn and Pandol, 1986). VIP also regulates the increase in epithelial barrier function following enteric nervous system stimulation, by decreasing the paracellular flux of small molecules and inducing expression of the tight junction-associated protein, ZO-1 (Neunlist *et al.*, 2003). When SP is added to the serosal surface of rabbit colonic mucosa, chloride-dependent short circuit current is increased, indicating active ion transport (Reigler *et al.*, 1999). Conversely, NPY inhibits cAMP-stimulated and Ca^{2+}-stimulated ion secretion in

HT-29 colonic epithelia by reducing apical Cl^- and basolateral K^+ conductance (Bouritis *et al.*, 1998).

Other mediators found in the intestinal milieu include: prostaglandins and leukotrienes, derived from epithelia, immune cells in the lamina propria, and mast cells; histamine, released by activated mast cells; and adenosine, which is produced by many cell types (Perdue and McKay, 1994). Each of these stimuli are capable of inducing active ion secretion by intestinal epithelia, which may be protective since it serves as a "flushing" mechanism designed to remove noxious stimuli from the GI tract (McKay and Perdue, 1993). In addition, these factors may also regulate barrier function.

Bacteria and Bacterial Products

Enteric bacteria, as well as their products, can directly affect epithelial barrier and ion transport function. One well-characterized example is the induction of sustained epithelial chloride secretion by cholera toxin produced by *Vibrio cholerae*, an event that causes massive secretory diarrhea *in vivo* (Sack *et al.*, 2004). Mechanistically, cholera toxin binding to the apical epithelial surface leads to translocation of the A subunit, subsequent activation of adenylate cyclase, and sustained cAMP-driven chloride secretion in T84 cells (Lencer *et al.*, 1995). Infection with enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) also induce a watery diarrhea *in vivo*, but through imprecisely understood mechanisms. Cell culture studies have yielded conflicting results regarding the effects of EPEC on epithelial ion transport. In Caco-2 cells, EPEC infection causes a significant increase in short circuit current responses

(Collington, Booth, and Knutton, 1998), while infected T84 monolayers demonstrate reduced secretory responses to cAMP and Ca^{2+} secretagogues (Hecht and Koutsouris, 1999). In terms of epithelial barrier function, EPEC and EHEC both cause increased paracellular permeability in colonic epithelial monolayers by disrupting tight junction protein expression (occludin and ZO-1) and rearranging the actin cytoskeleton (Simonovic *et al.*, 2000, Philpott *et al.*, 1998). As another example, *Clostridium difficile* toxins A and B increase intestinal monolayer permeability by a mechanism involving F-actin rearrangement and dissociation of occludin, ZO-1, and ZO-2 from the tight junction (Nusrat *et al.*, 2001). While as yet uncharacterized in intestinal epithelia, *Clostridium perfringens* enterotoxin removes claudin-3 and claudin-4 from tight junction strands of MDCK (kidney) cells, inducing decreased TER and concomitant paracellular flux of small dextrans to again demonstrate that bacterial proteins are direct regulators of barrier function (Sonoda *et al.*, 1999).

Cytokines

More than 60 cytokines and chemokines can be found in the intestinal milieu, and are produced from a variety of sources including submucosal fibroblasts, immune cells, or the intestinal epithelium itself. Currently, a large body of literature exists regarding the specific effects of cytokines on intestinal epithelial function and the molecular mechanisms responsible, some of which are discussed below. For example, the pro-inflammatory cytokine IFNγ was the first cytokine shown to directly affect T84 barrier function (Madara and Stafford, 1989). Since then studies show IFNγ decreases

electrolyte secretion and impairs barrier function in T84 colonic epithelia through regulation of critical ion pumps and channels (Na^+/K^+/ATPase, NKCC1), and tight junction proteins (occludin, ZO-1) (Sugi *et al.*, 2001, Youakim and Ahdieh 1999). IL-4 reduces stimulated Cl^- secretion in T84 monolayers by decreasing CFTR expression (Zund *et al.*, 1996), while IL-4 and IL-13 both increase epithelial permeability through phosphatidylinositol 3-kinase activation (Ceponis *et al.*, 2000). TNFα treatment alone increases Caco-2 epithelial permeability to mannitol fluxes by activating the NF-κB signaling pathway and down-regulating ZO-1 expression (Ma *et al.*, 2004). Interestingly, epithelia exposed to a mixture of TNFα and IFNγ display even greater barrier defects and altered ion transport (Schmitz *et al.*, 1999, Fish, Proujansky, and Reenstra, 1999), demonstrating the synergistic effects of cytokines in modulating epithelial function.

Through their non-mitogenic effects, growth factors also regulate epithelial function. Epidermal growth factor (EGF), transforming growth factor-α (TGFα), and insulin-like growth factor-1 (IGF-1) all reduce stimulated chloride secretion in colonic epithelial monolayers (Uribe *et al.*, 1996a, Beltinger, Hawkey, and Stack, 1996, Chang *et al.*, 2001). Both EGF and TGFα preserve Caco-2 monolayer barrier integrity by inhibiting oxidant-induced signaling pathways and F-actin destabilization (Banan *et al.*, 2000). Treatment with another cytokine/growth factor, transforming growth factor-β (TGFβ), not only increases colonic epithelial TER, but also antagonizes the barrier-disruptive effects of pro-inflammatory cytokines (e.g. TNFα, IFNγ) (Planchon *et al.*, 1994, McKay and Singh, 1997). TGFβ is one of the few cytokines known to increase colonic epithelial barrier function. However, the molecular mechanisms underlying the

effects of TGFβ on TER are unknown. Taken together, these data demonstrate the clear role cytokines play in the modulation of intestinal epithelial barrier and secretory functions.

Signal transduction pathways regulating epithelial ion transport and barrier function

As alluded to above, modulation of signaling pathways is involved in the regulation of epithelial function. Cytokines and growth factors in particular activate intracellular signaling cascades by binding to specific receptors on the epithelial cell surface. The pathways initiated may be unique to a cytokine and its receptor (e.g. IFNγ and STAT1: Signal Transducer and Activator of Transcription) or may be more widely employed pathways activated by a range of stimuli (e.g. mitogen activated protein kinases (MAPK), see below). Nevertheless, the general schema for these signaling cascades involves ligation of the cell surface receptor by the ligand, with subsequent phosphorylation events that culminate in either direct manipulation of an intracellular factor, or in the activation of transcription factors that regulate gene transcription. As already mentioned, elucidation of these pathways and their putative targets (e.g. ion channels, tight junction proteins) in the regulation of epithelial secretory and barrier function is currently the goal of several GI laboratories.

In addition to regulation by the second messengers cAMP and Ca^{2+}, Cl^- secretion in intestinal epithelia can also be regulated by MAPKs, phosphatidylinositol 3-kinase (PI 3-K), and protein kinase C (PKC) signaling pathways. For example, Ca^{2+}-driven chloride secretion in T84 colonic epithelia is negatively regulated by p38 MAPK (Keely and

Barrett, 2003) and extracellular-signal regulated kinase (ERK) MAPK (Keely, Uribe, and Barrett, 1998). Insulin, IGF-1, and EGF all inhibit Cl^- secretion in colonic epithelia via a PI 3-K-depdendent mechanism (Chang *et al.*, 2001, Uribe *et al.*, 1996b), while PKC has been implicated in increased Isc caused by Caco-2 cell exposure to thermostable direct haemolysin, a product of the diarrhea-inducing organism *Vibrio parahaemolyticus* (Takahashi *et al.*, 2000). In T84 epithelia stimulated with a pro-inflammatory phorbol ester, reduced Cl^- secretion is caused by PKC-mediated down-regulation of $Na^+/K^+/2Cl^-$ cotransporter expression and function (Farokhzad *et al.*, 1999). While certainly not the only example, the differential effect of PKC on epithelial ion secretion illustrates the diversity and complexity of outcomes that signaling cascades mediate in response to physiological mediators, indicating a need to understand ion secretion in several individual contexts.

Multiple signaling cascades also regulate intestinal epithelial barrier function. These include PKC, PI 3-K, myosin light chain kinase (MLCK), phospholipase C (PLC), and MAPK. Studies by Banan *et al.* (2003a, 2003b, 2004a, 2004b) have determined EGF-induced barrier protection against oxidant injury in Caco-2 cells is mediated by multiple PKC isoforms (i.e. θ, β-1, and ζ), as well as PLC-γ. In contrast, PKC activation by bacteria (e.g. EHEC) or bacterial toxins (e.g. zonula occludens toxin from *Vibrio cholera*) is responsible for mediating intestinal epithelial barrier disruption (Fasano *et al.*, 1995, Philpott *et al.*, 1998). In EHEC- and EPEC-infected T84 cells MLCK activation induces monolayer permeability (Philpott *et al.*, 1998, Yuhan *et al.*, 1997) by causing MLC_{20} phosphorylation and subsequent contraction of the perijunctional actin-myosin

ring. While not completely understood, it is hypothesized that tension from PAMR contraction would lead to disruption of the tight junction (Madara, Moore, and Carlson, 1987). Specific regulation of tight junction proteins was recently shown by Kinugasa *et al.* (2000) who determined that up-regulation of claudin-2 mRNA expression by IL-17 is prevented by a pharmacological inhibitor of ERK/MAPK. Through improved understanding of the various ways that signaling cascades affect barrier function, novel therapeutic targets may be revealed for treating diseases exacerbated by epithelial hyperpermeability.

TRANSFORMING GROWTH FACTOR BETA

Transforming growth factor-beta (TGFβ) is a growth factor/cytokine that was discovered in a mouse cell line transformed by a sarcoma virus (De Larco and Torado, 1978). The production of TGFβ was associated with its ability to induce a 'transformed' phenotype, characterized by the loss of density-dependent growth in a monolayer, overgrowth, changes in cellular morphology, and anchorage independence and was categorized at the same time as TGFα (Kahn and Shin, 1979, Cifone and Fidler, 1980). While similar in nomenclature, TGFβ and TGFα exist as a 25 kDa homodimer and 5.6 kDa single peptide chain respectively, sharing no sequence homology and functioning as distinct peptides (Roberts and Sporn, 1988). In addition to unique functional properties, these two mediators have separate receptors at the cell surface and different intracellular signaling pathways. As receptors for TGFβ exist on virtually every cell type including the basolateral surface of human colonic epithelial crypt tissue sections, and it has potent

biological effects, tight regulation of TGFβ activity is necessary (Planchon *et al.*, 1999). Indeed, TGFβ is secreted from cells as a pro-peptide, and requires proteolytic cleavage of the latency-associated peptide before TGFβ can become biologically active (Gleizes *et al.*, 1997). A variety of factors can activate TGFβ including plasmin, thrombospondin-1, integrins, and reactive oxygen species (Annes, Munger, and Rifkin, 2003).

There are three main isoforms of TGFβ ($TGF\beta_1$, $TGF\beta_2$, and $TGF\beta_3$), which exert similar physiological effects despite varied tissue distribution (Roberts and Sporn, 1988). Of the three isoforms, $TGF\beta_1$ is the most extensively studied, and this was the case for the thesis research discussed herein. In the context of the gut, TGFβ is produced by various immune cells, fibroblasts, and also intestinal epithelial cells (Stadnyk, 1994). While TGFβ is multifunctional, three general biological processes governed by TGFβ are: 1) regulation of cellular growth and proliferation, where it inhibits the growth and proliferation of lymphocytes and epithelia, while stimulating epithelial differentiation (Roberts and Sporn, 1988); 2) immunomodulation, where it is generally considered immunosuppressive (decreases T cell and tissue macrophage activity), but paradoxically, is highly chemotactic for neutrophils and monocytes (Letterio and Roberts, 1998); and 3) wound repair, which in the setting of epithelia, promotes restitution by inducing epithelial migration to the wound edge, and along these lines it can protect compromised barrier function during exposure to pro-inflammatory cytokines (Dignass and Podolsky, 1993, Planchon *et al.*, 1999, McKay and Singh, 1997,).

TGFβ-mediated Intracellular Signaling Pathways

There are two main receptors for TGFβ which are classified as type I or type II (Mehra and Wrana, 2002). Type I (TGFβRI) and type II (TGFβRII) receptors are transmembrane serine-threonine kinases with molecular weights of 55 kDa and 70 kDa respectively. TGFβRII is constitutively active, while TGFβRI requires a phosphorylation event before activation. The mechanism of TGFβ binding, receptor activation, and intracellular signal transduction pathways (Figure 1.4) are discussed below.

Research has shown that TGFβ uses the prototypic MAPK pathway, ERK1/2 MAPK, in addition to the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and p38 MAPK pathways (Hartsough and Mulder, 1997). Members of the TGFβ superfamily (e.g. $TGF\beta_{1, 2, 3}$, activins, bone morphogenic proteins etc.) also employ a unique group of intracellular signaling proteins called SMADs (coined from SMA signaling proteins in the nematode *C. elegans* and the homologous MAD proteins found in *Drosophila*). The SMAD pathway has both positive and negative feedback signals (Piek, Heldin, and Ten Dijke, 1999). Briefly, the TGFβ type II receptor binds exogenous dimeric TGFβ and autophosphorylates on its cytoplasmic domain using intrinsic kinase activity, subsequently phosphorylating the TGFβ type I receptor and inducing its serine-threonine kinase activity. Next, TGFβRII and TGFβRI form a heteromeric complex and through the assistance of a docking protein (SARA: SMAD Anchor for Receptor Activation), cytosolic SMAD3 is recruited, serine phosphorylated and released from the receptor where it subsequently binds to a SMAD common to SMAD-involved signaling, SMAD4. Together the SMAD3/SMAD4 complex translocates to the nucleus where it

binds directly to DNA to regulate gene transcription (Mehra and Wrana, 2002). As TGFβ is capable of auto-inducing its own production, there is an intrinsic negative-feedback mechanism designed to control prolonged TGFβ exposure. Activation of SMAD signaling induces the inhibitory SMAD7, which binds to the phosphorylated TGFβRI, thereby preventing association, phosphorylation, and activation of SMAD3 (Hayashi *et al.*, 1997).

Although the mechanisms are incompletely understood, there is a growing body of experimental evidence that cross-talk exists between TGFβ-induced MAPK and SMAD signaling cascades. For example, TGFβ-induced ERK1/2 and p38 MAPK signaling prevent SMAD-mediated transcriptional activation during TGFβ up-regulation of collagenase expression in rat osteoblastic cells (Selvamurugan *et al.*, 2004). Similarly, TGFβ-dependent responses in human mesangial cells involve cross-talk between ERK MAPK and SMAD signaling pathways (Hayashida, Decaestecker, and Schnaper, 2003). Furthermore, there is evidence for cross-talk between ERK MAPK, p38 MAPK, and SMAD signaling pathways after TGFβ-induced gene transcription in a chondrogenic cell line (Watanabe, de Caestecker, and Yamada, 2001). Interestingly, TGFβ-mediated SMAD3/4 signaling was shown to down-regulate *Haemophilus influenzae*-induced p38 MAPK activation and subsequent mucin transcription in human airways epithelia (Jono, 2003). This suggests that cross-talk between the SMAD and MAPK signaling pathways depend on the context in which the signals are generated. Thus, the outcome of cross-talk between TGFβ signaling pathways will depend on the cell line or tissue type examined

and presence of other biological mediators (e.g. bacteria) which may be initiating their own signaling cascades.

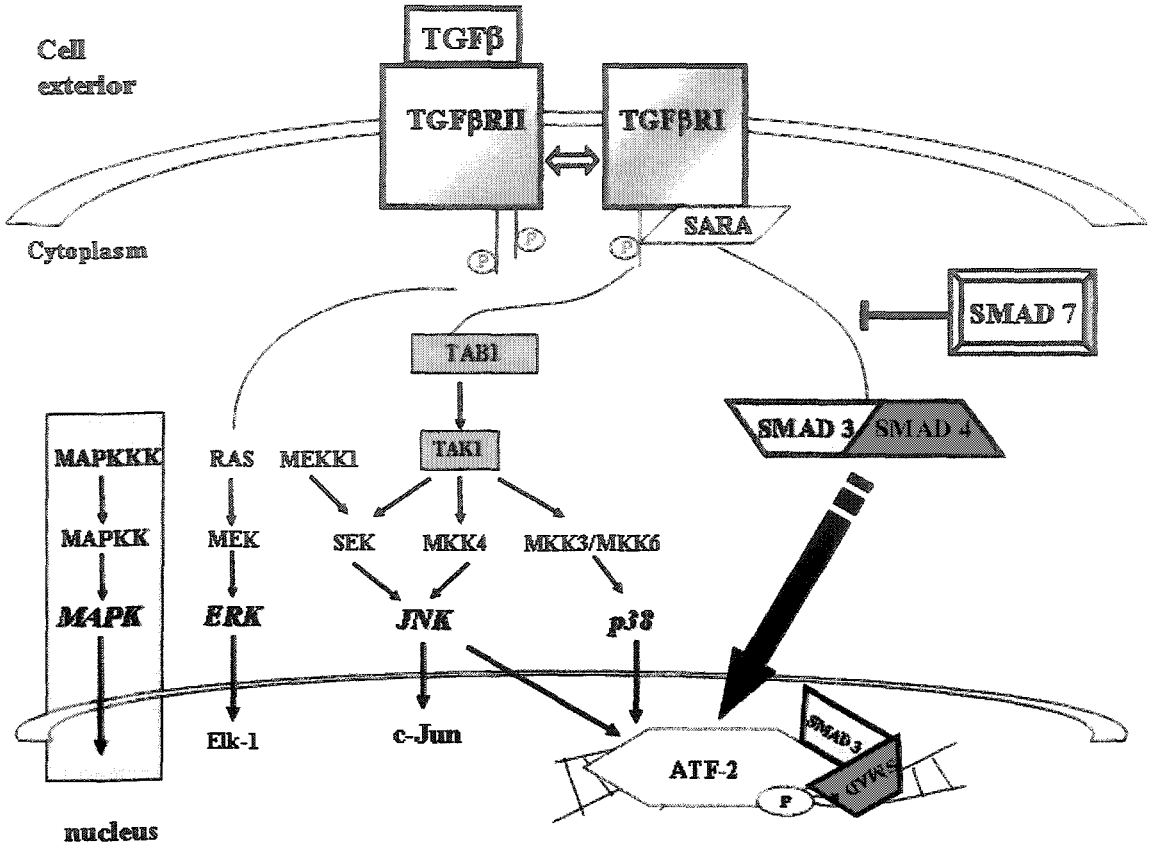

Figure 1.4. Generalized schema of pathways involved in TGFβ signaling. TGFβ binds to the type II receptor (TGFβRII) which autophosphorylates and phosphorylates the type I receptor (TGFβRI). Smad anchor for receptor activation (SARA) helps dock Smad3 which complexes with the common Smad4 and translocates to the nucleus where it can bind to DNA binding sites and initiate gene transcription. TGFβ also activates the ERK, JNK, and p38 MAPK pathways which leads to activation of transcription factors necessary for gene transcription such as Elk-1, c-Jun, and ATF2.

TGFβ and Intestinal Disease

Animal models investigating TGFβ have determined this cytokine is critical for controlling inflammatory responses since the $TGF\beta_1$ null mutation in mice leads to a multifocal inflammation resulting in early death (Kulkarni *et al.*, 1993). Likewise, TGFβ-mediated events appear to suppress and even abrogate experimental models of murine colitis (e.g. $CD45Rb^{low}$ $CD4^+$ T cell transfer and granulomatous colitis) (Powrie *et al.*, 1996, Neurath *et al.*, 1996). This has been further supported by the observation that increasing systemic levels of TGFβ can ameliorate experimentally induced colitis in rodents (Giladi *et al.*, 1995; Kitani *et al.* 2000), suggesting that targeted TGFβ delivery may offer new treatment options in patients with IBD.

In the context of TGFβ and human GI disease, studies have shown that tissue levels of TGFβ and its receptor are increased. In a study by diMola *et al.* (1999), it was determined that 72% of Crohn's disease tissue samples contained elevated levels of the cytokine and receptor. Research on both adolescent and adult IBD populations has shown increased TGFβ-positive cells in the lamina propria, including T-cells, neutrophils, monocytes, and macrophages (Xian *et al.* 1999, Babyatsky *et al.* 1996). Furthermore, in ulcerative colitis patients, elevated levels of $TGF\beta_1$ in mucosal tissue samples are correlated with milder disease (Ashwood *et al.*, 2004), while responders to anti-inflammatory therapy exhibit increased serum $TGF\beta_1$ (Sambuelli *et al.*, 2000). These observations are supported by studies implicating defective $TGF\beta_1$ signaling in the regulation of IBD. Montelone *et al.* (2001) found that overexpression of SMAD7 in IBD mucosal sections and purified T-cells correlated with decreased SMAD3 phosphorylation.

Notably, restoration of TGFβ signaling after treatment of these T-cells with SMAD7 antisense not only increased SMAD3 activation, but also enabled TGFβ-mediated inhibition of pro-inflammatory cytokine release such as IFNγ and TNFα in cells isolated from IBD tissue. It has been postulated that SMAD7 overexpression likely results from chronically inflamed tissue where elevated levels of IFNγ and TNFα may regulate SMAD7 gene transcription (Nakao, Okumura, and Ogawa, 2002). Indeed, IFNγ and TNFα have both been shown to antagonize TGFβ signaling by inducing SMAD7 expression (Ulloa, Doody, and Massague, 1999, Bitzer *et al.*, 2000). However, this hypothesis could be extended to suggest that in patients where elevated levels of TGFβ are associated with milder inflammation (and therefore likely to exhibit normal SMAD signaling), genetic factors may play a role in limiting the degree of inflammation in the early stages of disease pathogenesis. This would result in appropriate TGFβ production which then limits pro-inflammatory cytokine expression and prevents SMAD7 overexpression. *In vitro* evidence from our laboratory indicates $TGF\beta_1$ antagonizes IFNγ and TNFα signaling pathways (Reardon, Howe and McKay, unpublished observations), with the latter being supported by *in vivo* studies showing $TGF\beta_1$ is a negative regulator of NF-κB signaling in IBD tissue-derived fibroblasts (Monteleone *et al.*, 2004). Together, these data indicate expression of TGFβ and functional SMAD signaling may be important in limiting pro-inflammatory signal transduction events and IBD pathogenesis.

AIMS OF THESIS RESEARCH

Intestinal epithelial barrier and secretory functions are crucial for maintaining gut homeostasis and are affected by cytokines. Despite the wealth of data regarding direct cytokine effects on epithelial function, the mechanisms behind these effects have only recently begun to be elucidated (McKay and Baird, 1999). Previous studies have shown TGFβ is elevated in IBD and has epithelial barrier-enhancing as well as barrier-protective effects, pointing to a need to understand TGFβ-effects on the enterocyte. However, neither the mechanisms behind TGFβ regulation of intestinal epithelial barrier, nor the direct effects of this cytokine on intestinal chloride secretion are known. **Thus, the overall aim of this thesis was to determine the mechanism of TGFβ regulation of Cl^- secretion and barrier function in a model epithelium.**

In the first study, the aim was to examine the effects of acute and chronic TGFβ exposure on stimulated Cl^- secretion and transepithelial electrical resistance (TER) in T84 colonic epithelial monolayers. Ussing chambers were used to assess net active ion secretion (ΔIsc) following stimulation with cAMP and Ca^{2+} secretagogues, while TER was recorded using a voltmeter and chopstick electrodes. For the acute studies (≤ 72h cytokine exposure) the kinetics of recombinant TGFβ stimulation were characterized by assessing ΔIsc and TER after various times and doses of exposure. Chronic TGFβ exposure (6 days) was induced by adenoviral delivery of active TGFβ to epithelial monolayers, with ΔIsc and TER recorded six days post-infection. In addition to observing differential effects of TGFβ on epithelial barrier and ion transport function, the signaling pathway responsible for mediating TGFβ-induced alterations in epithelial

chloride secretion was examined using pharmacological inhibitors of several signaling cascades. This study not only defined the novel and direct effect of TGFβ on intestinal ion transport, but also delineated for the first time the signaling pathways induced by TGFβ in intestinal epithelia.

Cytokines can alter epithelial ion transport by regulating pump, ion channel or co-transporter protein expression (e.g. IFNγ). **Therefore, the next study aimed to determine the structural mechanism behind TGFβ-induced altered epithelial Cl^- secretion by examining TGFβ regulation of the main apical Cl^- channel, the CFTR.** Using colonic epithelial cell lines and a kidney epithelial cell line expressing stably-transfected CFTR tagged with green fluorescent protein, the effects of TGFβ on CFTR mRNA and protein expression, in addition to CFTR subcellular localization were assessed. The methodologies used for these studies included the Ussing chamber, confocal microscopy, biotinylation of epithelial membranes, and reverse transcriptase-polymerase chain reaction (RT-PCR). Here, it was determined TGFβ is an important regulator of epithelial chloride secretion in multiple epithelial cell types through differential regulation of the secretory apparatus.

Recent evidence has focused on the integral role of tight junction proteins, particularly claudins, in the regulation of epithelial barrier function. **Thus, the objective of the third and final group of experiments was to determine the mechanism behind TGFβ-induced barrier enhancement in colonic epithelial monolayers by testing the hypothesis that TGFβ increases TJ protein expression.** The delineation of TGFβ signaling pathways mediating barrier enhancement was also sought. Activation of

SMAD and MAPK signaling cascades, and TJ protein expression after TGFβ stimulation were assessed by immunoblotting techniques. **Furthermore, based on the barrier-protective effects of TGFβ in the face of pro-inflammatory cytokines, it was tested whether TGFβ could prevent the barrier defect caused by epithelial infection with the enteric pathogen, EHEC O157:H7.** Together, the results of this study have demonstrated claudin TJ proteins are critical in the maintenance of intestinal epithelial barrier function and revealed new mechanisms of EHEC-induced barrier dysfunction.

As TGFβ is important to normal GI homeostasis, these studies have begun to fulfill the need to provide basic information on how TGFβ affects epithelial function so that cytokine-epithelial interaction in health and disease may be better appreciated.

Chapter 2

TGFβ Effects on Epithelial Ion Transport and Barrier: Reduced Cl^- Secretion Blocked by a p38 MAPK Inhibitor.

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American Journal of Physiology (Cell Physiology) **2002 283(6):C1667-C1674**

ABSTRACT

Growth factors have been shown to affect a variety of epithelial functions. Here we examined the ability of transforming growth factor β (TGFβ) to modulate epithelial ion transport and permeability. Filter-grown monolayers of human colonic epithelia, T84 and HT-29 cells, were treated with TGFβ (0.1-100 ng/ml, 15 min-72 h), or infected with an adenoviral vector encoding TGFβ (Ad-TGFβ) for 144 h. Ion transport (i.e., short-circuit current, Isc) and transepithelial resistance (TER) were assessed in Ussing chambers. Neither recombinant TGFβ nor Ad-TGFβ infection affected baseline Isc, however exposure to ≥1 ng/ml TGFβ led to a significant (30-50%) reduction in the Isc responses to forskolin, vasoactive intestinal peptide and cholera toxin (agents that evoke chloride secretion via cAMP mobilization) and the cell permeant dibutyryl cAMP. Pharmacological analysis of signaling pathways revealed that the inhibition of cAMP-driven epithelial chloride secretion by TGFβ was blocked by pre-treatment with SB203580, a specific inhibitor of p38 MAP kinase, but not by inhibitors of JNK, ERK 1/2 MAP kinase, or phosphatidylinositol-3' kinase. TGFβ enhanced the barrier function of the treated monolayers by up to 3-fold as assessed by TER; however, this event was temporally displaced from the altered Isc response, being statistically significant only at 72 h post-treatment. Thus, in addition to TGFβ promotion of epithelial barrier function, we show that this growth factor also reduces responsiveness to cAMP-dependent

secretagogues in a chronic manner, and speculate that this serves as a braking mechanism to limit secretory enteropathies.

Keywords: short-circuit current, T84 epithelia, growth factor, transepithelial resistance

INTRODUCTION

A number of essential functions are provided by the epithelial lining of the gastrointestinal (GI) tract. In addition to nutrient digestion/absorption and immune surveillance roles, the gut epithelium also secretes electrolytes; it is this vectorial electrolyte secretion that establishes a driving force for directed water movement. Excess water in the lumen can lead to diarrhea (essentially a protective process), which if recurrent or prolonged, may result in dehydration and even death. Given the association of GI epithelia with the enteric nervous system, neighbouring enteroendocrine cells, and the luminal contents, it is not surprising that nervous and endocrine input, as well as bacteria and/or their products constitute the major regulators of epithelial function. Recent literature however, particularly from *in vitro* studies, has shown that immune mediators including cytokines can also modify epithelial function (24).

Growth factors are cytokines that have been shown to have both mitogenic and non-mitogenic effects. Specifically, in terms of ion transport, treatment of colonic epithelial cell lines with transforming growth factor-α (TGFα) or epidermal growth factor (EGF) resulted in decreased secretory responses to stimulants of chloride secretion (6, 39). Transforming growth factor-β (TGFβ) is a multifunctional cytokine, the bioactivity of which can be grouped into three main properties: 1) regulation of cell growth and proliferation, 2) immunomodulation, and 3) stimulation of wound repair (epithelial restitution). Studies have shown that TGFβ is upregulated in many diseases, including inflammatory bowel disease (IBD) (2), an enteropathy often characterized by perturbed water movement. Given that altered epithelial electrolyte transport can lead to aberrant

water balance in the gut, that other growth factors have been shown to affect this process, and that TGFβ is upregulated during enteric disease, the primary aim of this study was to determine the effect of TGFβ on epithelial ion transport function.

Using monolayers of the human T84 or HT-29 colonic epithelial cell lines as model epithelia, the data herein illustrate that exposure to TGFβ leads to a ~30% decrease in epithelial secretory responses to stimuli that initiate cAMP-dependent chloride secretion. This disruption of normal electrolyte transport events is temporally distinct from the ability of TGFβ to increase epithelial barrier function and can be restored by treatment with SB203580, an inhibitor of the p38 mitogen activated protein kinase (MAPK) signaling pathway.

MATERIALS AND METHODS

Cell culture

The human colonic adenocarcinoma-derived T84 epithelial cell line was maintained in media containing equal volumes of Dulbecco Modified Eagle medium (DMEM) and Ham's F-12 medium, supplemented with 10% v/v fetal calf serum, 1.5% v/v HEPES and 2% v/v penicillin-streptomycin (all from Life Technologies, Grand Island, NY) at 37°C, 5% CO_2. The HT-29cl.19A cell line (HT-29; was a kind gift from Dr. J.A. Groot, University of Amsterdam) was maintained in DMEM supplemented with 5% v/v fetal calf serum, 0.1% v/v L-glutamine, 2% v/v penicillin-streptomycin, and 5% v/v sodium bicarbonate. Cells were seeded onto semipermeable filter supports (0.4 μM pore size, Costar Inc., Cambridge, MA) having a surface area of 1 cm^2 (10^6 cells) for physiological assessment and grown to confluence as determined by transepithelial resistance (minimum 6 days growth).

Physiological assessment studies

Recombinant human TGFβ (R&D Systems, Minneapolis, MN) was added to the basolateral compartment of the semipermeable filter supports at concentrations of 0.1, 1, 10 or 100 ng/ml. The basolateral surface of T84 cells was exposed to TGFβ for 4, 8, 16, 24, and 72 h, at which point cells were mounted in specialized Ussing chambers (Precision Instrument Design, Tahoe City, CA) under voltage-clamped conditions as previously described (25) and short circuit current (Isc) responses to known stimuli of Cl^-

secretion were measured. In other studies T84 monolayers were exposed to TGFβ for 15 min, then rinsed (x2) in fresh media, and Isc responses examined 16 h later. Comparative studies examined Isc responses to epidermal growth factor (EGF, 10 or 100 ng/ml; R&D Systems) following 16 h of exposure. Acute experiments exposed T84 cells to TGFβ for 30 min (1 or 10 ng/ml) while mounted in the Ussing chambers, followed by measurement of Isc responses. In all experiments, baseline Isc was obtained following 10 min of equilibration. Stimulated chloride secretion was induced by forskolin (10^{-5} M), vasoactive intestinal peptide (VIP, 10^{-7} M), cholera toxin (10 μg/ml), dibutyryl cAMP (200 μM) (all from Sigma Chemical, St. Louis, MO) and the maximal change in Isc recorded. These secretagogues were chosen because of their known ability to elicit chloride secretion via raising intracellular cAMP (13, 23, 44). Transepithelial resistance (TER, Ω/cm^2) of the T84 monolayers was recorded with chopstick electrodes and a voltmeter (Millipore Corporation, Bedford, MA) as a measure of paracellular permeability.

Adenoviral infection

Twenty-four hours after seeding (10^6 cells/ml on semipermeable filter supports), T84 cells were infected with replication-deficient adenovirus contructs encoding active TGFβ (Ad-TGFβ (35)) at a multiplicity of infection (moi) of 10, 20 or 50 virus particles per cell. Sixteen hours later, T84 cells were rinsed twice with media to remove any residual virus and cultured for 6 days, whereupon they were mounted in Ussing chambers

for analysis of secretory responsiveness to forskolin. Changes in barrier function were monitored daily throughout the 6-day post-infection period by recording transepithelial resistance.

Epithelia infected with either: 1) the deletion variant (empty vector) of the replication-deficient adenovirus (Ad-delete (16)) or 2), adenovirus encoding latent TGFβ (Ad-latent TGFβ (47)), were used as additional controls for these experiments. Infectivity of the adenovirus was verified in T84 cells using virus expressing the marker genes, β-galactosidase or luciferase (27). Finally, synthesis of the active form of TGFβ from Ad-TGFβ-infected cells and non-infected control epithelia was determined by the presence of TGFβ in the media at 24 h intervals post-infection (24-120 h) using ELISA (R&D Systems). Upon completion of the experiments, epithelial cells were trypsinized off the filter supports and viability assessed using the trypan blue exclusion technique.

Pharmacological Inhibition of Intracellular Signaling

Physiological assessment experiments were conducted using a single dose (10 ng/ml) and time (16 h) of TGFβ exposure to T84 monolayers. T84 cells were pretreated with inhibitors of: 1) p38 MAPK (1 h, 10 μM, SB203580; Calbiochem, CA (18, 20, 33)); 2) c-Jun NH_2-terminus kinase (JNK) (30 min, 10 μM, SP600125; Calbiochem, CA(17)); 3) extracellular regulated kinase 1/2 (ERK 1/2) signaling, via inhibition of MEK, the enzyme upstream of ERK 1/2 (1 h, 25 μM, PD98059; Calbiochem, CA (4, 8)); or 4) phosphatidylinositol 3' kinase (PI 3-K) (15 min, 20 μM, LY294002; Sigma Chemical Co.

(42)). TGFβ was subsequently added to the basal compartment of the culture well (inhibitor not washed out) and after a defined incubation time the monolayers were mounted in Ussing chambers and Isc responses to forskolin were recorded. Pharmacological inhibition of the p38 MAPK signaling cascade in HT-29 cells was accomplished by pretreating monolayers with SB203580 for 30 minutes (0.1-50 μM), subsequently followed by TGFβ application (100 ng/ml, 24 h).

Statistical and data analysis

Data are normalized to time-matched controls (i.e., % of control response) and are presented as means ± SE. Data were analyzed using one-way ANOVA and $P < 0.05$ was accepted as the level of statistical significance.

RESULTS

TGFβ reduces Isc responses to cAMP secretagogues

Treatment with TGFβ (0.1-100 ng/ml, 4-72 h) did not significantly affect baseline Isc compared to naïve time-matched controls (0.17 ± 0.17 vs. 0.33 ± 0.33 $\mu A/cm^2$ for control and TGFβ-treated (10 ng/ml, 16 h), respectively; n=6 monolayers from a representative experiment). Addition of TGFβ to the Ussing chamber for 30 min (0.1, 1, 10 ng/ml) had no effect on epithelial baseline Isc or ΔIsc evoked by forskolin (data not shown). Treatment with TGFβ for 16 h did, however, cause a statistically significant decrease in Isc responses to forskolin; this was evident with ≥1 ng/ml (data not shown). Figure 1 shows that T84 cells exposed to TGFβ (10 ng/ml) for 16 or 24 h demonstrated a statistically significant decrease in forskolin-induced Isc responses of ~30% compared to controls ($P < 0.0001$). This reduced responsiveness to forskolin was further enhanced by 72 h post-TGFβ treatment, being on average only 50% of the magnitude of the response observed in time-matched control monolayers ($P < 0.05$ compared to 16 and 24 h TGFβ exposure). Following 72 h of TGFβ treatment there was a significant increase in transepithelial resistance (1405 ± 254 Ω/cm^2 compared to control at 846 ± 204 Ω/cm^2; $P < 0.05$, n = 9-12 monolayers) that was not observed with shorter TGFβ exposure periods. In "washout" experiments, cells exposed to TGFβ (10 ng/ml) for 16 h and subsequently rinsed free of TGFβ also displayed a similar decrease in Isc responses to forskolin at 72 h post-treatment (40.6 ± 1.7% of control response, $P < 0.0001$, n = 3 monolayers), comparable to those seen after 72 h of persistent TGFβ exposure (49.3 ± 7.5% of control

responses, $P < .0001$, n = 9 monolayers). This washout treatment also resulted in increased TER (1598 ± 48 Ω/cm^2 vs. controls at 847 ± 20 Ω/cm^2; $P < 0.001$, n = 3). T84 cells exposed to TGFβ for 16 h at the highest dose used (i.e., 100 ng/ml) displayed a ~30% decrease in forskolin-induced increases in Isc, which was not statistically different from the inhibition of Isc observed with lower doses of cytokine (i.e., 10 ng/ml). Furthermore, additional washout experiments revealed that exposure to TGFβ for 15 min was sufficient to induce diminished responsiveness to forskolin upon examination 16 h later (untreated controls 37.3 ± 4 vs. TGFβ-treated cells 17 ± 2.1 μA/cm^2; $P < 0.001$, n = 6-7 monolayers): in this case a 55% reduction in ΔIsc.

TGFβ treatment (10 ng/ml, 16 h) also resulted in a significant diminution of epithelial responsiveness to cAMP-driven chloride secretion initiated by VIP and cholera toxin (Fig. 2). Similarly, cells exposed to TGFβ displayed reduced ΔIsc responses to the cell permeant cAMP analogue, dibutyryl cAMP (Fig. 2). In contrast to the effects of TGFβ on Isc responses, T84 monolayers treated with EGF for 16 h (10 or 100 ng/ml) displayed Isc responses to forskolin that were not different from controls (Table 1).

In additional experiments, HT-29 epithelial cells treated with TGFβ (100 ng/ml, 72 h) displayed reduced Isc responses to forskolin (control 118.7 ± 23.7 vs. TGFβ-treated 64.3 ± 12.3 μA/cm^2; $P < 0.05$, n = 3 monolayers). Following 72 h of exposure, TGFβ-treated HT-29 monolayers also exhibited a small, but significant, increase in TER compared to controls (control 287.7 ± 6.8 vs. TGFβ 302.0 ± 3.1 Ω/cm^2; $P < 0.05$, n = 3 monolayers).

Exposure to $TGF\beta_1$ delivered via adenoviral gene transfer causes decreased forskolin responsiveness

We sought to determine what effect chronic exposure to TGFβ would have on epithelial function using adenoviral gene transfer of the biologically active form of TGFβ. Cell infectivity was verified by use of adenoviral vectors encoding marker genes (Fig. 3A). Cells infected with the highest dose of virus (50 moi) displayed no significant decrease in viability, as determined by the trypan blue exclusion technique (data not shown), and exhibited increased TGFβ production compared to uninfected controls (e.g. 912.2 ± 202.4 pg/ml TGFβ in supernatants collected 72 h after infection with Ad-TGFβ vs. 217.9 ± 8.5 pg/ml in supernatants of naïve controls, $P < 0.05$, n = 3). Assessment of Isc responses to forskolin 6 days post-Ad-TGFβ infection revealed a statistically significant decrease in secretory responsiveness compared to naïve controls and T84 cells infected with Ad-delete or Ad-latent TGFβ (Fig. 3B). In addition, Ad-TGFβ infection resulted in a statistically significant increase in TER by the end of the 6-day (144 h) post-infection period (Fig. 3C), which was first apparent 72 h post-infection (Fig. 3D). Similar to the experiments with recombinant TGFβ, HT-29 cells infected with Ad-TGFβ (50 moi) displayed reduced Isc responses to forskolin 6 days post-infection (66 ± 18.7 compared to controls at 118.7 ± 23.7 $\mu A/cm^2$; $P < 0.05$, n = 3).

Inhibition of p38 MAPK, but not JNK, ERK 1/2 MAPK, or PI 3-kinase, reduced the TGFβ effect on cAMP-mediated Isc

Pretreatment of HT-29 cells with SB203580 (≥1 μM), a potent inhibitor of p38 MAPK activity, 30 min before TGFβ application completely restored the Isc responses to forskolin to control values (Fig. 4), while similarly treated T84 monolayers resulted in a significant, but only partial, improvement in Isc responses to forskolin (Table 2). Other signaling pathways in T84 epithelial cells did not appear to contribute to the diminished Isc, since pretreatment with the JNK inhibitor (SP600125), ERK1/2 pathway inhibitor (PD98059) and the PI 3-kinase inhibitor (LY294002) did not inhibit the observed effect of TGFβ on ion transport: in fact, exposure to PD98059 or LY294002 alone caused a significant decrease in Isc responses to forskolin ~16 h later (Table 2).

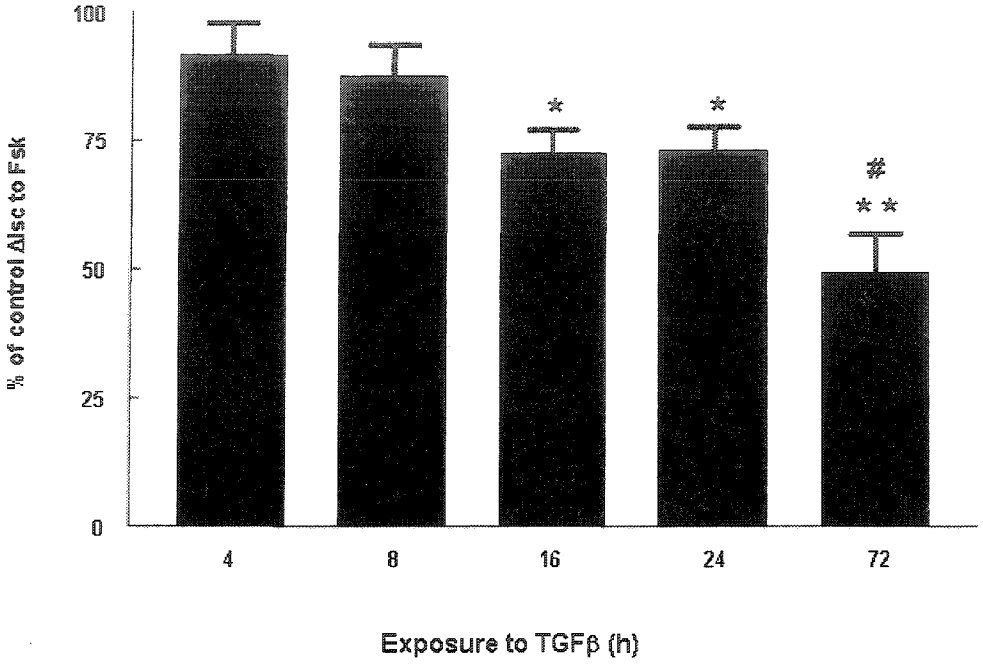

Figure 2.1. Bar chart showing the time-dependent decrease in T84 epithelial ΔIsc responses to forskolin (Fsk, 10^{-5} M) following exposure to TGFβ (10 ng/ml). Values are given as a percent of control monolayer responses (*$P < 0.05$ and **$P < 0.001$ compared to control; $^{\#}P < 0.05$ compared to 16- and 24-h TFGβ exposure; n = 6-10 monolayers; mean ± SE). Control ΔIsc responses to forskolin ranged from 40-80 μA/cm^2.

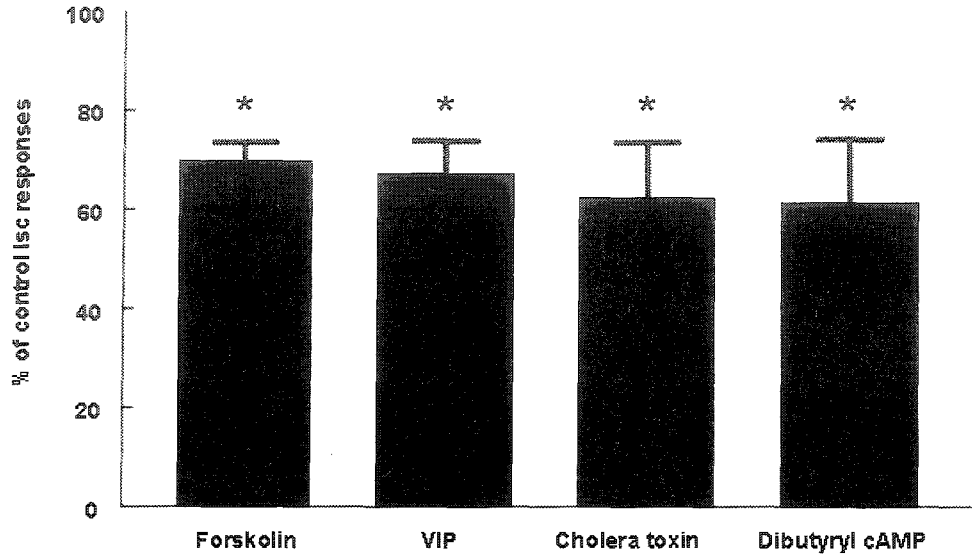

Figure 2.2. Bar chart showing reduced epithelial responses to cAMP secretagogues (Forskolin 10^{-5} M, VIP 10^{-7} M, Cholera toxin 10 μg/ml) and a cell-permeant cAMP analogue (dibutyryl cAMP, 200 μM) in T84 monolayers exposed to TGFβ (10 ng/ml, 16 h). Values are given as a percent of control monolayer responses (*P < 0.05 compared to control; n = 4-12 monolayers; mean ± SE).

TABLE 2.1: EGF-treated (16h) T84 cells do not display reduced Isc responses to forskolin

Control	EGF (10 ng/ml)	EGF (100 ng/ml)
69 ± 8	86 ± 12.7	71.8 ± 15.2
n=8	n=7	n=4

Values are means ± SE (in $\mu A/cm^2$). Isc, short-circuit current; *n*, no. of monolayers.

Figure 2.3 (following pages). *A*: T84 monolayers infected with luciferase-encoding adenovirus (Ad-luciferase) for 72 h at 10, 20 or 50 multiplicity of infection (moi) display increased production of luciferase [30-50% of T84 cells showed positive β-galactosidase staining after 72 h of infection with β-galactosidase encoding adenovirus (20 moi), data not shown]. *B* and *C*: decreased epithelial Isc responses to forskolin (Fsk, 10^{-5} M; *B*) and the increase in transepithelial resistance (TER; *C*) following adenoviral infection with active TGFβ (Ad-TGFβ; 10, 20, or 50 moi, 144 h), but not inactive TGFβ (Ad-latent TGFβ) or a deletion variant (Ad-delete) (both 50 moi). Data are percentages of control monolayers (*$P < 0.05$ compared with untreated controls; n = 6-12) and are expressed as means ± SE. *D*: line graph from a representative experiment (n = 3 monolayers) depicting the change in transepithelial resistance (TER) over the 6-day post-infection (50 moi) period as a percentage of uninfected control TER values (controls 500–2,000 Ω/cm^2; n = 3-5 experiments; *$P < 0.05$ compared with controls), expressed as means ± SE.

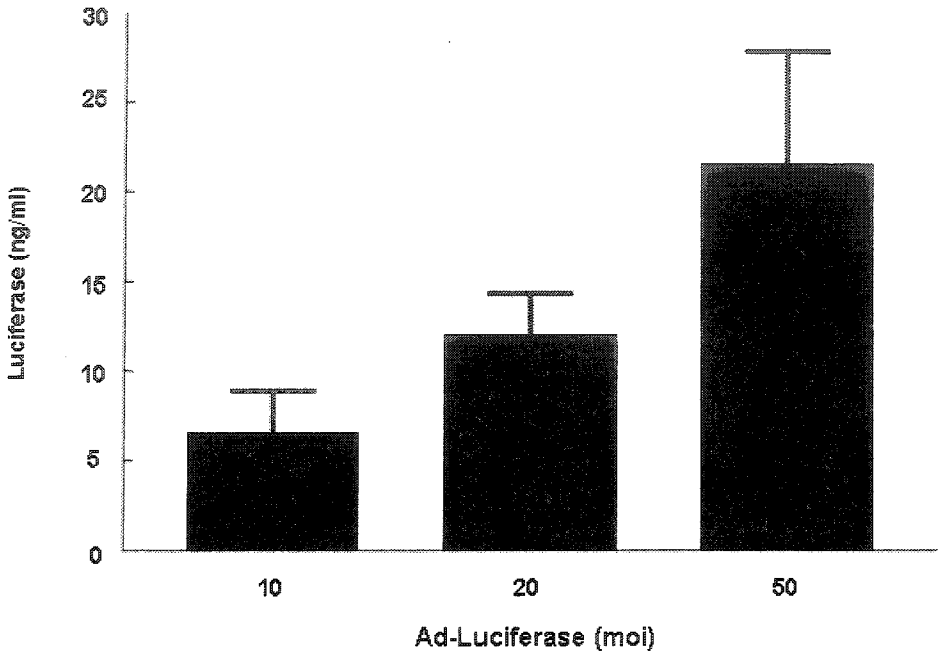

Figure 2.3a

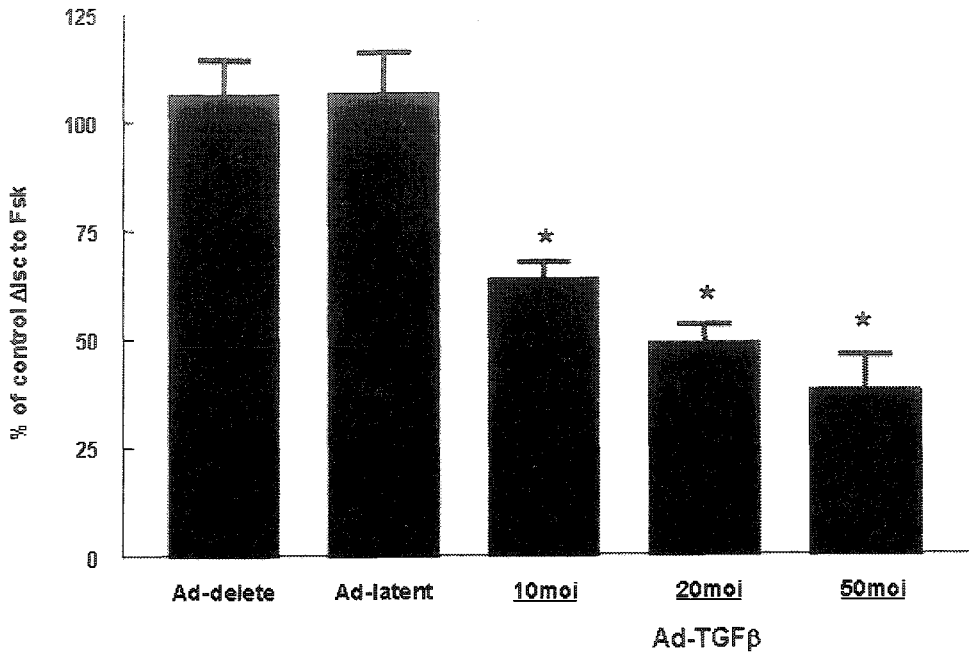
125
100
75
50
25
0
% of control Δlsc to Fsk
*
*
*
Ad-delete
Ad-latent
10moi
20moi
50moi
Ad-TGFβ

Figure 2.3b

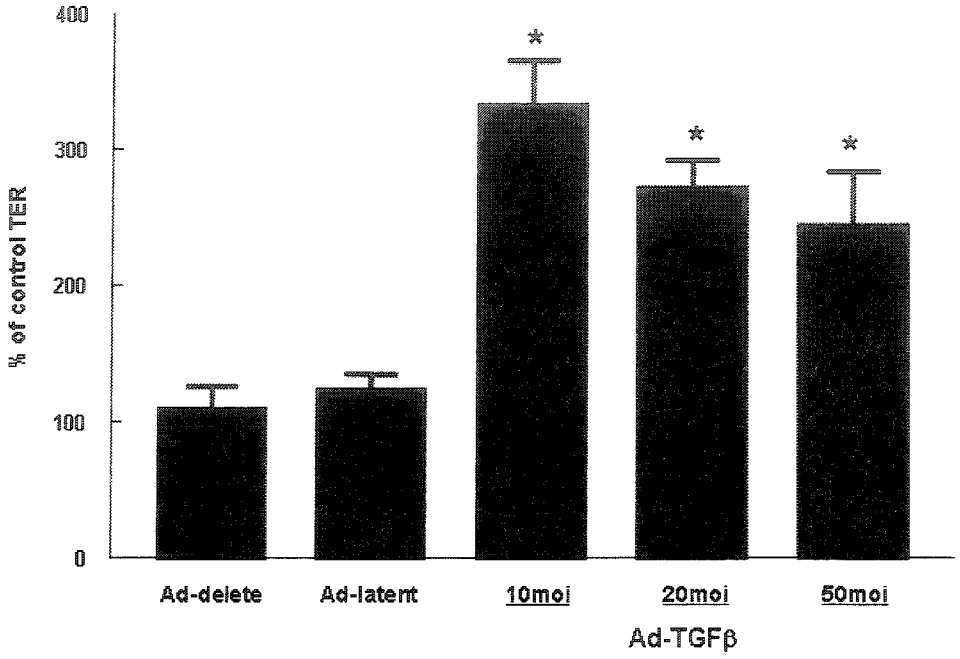
400
300
200
100
0
% of control TER
*
*
*
Ad-delete
Ad-latent
10moi
20moi
50moi
Ad-TGFβ

Figure 2.3c

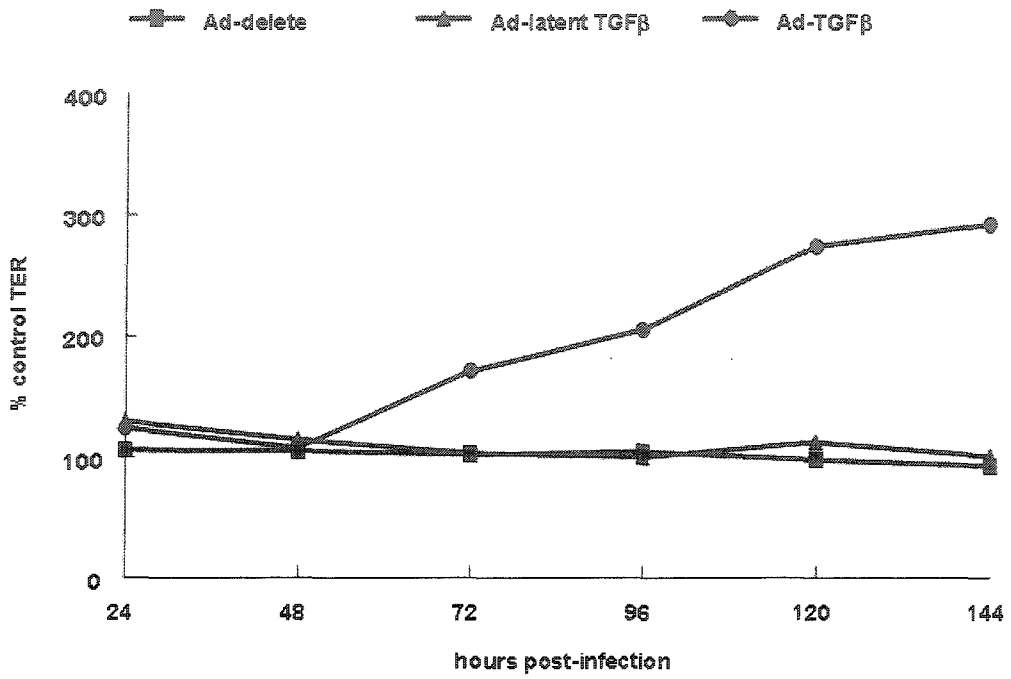
Ad-delete
Ad-latent TGFβ
Ad-TGFβ
400
300
200
100
0
% control TER
24
48
72
96
120
144
hours post-infection

Figure 2.3d

TABLE 2.2: Pharmacological inhibition of p38, but not JNK, ERK 1/2 or PI 3-kinase reduces TGFβ inhibition of ΔIsc to forskolin in T84 epithelia.

Treatment	% control ΔIsc to forskolin
TGFβ (10 ng/ml, 16 h)	64 ± 3.3*
p38*i* (SB203580; 10 μM)	97 ± 5.3
p38*i* + TGFβ	81.6 ± 2.7#
JNK*i* (SP600125; 10 μM)	93.8 ± 8.7
JNK*i* + TGFβ	61.3 ± 5.0*
ERK 1/2*i* (PD98059; 25 μM)	61.5 ± 1.5*
ERK 1/2*i* + TGFβ	52 ± 1.9*
PI 3-K*i* (LY294002; 20 μM)	61.3 ± 5.3*
PI 3-K*i* + TGFβ	57.6 ± 2.3*

Values are means ± SE; n = 3-9 monolayers. PI 3-K, phosphatidyl 3' kinase; *i*, inhibitor. *$P < 0.001$ compared with control (i.e., 100%); #$P < 0.05$ compared with control and TGFβ-treated cells.

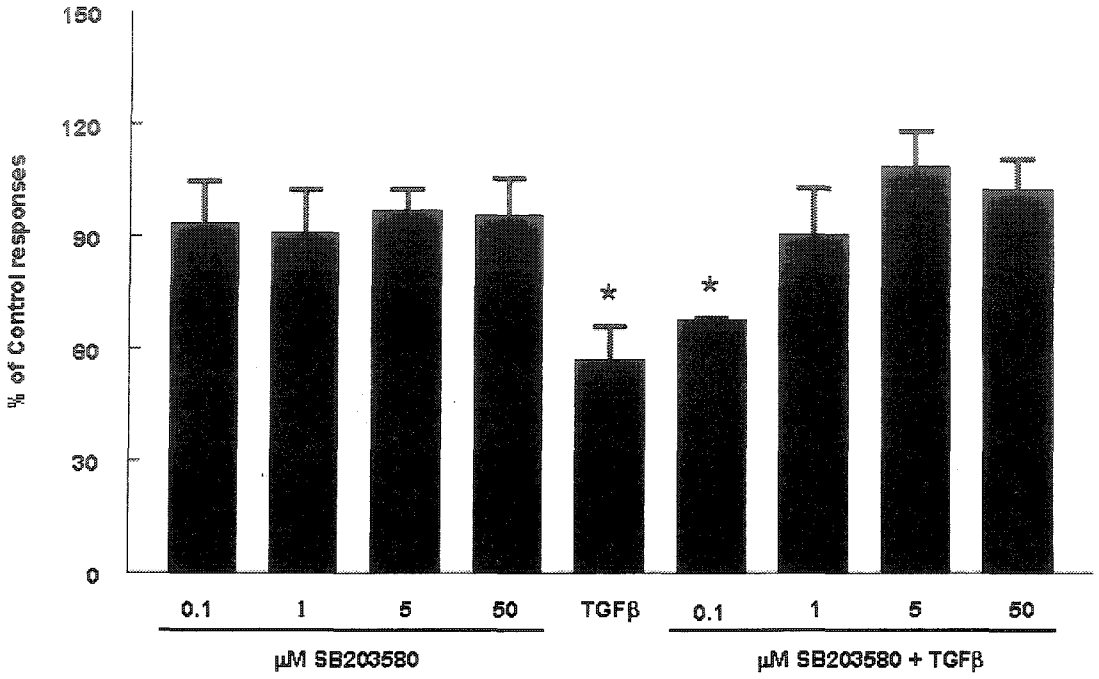

Figure 2.4. Bar chart depicting a dose-response to the recovery of TGFβ-induced (100 ng/ml, 24 h) diminished Isc responses to Fsk (10^{-5} M) in HT-29 epithelia pretreated with the p38 MAP kinase inhibitor SB203580. Values are percentages of control responses to Fsk (*$P < 0.05$ compared with naïve controls; n = 8-9 monolayers) and are expressed as means ± SE.

DISCUSSION

Transforming growth factor β is a multifunctional peptide that affects cell proliferation and differentiation, and has immunosuppressive properties. Using human enteric epithelial cell lines, this study shows: 1) exposure to TGFβ significantly decreases cAMP-driven Cl^- secretion; 2) that this effect can be blocked by an inhibitor of p38 MAPK but not pharmacological inhibitors of JNK, ERK 1/2 MAPK, or PI 3-K activity; and 3) the increased barrier function caused by TGFβ is temporally distinct (i.e., delayed) from the altered ion transport characteristics of the treated epithelium.

Ion transport is an important element of gut homeostasis. As the driving force for water movement it facilitates surface hydration, which if dysregulated, can result in debilitating diarrhea or constipation. TGFβ had no effect on tonic epithelial ion transport (i.e., baseline Isc) but significantly reduced secretory responses to 3 cAMP-dependent secretagogues that operate via different mechanisms. This effect likely lies downstream of cAMP generation since TGFβ-treated cells stimulated with dibutyryl cAMP displayed similarly reduced Isc events. The responses to forskolin, VIP, cholera toxin and dibutyryl cAMP were all reduced by a similar magnitude (i.e., ~30%), suggesting that while TGFβ can dampen cAMP-driven Cl^- secretion, the pathophysiology that would be associated with total blockade of Cl^- secretion is avoided. The inhibition of secretory responsiveness required a minimum of 1 ng/ml TGFβ and was statistically significant 16 h post-treatment; however, constant exposure to TGFβ was not essential as a 15 min exposure resulted in reduced ΔI_{sc} to forskolin 16 h later. Higher doses of TGFβ did not affect the

time required to observe a reduced ΔIsc to forskolin, neither did they affect the magnitude of the response. This lack of a dose-response has been noted for TGFα inhibition of bradykinin-induced ΔIsc in HCA-7 colonocytes, although in this instance the Isc responses were reduced by 6 h post-treatment (6).

The effects of recombinant TGFβ were reproduced in epithelia infected with adenovirus encoding the gene for the active form of TGFβ; diminished ΔIsc to forskolin were apparent 144 h post-infection (i.e., end of the experiment). However, unlike other growth factors, such as EGF and IGF, which induce acute effects on epithelial ΔIsc (39, 11), addition of TGFβ to T84 monolayers in Ussing chambers for 30 min had no effect on subsequent forskolin-evoked ΔIsc. Thus, TGFβ can be added to the list of cytokines that directly modulate epithelial ion transport (reviewed in (24)). In addition, the effect of TGFβ is set apart from those of other growth factors that rapidly affect ion transport; rather, the TGFβ effect is reminiscent of IFNγ or IL-4 diminution of epithelial secretory responsiveness which typically requires 24 h or longer to become apparent (1, 10).

Upon identifying that TGFβ exerts an effect on epithelial ion transport, a number of mechanistic issues arose. One option was to investigate the physiological changes mediated by TGFβ, such as the possible change in amount, location, or activity of the apical chloride channel, CFTR. Indeed, growth factor regulation of specific ion channels (3, 34) and cytokine modulation of CFTR gene expression has been reported (7, 9, 30). Alternatively, the TGFβ signaling pathway leading to the decreased secretory response could be addressed. Data from many cell types shows that TGFβ binding to its surface

receptor causes mobilization of classical MAP kinases, and a unique series of molecules designated SMADs, and that these signaling cascades regulate specific aspects of the biological effects of TGFβ (29, 46, 41, 37). Little is known, however, of the TGFβ signaling pathways in epithelia in general (36), with a distinct lack of data relevant to enteric epithelial cells.

Using a pharmacological approach, we found that the TGFβ effects on ion transport were reduced in two cell lines by pretreatment with the established inhibitor of the p38 MAPK pathway, SB203580. This is the first time p38 MAPK has been implicated in governing a TGFβ function in gut epithelia and these findings are complementary to a recent report showing that hyperosmolar stress-induced reduction in colonic CFTR mRNA was blocked by inhibition of p38 MAPK activity (5). While low-dose SB203580 (i.e., 1 μM) completely restored the TGFβ-induced diminished ion transport events in HT-29 cells, higher doses of the pharmacological inhibitor only partially corrected the defect in T84 epithelia. This indicates cell line-specific differences and in the case of T84 cells suggests either reduced sensitivity to SB203580, or that other signaling pathways participate in the TGFβ effect. Thus, we adopted the same pharmacological approach to assess the putative involvement of other signaling molecules in TGFβ modulation of ion transport. c-Jun NH_2-terminal kinase (JNK) is likely activated in tandem with p38 MAPK in response to TGFβ (22, 43) and can affect the $Na^+/K^+/2Cl^-$ transporter (19). Interference with the activity of this transporter would impact on apical Cl^- secretion events. Furthermore, high dose SB203580 (i.e., 10-20 μM) may inhibit JNK activity (45), yet use of a reputedly specific inhibitor of JNK activity did

not ameliorate the TGFβ-induced diminution of T84 ΔIsc to forskolin. ERK, the third member of the MAPK family, is mobilized in a variety of cell types by TGFβ (14, 15, 28) - pharmacological inhibition of the enzyme upstream of ERK, MEK, did not affect the decreased ΔIsc induced by TGFβ. Lastly, use of an established inhibitor of PI 3-K activity excluded the involvement of this ubiquitous signaling molecule in the TGFβ-induced perturbation of cAMP-driven Cl^- secretion. The latter observation contrasts with the diminished carbachol-induced increases in Isc in T84 cells caused by EGF that are PI 3-K sensitive (40). In this context, we observed that TGFβ did not consistently alter carbachol-elicited increases in Isc in T84 or HT-29 epithelia (personal observation).

Exposure to the MEK and PI 3-K inhibitors alone reduced T84 ΔIsc to forskolin (Table 2), suggesting that longer inhibition of ubiquitous signaling pathways may affect multiple energy-dependent processes such as epithelial ion transport. The inhibition of forskolin-induced ΔIsc by LY294002 appears to contradict the work of Dickson *et al.* (12). The discrepancy may be due to the fact that in the latter study the epithelium was exposed to LY294002 in Ussing chambers for ≤30 minutes and Isc responses immediately assessed. Indeed, when these investigators inhibited PI 3-K via wortmannin for 24 h they observed a ~50% decrease in forskolin-stimulated Isc, which is compatible with the data in the current study.

Agent specificity must be considered in any pharmacological study. Although accepted as a specific p38 MAPK inhibitor, SB203580 has recently been shown to block ALK5 activity, a kinase that phosphorylates SMAD3 (21). Thus, involvement of SMAD3 in TGFβ inhibition of ΔIsc to forskolin is a possibility. We have been unable to

convincingly and consistently show increased amounts of phosphorylated p38 MAPK in nuclear or whole-cell extracts of serum-starved TGFβ-treated epithelial cells, or elevated p38 MAPK activity (data not shown). This may be due to: 1) the relatively high constitutive phospho-p38 MAPK found in control extracts; 2) the possibility that there may in fact be only very subtle increases in activated p38 MAPK, since TGFβ causes only a partial ablation of the Isc response; or 3) the TGFβ effect on ion transport is via a specific p38 MAPK isoform (i.e. α, β, γ, δ), the increases of which are masked by constitutive expression of the other isoforms. Indeed, in light of the data from Laping *et al.* (21) a finding of increased phospho-p38 MAPK in TGFβ-treated epithelia would be suggestive of, but not unequivocal proof of, involvement of this enzyme in the altered ion transport. Thus, while we have ruled out JNK, ERK and PI 3-K, definitive statements on the intracellular signals that mediate the TGFβ effect on enteric epithelial ion transport require the development of p38 MAPK and SMAD3 knock-out (and preferably inducible knock-out) cell lines suitable for the analysis of vectorial ion transport.

Recombinant or adenovirally delivered TGFβ was shown to increase TER, an accepted index of the paracellular permeability pathway. This finding is in accordance with studies where TGFβ was shown to increase epithelial barrier function and/or preserve barrier integrity compromised by inflammatory cytokines (26, 31, 32). Intriguingly, we found that the effects of TGFβ on barrier function were temporally distinct from those on ion transport – TER was, unlike Isc, unaltered by 16-24 h post-TGFβ treatment. This divergence in the timing of TGFβ effects on two of the primary roles of the enteric epithelium implies utilization of different or additional signaling

pathways in the modulation of epithelial barrier and ion transport. Research directed towards understanding the structural basis for the TGFβ-induced increased TER, the signaling events underlying this process, and defining the mechanism(s) responsible for the temporal separation of enhancement of the epithelial barrier and diminished cAMP-driven Cl^- secretion are required. As noted, TGFβ can antagonize IFNγ-mediated disruption of the barrier function of T84 monolayers (32). Since IFNγ can reduce TER within 24 h of exposure and TGFβ-induced increases in TER are not apparent at this time (i.e., 24h), it suggests that the TGFβ inhibition of IFNγ-induced decreases in TER occurs via a distinct mechanism – that is, inhibition of the IFNγ signaling cascade. The reciprocal event (i.e., IFNγ interference of TGFβ SMAD signaling) has been shown (38).

In conclusion, irrespective of the intracellular signaling mechanism, exposure of model gut epithelia to TGFβ led to not only the expected increase in TER (i.e., at 72 h post-treatment), but also resulted in diminished responsiveness to cAMP-dependent secretagogues 16 h post-treatment. Prolonged exposure to TGFβ (using adenoviral vectors encoding the gene for the active form of TGFβ) maintained the Cl^- secretion abnormality. The temporal separation of TGFβ modulation of epithelial ion transport and barrier functions adds to our appreciation of the complexity and spectrum of biological activities coordinated by this pleiotropic cytokine. Finally, we speculate that in addition to its ability to maintain or enhance epithelial barrier function, TGFβ may reduce pathophysiological complications resulting from excess water movement into the gut lumen by limiting cAMP-driven Cl^- secretion.

ACKNOWLEDGEMENTS

KH has been a recipient of an Ontario Graduate Scholarship in Science and Technology (2000) and a Natural Sciences and Engineering Research Council studentship (2001-2003). DMM is a Canadian Institutes of Health Research (CIHR) scholar (1998-2003). This work was funded by a CIHR grant (MT-13421) to DMM. We thank Jun Lu and Imelda Gunawan for technical assistance.

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Chapter 3

TGFβ Down-regulation of the CFTR: A Means to Limit Epithelial Chloride Secretion.

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Experimental Cell Research **(2004) 298(2):473-484**

ABSTRACT

Transforming growth factor β (TGFβ) is a multifunctional cytokine with effects on many cell types. We recently showed that in addition to epithelial barrier enhancing properties, TGFβ causes diminished cAMP-driven chloride secretion in colonic epithelia, in a manner that is p38 MAPK-dependent. In this study we sought to further delineate the mechanism behind TGFβ diminution of chloride secretion. Using colonic and kidney epithelial cell lines, we found that exposure to TGFβ causes dramatic changes in the expression and localization of the apical membrane chloride channel, cystic fibrosis transmembrane conductance regulator (CFTR). In TGFβ-treated colonic epithelia (T84 and HT-29), CFTR mRNA was significantly reduced 2-24h post-cytokine exposure. At a time consistent with decreased colonic epithelial secretory responses (16h), TGFβ treatment caused diminished intracellular CFTR protein expression (confocal microscopy) and reduced channel expression in the apical membrane during stimulated chloride secretion (biotinylation assay). In comparison, polarized kidney epithelia (MDCK) treated with TGFβ displayed similarly reduced secretory responses to cAMP stimulating agents; however, a perinuclear accumulation of CFTR was observed, contrasting the diffuse cytoplasmic CFTR expression of control cells. Our data indicate that TGFβ has profound effects on the expression and subcellular localization of an important channel involved in cAMP-driven chloride secretion, and thus suggest TGFβ represents a key regulator of fluid movement.

Keywords: ion transport, cAMP, actin, T84, HT-29, MDCK

INTRODUCTION

In many organ systems, such as the intestine and kidney, polarized epithelia are responsible for establishing a physical barrier and vectorial electrolyte secretion – the latter providing the driving force for directed water movement. In the intestine, fluid movement is necessary for digestive processes, lubrication, and the "flushing" of potentially noxious substances [1], while the renal system depends on water movement for its osmoregulatory function. Maintenance of gut and kidney homeostasis depends on the precise co-ordination of several epithelial ion transport pathways. Indeed, dysregulation of any one pathway causes perturbed water balance and can potentially have pathological consequences. For example, the secretory diarrhea following cholera infection, intestinal obstructions in patients with cystic fibrosis, or fluid accumulation in autosomal dominant polycystic kidney disease, all result from dysregulated epithelial chloride secretion and are key illustrations of the pivotal role chloride plays in directing water movement [2, 3, 4, 5, 6].

A major portion of chloride movement across polarized epithelia is via a cyclic adenosine monophosphate (cAMP)-dependent chloride channel, the cystic fibrosis transmembrane conductance regulator (CFTR). Through activation of adenylate cyclase in the epithelial cell membrane, an increased amount of cAMP is generated, leading to protein kinase A (PKA) phosphorylation, and ultimately CFTR activation, such that there is an efflux of chloride ions into the lumen of the organ. Numerous regulators of intestinal chloride secretion have been identified, including cytokines (interleukin-4, interferon-γ), neurotransmitters (vasoactive intestinal polypeptide, substance P), mast cell

mediators (histamine), and growth factors (transforming growth factor-α, epidermal growth factor) [7, 8, 9, 10, 11, 12, 13]. Of these, interferon-γ has been shown to directly alter the function and expression of critical ion transport proteins, such as the $Na^+/K^+/2Cl^-$ co-transporter and Na^+/K^+/ATPase pump [8, 9].

Transforming growth factor β (TGFβ) is a multifunctional cytokine with effects on epithelia that include control of cell growth and differentiation, restitution, and enhancement of barrier function [14, 15, 16, 17]. We recently demonstrated that human TGFβ diminishes cAMP-driven chloride secretion (as determined by short circuit current, Isc) in model intestinal epithelial cell monolayers and implicated the p38 mitogen-activated protein kinase (MAPK) signaling pathway in this process [14]. The observation that reduced epithelial responsiveness to cAMP secretagogues occurred several hours post-treatment suggested that TGFβ might not mobilize a rapid response to inactivate CFTR in the apical membrane, but rather exert its effects by modulating epithelial gene expression. Thus, the present study aimed to determine whether the effects of TGFβ on cAMP-driven chloride secretion were due to altered CFTR expression, reduced cAMP levels, or possibly via effects on epithelial cell differentiation.

MATERIALS AND METHODS

Epithelial cell culture

The human colonic adenocarcinoma-derived T84 epithelial cell line was maintained in media containing equal volumes of Dulbecco Modified Eagle medium (DMEM) and Ham's F-12 medium, supplemented with 10% v/v fetal calf serum, 1.5% v/v HEPES and 2% v/v penicillin-streptomycin (all from Life Technologies, Grand Island, NY) at 37°C, 5% CO_2. The HT-29 cell line (HT-29cl.19A, a kind gift from Dr. J.A. Groot, University of Amsterdam) was maintained in DMEM supplemented with 5% v/v fetal calf serum, 0.1% v/v L-glutamine, 2% v/v penicillin-streptomycin, and 5% v/v sodium bicarbonate. Cells were seeded onto semipermeable filter supports of varying surface area (described in each section) (0.4μM pore size, Costar Inc., Cambridge, MA) and grown to confluence (minimum 6 days growth).

Madin-Darby canine kidney (MDCK) type I epithelial cells stably transfected with green fluorescence protein (GFP)-conjugated CFTR were grown as described elsewhere [18]. Briefly, cells were maintained in minimum essential medium with Earle's salts, supplemented with fetal calf serum (10%), penicillin (50U/ml), streptomycin (50μg/ml), L-glutamine (2mM) and geneticin (G418, 150μg/ml, removed 2 days prior to experimentation; all from Life Technologies) at 37°C, 5% CO_2. Enhanced GFP-CFTR visualization and measurable chloride secretion was dependent on addition of sodium butyrate (5mM; Sigma-Aldrich, St. Louis, MO) for 16h prior to experimentation to

increase GFP-CFTR protein expression levels [18]. (Pilot studies in sodium butyrate-free conditions results in monolayer preparations with very low current responses and hence the inclusion of this agent in our physiological investigations).

Physiological assessment of ion transport

MDCK epithelia were seeded ($1.5x10^5$/well) onto semipermeable filter supports (surface area $1cm^2$) and grown as described above. Recombinant human transforming growth factor beta 1 (TGFβ; R&D Systems, Minneapolis, MN) was added to the basolateral compartment (10 or 50ng/ml, 16h) and monolayers were then mounted in specialized Ussing chambers (Precision Instrument Design, Tahoe City, CA) under voltage-clamped conditions, as previously described [19]. Briefly, the potential difference across each monolayer was maintained at 0V and the net active ion transport measured by injected short circuit current (Isc, $\mu A/cm^2$). Baseline Isc was recorded after a 15min equilibration period, while stimulated ion transport was recorded following the maximum change in Isc (ΔIsc). Addition of the cAMP-stimulating agent, forskolin (Fsk, $2x10^{-5}M$, Sigma-Aldrich), and the cyclic nucleotide phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX, $10^{-4}M$, Sigma-Aldrich) to the basolateral compartment, generates Isc responses that are attributable to chloride secretion [18]. In all experiments, amiloride ($10^{-5}M$) was present in the apical bath to inhibit electrogenic Na^+ absorption. Physiological assessment of TGFβ-treated colonic epithelia (T84 and HT-29) has been described elsewhere [14]. In brief, monolayers were treated with TGFβ (1, 10, or

100ng/ml, 4-24h, basolateral compartment), subsequently mounted in Ussing chambers, stimulated with forskolin (10^{-5}M), and maximum ΔIsc occurring within 15min was recorded.

Cyclic adenosine monophosphate (cAMP) assay

Intracellular cAMP levels were compared using a low pH cAMP assay with a specific sensitivity of 0.39pmol/ml (R&D Systems). T84 cells were seeded (10^6/well) onto semipermeable filter supports (surface area 1cm^2). Six days later, cells (± TGFβ, 10ng/ml or 100ng/ml, 16h) were either stimulated with forskolin for 15min (10^{-5}M) or left non-stimulated, lysed in 0.1M HCl (10min), centrifuged at 600*g* and supernatants collected. Analysis of cAMP levels in the supernatant was carried out immediately following the manufacturer's instructions.

RT-PCR for CFTR mRNA

Colonic epithelia were seeded ($2x10^6$/well) onto semipermeable filter supports (surface area 4cm^2) and treated with TGFβ (1, 10, or 100ng/ml; 1, 2, 4, 16, or 24h). Cells were extracted using the RNAqueous™-4PCR kit lysis buffer (Ambion Inc., Austin, TX) and total RNA isolated according to the manufacturer's protocol. cDNA was generated (iScript™ cDNA Synthesis kit, Bio-Rad Laboratories, Hercules, CA) using 1μg total RNA. Following 30 cycles of PCR (Platinum Taq, Invitrogen, Burlington, ON),

amplified fragments were electrophoresed through 2% agarose gels (in Tris/Acetate/EDTA (TAE)) in TAE buffer containing ethidium bromide and visualized under UV light. Densitometry was conducted using a Kodak EDAS 290 gel doc system (Kodak, Rochester, NY). Primers were obtained by using PubMed Blast with published CFTR and β-actin mRNA sequences (GenBank). Primers were synthesized by the Molecular Biology Institute, McMaster University, Hamilton, ON (human CFTR (forward 5' - CTG GAA TCT GAA GGC AGG AG; reverse 5' - GGC ATT TCC ACC TTC TGT GT) and human β-actin (forward 5' - CCA GAG CAA GAG AGG TAT CC; reverse 5' - CTG TGG TGG TGA AGC TGT AG)).

Sample preparation for confocal microscopy

CFTR:

MDCK and colonic epithelia were seeded ($5x10^4$/well and $3x10^5$/well, respectively) onto semipermeable filter supports (surface area $0.33cm^2$) and grown for 5 days as described earlier. *MDCK*: Cells (± TGFβ, 10 or 50ng/ml, 16h) were rinsed in PBS, fixed in 80% acetone (5min), filters excised from the polystyrene filter supports, and mounted on glass slides in Gel/Mount anti-fade reagent (Biomeda Corporation, Foster City, CA). *T84 and HT-29*: Cells (± TGFβ, 10ng/ml or 100ng/ml, 16h) were fixed in 4% formalin, permeabilized with 0.1% TritonX-100 (Mallinckrodt Inc., Paris, KY), and incubated for 1h at room temperature for both primary and secondary antibodies (mouse anti-human CFTR: 1:100, R&D Systems and Alexa Fluor® 488 goat anti-mouse IgG: 1.25μg/ml, Molecular Probes, Eugene, OR, respectively) diluted in blocking

solution (5% BSA/10% goat serum (Sigma-Aldrich) in 0.1% Triton X-100). Nuclei were visualized with propidium iodide (0.5mg/ml; Sigma-Aldrich). Monolayers on filter membranes were excised and mounted as described above.

Actin

MDCK cells were seeded ($5x10^4$/well) onto semipermeable filter supports (surface area $0.33cm^2$), treated as described above, and fixed in 4% formalin (T84 cells seeded at 10^5/well onto 8-well chamber slides from Lab Tek (Nalge Nunc International, Naperville, IL)). Cells were rinsed in PBS (x3), permeabilized in 0.1% Triton X-100 (4min), rinsed in PBS (x3), and stained for actin using Texas red-phalloidin (1:40 dilution, Molecular Probes) in 1% BSA/PBS (20min). Monolayers on filter membranes were excised and mounted on glass slides with Gel/Mount anti-fade, sealed, and stored at 4˚C until confocal microscopy.

Confocal microscopy

Images were acquired using an inverted Zeiss laser scanning microscope (LSM 510, Axiovert 100M; Oberkochen, Germany) equipped with argon (450-514nm) and helium-neon (543nm, 633nm) lasers. Both GFP-CFTR and immunoreactive CFTR fluorescence was excited using the 488nm laser line and collected using a standard fluorescein isothiocyanate (FITC) filter set, while propidium iodide-labeled nuclei were excited using the 633nm laser line and collected with a Syto-red filter set. A series of z-

stacks, taken at 1μm increments, beginning at the apical membrane, were used to generate cross-sectional images of epithelial monolayers. Pixel counts were used to determine CFTR fluorescence intensity for each 1μm z-section and exported to Microsoft Excel for tabulation (software program, design and use courtesy of Dr. L. Arsenault, McMaster University). Perinuclear rings was quantified by counting the percentage of cells displaying perinuclear GFP-CFTR rings in 5 randomly selected fields of view on 3 separate monolayers under blinded conditions. Actin fluorescence was excited using the 543nm laser line and collected using a Rhodamine Red filter set. For each experiment, image acquisition (i.e. confocal microscope settings) and processing was identical between control and TGFβ-treated cells.

Cell-surface biotinylation

Cell surface expression of CFTR in confluent T84 monolayers was determined by biotinylating apical membrane proteins and immunoblotting for CFTR (after Lisanti *et al.* [20]) following stimulation with the cAMP secretagogue, forskolin (10^{-5}M). Briefly, filter-grown T84 cells (seeded at $2x10^6$/well, surface area $4cm^2$) ± TGFβ (10ng/ml, 16h) were washed with ice cold PBS/Mg^{2+}/Ca^{2+} (1mM $MgCl_2$, 0.1mM $CaCl_2$ in PBS) and subsequently incubated with biotin (0.75mg/ml NHS S-S Biotin, Pierce, Rockford, IL) added to the apical surface for 60min (on ice). Following removal of the biotin and further washing (PBS/Mg^{2+}/Ca^{2+} x3, PBS x2), cells were exposed to 200μl of lysis buffer (50mM TrisHCl/150mM NaCl/1% IGEPAL CA630 (Sigma-Aldrich), protease inhibitor

cocktail (Complete tablets, Roche, Laval, QE)) for 15min and cell supernatants collected. Biotin-labeled proteins were collected by incubating the samples with immobilized streptavidin beads (Pierce, Rockford, IL) overnight. Beads were washed with PBS solution, placed in Laemli sample buffer (0.24M Tris-HCl, 5.6% SDS, 0.008% Bromophenol blue, 16% glycerol, 80mM DTT), and heated for 10min at 95°C. Supernatants were collected and immunoblotted for CFTR.

Western Blotting

Samples were loaded onto a 4-15% SDS-PAGE gradient gel (Bio-Rad Laboratories), run for 2.5h at 111V, and proteins transferred onto a PVDF membrane (Pall Corporation, Ann Arbor, MI) for 2h at 90V. Membranes were blocked with 5% BSA (in Tris-Buffered Saline with 0.1% TritonX-100, TBST) and incubated with primary antibody overnight at 4°C (mouse anti-human CFTR, clones M3A7 and L12B4, 1μg/ml; Upstate Biotech, Lake Placid, NY). Immunoblots were washed thoroughly with TBST and exposed to secondary antibody conjugated to HRP for 1h at room temperature (goat anti-mouse, 1:500; Santa Cruz Biotechnology Inc., Santa Cruz, CA). The membrane was washed extensively and immuno-reactive proteins were visualized using chemiluminescence (ECL, Amersham Pharmacia, Piscataway, NJ) and exposure to Kodak XBL film (Eastman Kodak Company, Rochester, NY). Densitometry was conducted using a Kodak EDAS 290 gel doc system (Kodak).

Alkaline Phosphatase Assay

Filter-grown T84 monolayers (seeded at 10^6cells/well, surface area 1cm^2) ± TGFβ (10ng/ml, 16h) were lysed using 7% BSA in 0.1% Triton X-100 for 15min. Samples were pipetted vigorously, vortexed, and centrifuged at 5000x*g* for 2 minutes. Supernatants were collected and sent to the Clinical Laboratory of Hamilton Health Sciences (McMaster University Medical Centre) where alkaline phosphatase activity was tested using the Roche/Hitachi Modular System (Roche Diagnostics, Laval, QC).

[^{3}H]-Thymidine Incorporation (Proliferation) Assay

T84 cells were seeded (10^6/well) onto semipermeable filter supports (surface area 1cm^2) and grown for 7 days. [^{3}H]-thymidine (1μCi/well; DuPont-New England Nuclear, Wilmington, DE) was added for the duration of TGFβ treatment (1, 10, or 100ng/ml; 4 or 16h). Cells were harvested by lysing in 0.1% TritonX-100 in PBS for 10min, after which they were pipetted vigorously, and samples collected. Sample aliquots (0.5ml) were assayed in 4.5ml aqueous counting scintillant for beta-ray emission and counts per minute determined in a scintillation counter (Becton Dickinson, Mississauga, ON).

Statistical Analysis

Data are presented as means ± standard error of the mean (SEM). Data were analyzed using one-way ANOVA followed by post-hoc comparisons using Tukey's test,

or Student's t-test, as indicated, with $p<0.05$ accepted as the level of statistical significance.

RESULTS

We previously determined that TGFβ decreases cAMP-driven chloride secretion in colonic epithelia [14], and Figure 1a shows the extent of this effect after various doses and times of cytokine exposure in T84 cells. Chloride secretory responses to forskolin were similarly reduced by 1, 10, and 100ng/ml TGFβ following 16 and 24h treatment, suggesting dose-independent effects at these timepoints. These observations were extended by determining that TGFβ stimulation causes diminished chloride secretion in other secretory epithelial cell lines, i.e. HT-29 colonic and MDCK kidney epithelia, and in contrast to T84 cells, the effect was dose-dependent (Figure 1b). To define the mechanism behind TGFβ-induced diminished secretory responses in epithelia, we considered three hypotheses under conditions that elicit a consistent decrease in chloride secretion: 1) TGFβ impairs cAMP production and thus limits an activator of the chloride transport pathway; 2) TGFβ regulates protein expression and/or subcellular localization of the CFTR; and 3), TGFβ inhibits proliferation and promotes differentiation of epithelia from a pro-secretory to a pro-absorptive phenotype.

TGFβ has a dose-dependent effect on intracellular cAMP levels in stimulated T84 cells

To determine if cAMP generation is affected by TGFβ treatment, we assayed intracellular cAMP levels in T84 colonic epithelia. Under basal (i.e. non-stimulated) conditions there were no significant differences in cAMP levels between controls and

epithelial monolayers treated with 10 or 100ng/ml TGFβ (Table 1). In contrast, following treatment with TGFβ at 100ng/ml, but not 10ng/ml, stimulation with forskolin (10^{-5}M, 15min) elicited a significantly lower level of intracellular cAMP as compared to control (Table 1), even though both doses of TGFβ consistently reduced ΔIsc to forskolin (Figure 1a).

TGFβ causes a decrease in colonic epithelial CFTR mRNA expression

We determined if TGFβ altered CFTR gene expression by RT-PCR analysis of T84 and HT-29 colonic epithelial monolayer RNA extracts. Figure 2a shows that CFTR mRNA in T84 cells is similarly reduced by 1, 10, or 100ng/ml TGFβ treatment (16h). The kinetics of this effect are depicted in Figure 2, where it is shown that CFTR mRNA is reduced after 2, 4, 16, and 24h TGFβ exposure to 10ng/ml (Figure 2b) and 100ng/ml (Figure 2c, left panel). Densitometric analysis conducted to semi-quantify CFTR mRNA levels in TGFβ-treated T84 epithelia illustrated that 2-24h of exposure to cytokine causes a 40-50% decrease compared to untreated controls (Figure 2d). Similar to T84 colonic epithelia, HT-29 cells treated with TGFβ (100ng/ml) display reduced CFTR mRNA levels after 2, 4, and 16h exposure (Figure 2c, right panel).

TGFβ affects CFTR distribution and cytoskeletal arrangement

Polarized kidney epithelia

Using polarized Madin-Darby canine kidney (MDCK) cells (type I) that were stably transfected with GFP-tagged CFTR, CFTR localization was compared between TGFβ-treated cells and untreated controls. TGFβ treatment (50ng/ml, 16h) resulted in a perinuclear accumulation of CFTR that was not observed in untreated control MDCK epithelia (Figure 3a). We quantified this observation by counting the percentage of cells displaying perinuclear GFP-CFTR rings in 5 randomly selected fields of view on 3 separate monolayers. TGFβ treatment resulted in perinuclear CFTR rings in 70 ± 7% of cells examined, a statistically significant increase over control levels of 23 ± 3% (Mean ± SEM; $p<0.05$ as determined by Student's t-test).

In the event that MDCK perinuclear CFTR accumulation results from ultrastructural changes within the cell, and since CFTR requires an intact cytoskeleton to function, we compared F-actin organization between TGFβ-treated and untreated control epithelia. Exposure to TGFβ (10ng/ml, 16h) caused a reorganization of F-actin into elongated stress fibres, appearing as rope-like strands located throughout the cell, in marked contrast to the more diffuse F-actin network observed in untreated controls (Figure 3b).

Polarized intestinal epithelia

To determine if diminished CFTR gene expression also correlates with a reduction in CFTR protein levels in intestinal epithelia, T84 and HT-29 monolayers were immunostained for CFTR and examined using confocal microscopy. Serial images, taken in 1μm increments, were obtained for each monolayer, beginning at the apical surface. From the representative z-series shown in Figure 4a, right panel (TGFβ), TGFβ-treated T84 cells displayed a significant decrease in CFTR expression throughout the apical and mid-portion of the cell compared to untreated control cells (Figure 4a, left panel; Control). The computer-generated reconstructed images in Figures 4b and 4c provide an alternate view of CFTR distribution within the T84 and HT-29 monolayer, respectively, and clearly demonstrate the significant reduction of CFTR protein levels in the apical regions of TGFβ-treated colonic epithelial cell lines (right panels). Furthermore, as a semi-quantitative measurement of CFTR protein expression, CFTR fluorescence intensity was compared between untreated control and TGFβ-treated T84 monolayers by tabulating the number of green pixels counted per z-section. Figure 4d shows the reduction in CFTR fluorescence intensity, indicative of protein expression, in each region of the TGFβ-treated T84 monolayer (data correspond to monolayer from Figure 4a).

To determine if TGFβ also affected actin organization in colonic epithelia, T84 monolayers (± TGFβ, 10ng/ml, 16h) were stained with Texas red-conjugated phalloidin and visualized with confocal microscopy. Despite the high level of actin staining observed throughout the cells, and in contrast to MDCK epithelia, TGFβ treatment did

not result in obvious reorganization of F-actin into elongated stress fibres (data not shown).

TGFβ reduces cell surface expression of CFTR in T84 epithelia

To correlate the decrease in CFTR expression with the diminished ion transport function of TGFβ-treated colonic epithelia, we assessed the level of T84 CFTR protein expression at the apical membrane following cAMP stimulation with forskolin. We used a biotinylation/immunoblot methodology since the resolution limits of confocal microscopy prevent distinction between CFTR inserted in the apical membrane and that located in sub-apical pools. In figure 5 (inset) we show that treatment with TGFβ causes a reduction in CFTR protein expression at the apical membrane during stimulated chloride secretion in colonic epithelia. Additionally, densitometric analysis of the immunoblots showed that exposure to TGFβ for 16h caused an ~40% decrease in CFTR protein expression at the apical membrane during stimulated chloride secretion (Figure 5).

TGFβ does not affect epithelial differentiation or proliferation

To determine if TGFβ causes intestinal epithelial cellular differentiation, we measured activity of the brush border enzyme, alkaline phosphatase, typically expressed by pro-absorptive, differentiated intestinal epithelia. TGFβ (1, 10, 100ng/ml; 16h) had no consistent effect on T84 cell alkaline phosphatase activity and hence provided no

evidence that differentiation was occurring. At the highest dose of TGFβ tested (i.e. 100ng/ml), alkaline phosphatase activity was 94.4 ± 10.2% of controls (Mean ± SEM; p=0.30 as determined by Student's t-test; n=6 monolayers).

We assessed thymidine incorporation into DNA to establish if TGFβ affected cellular proliferation in T84 monolayers after 4 or 16h cytokine exposure (1, 10, and 100ng/ml). There was no significant difference in [^{3}H]-thymidine detected between TGFβ-treated T84 cells and untreated controls for any of the times or doses of TGFβ tested (Table 2), suggesting that TGFβ-induced effects on epithelial chloride secretion are independent of cell proliferation.

Figure 3.1 (following pages). Bar charts showing the decrease in T84, HT-29, and MDCK epithelial ΔIsc responses following exposure to TGFβ. T84 and HT-29 were stimulated with forskolin (Fsk, 10^{-5}M), while MDCK were stimulated with Fsk ($2x10^{-5}$M) plus the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX, 10^{-4}M) to prevent cAMP degradation. **A**: Time- and dose-dependent effect of TGFβ (4-24h, 1-100ng/ml) on T84 stimulated chloride secretion (values are given as a percent of control monolayer responses normalized to time-matched controls, where average values ranged from 45.0 ± 2.1 to 61.9 ± 8.0 μA/cm^2 (4 and 24h controls, respectively); mean ± SEM; *, $p<0.05$ compared to time-matched control as determined by one-way ANOVA; n=13-20 monolayers). **B**: Stimulated Isc responses in HT-29 and MDCK cells ± TGFβ (10, 50, or 100ng/ml; 16h). Mean ± SEM; *, $p<0.05$ compared to control, **, $p<0.01$ compared to control as determined by one-way ANOVA; n=3-9 monolayers.

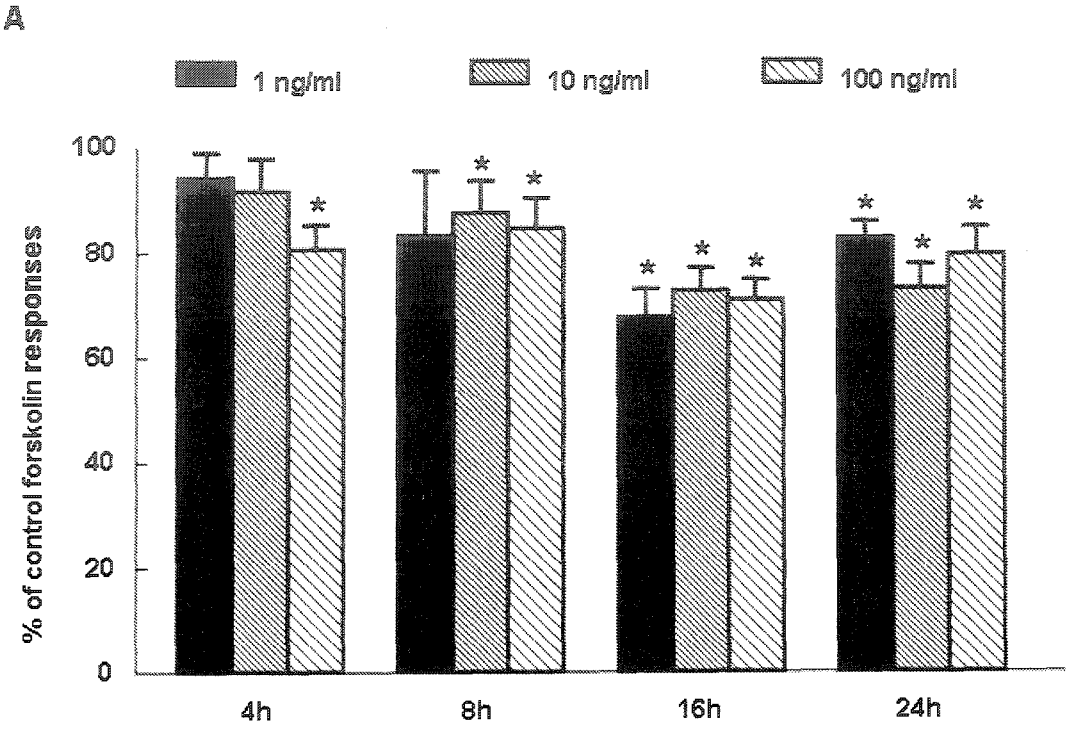
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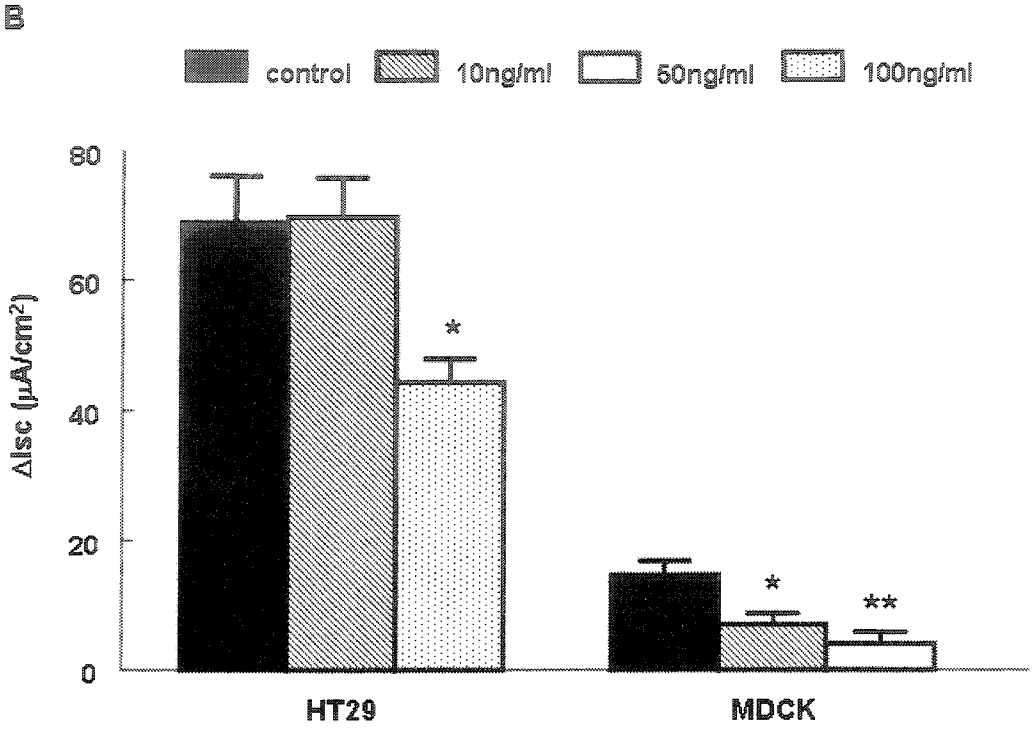
Figure 3.1a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HT29
MDCK

Figure 3.1b

TABLE 3.1: Dose-dependent effect of TGFβ (16h) on intracellular cAMP levels in forskolin-stimulated colonic epithelia.

	Control	**TGFβ (10ng/ml)**	**TGFβ (100ng/ml)**
Unstimulated	3.5 ± 0.6	4.0 ± 1.0	4.1 ± 0.3
Forskolin (10^{-5}M)	39.7 ± 8.9	46.2 ± 9.2	11.3 ± 2.9*

(Mean ± SEM; data are reported in pmol/ml concentration; *, $p<0.01$ compared to Fsk-stimulated control as determined by one-way ANOVA; n=4-11 monolayers)

Figure 3.2 (following pages). **A**: Representative image showing CFTR mRNA is similarly reduced in T84 colonic epithelia treated with 1, 10, or 100ng/ml TGFβ (16h; n=3 experiments). Representative images showing CFTR mRNA is significantly reduced in T84 epithelia after 2-24h TGFβ exposure at 10ng/ml (**B**: n=2 experiments) or 100ng/ml (C: left panel, n=3 experiments), as well as TGFβ-treated HT-29 epithelia (**C**: right panel, n=2 experiments). **D**: Densitometric analysis of CFTR:β-actin mRNA levels in TGFβ-treated T84 colonic epithelia (1-24h, 100ng/ml). Values are relative to control CFTR:β-actin mRNA levels normalized to each experiment (*, $p<0.05$ as determined by one-way ANOVA; n=3 experiments). For each experiment the level of β-actin mRNA was used as an internal control.

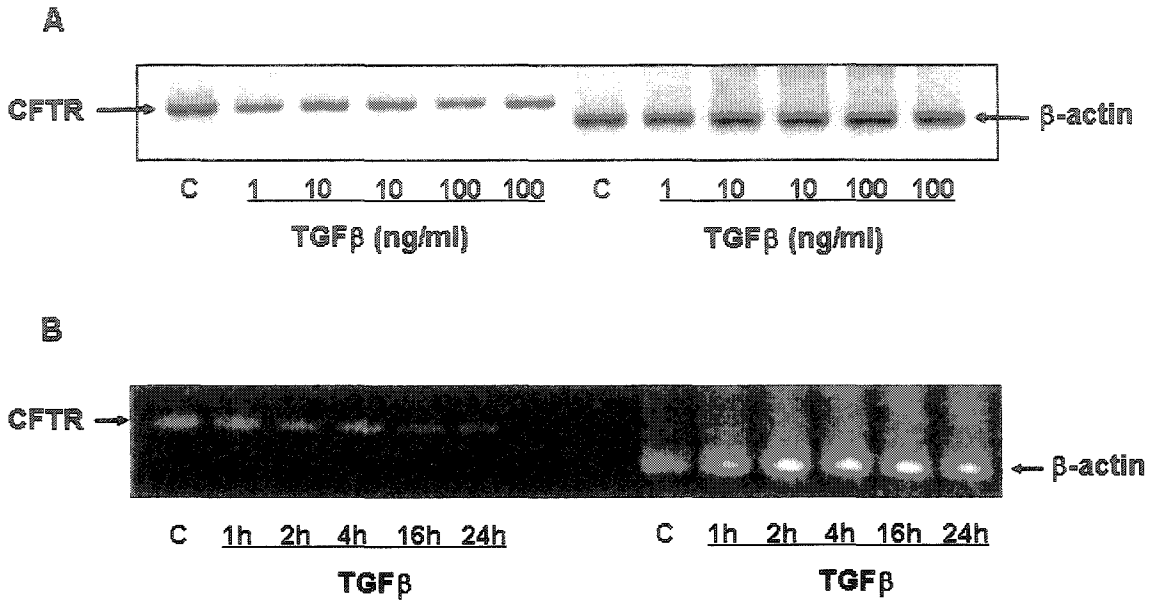
A
CFTR
β-actin
C 1 10 10 100 100 C 1 10 10 100 100
TGFβ (ng/ml)
TGFβ (ng/ml)
B
CFTR
β-actin
C 1h 2h 4h 16h 24h C 1h 2h 4h 16h 24h
TGFβ
TGFβ

Figure 3.2a,b

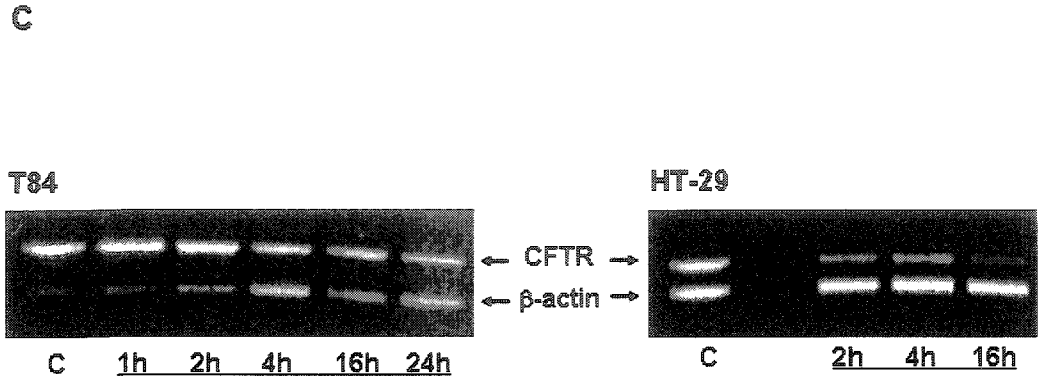

Figure 3.2c

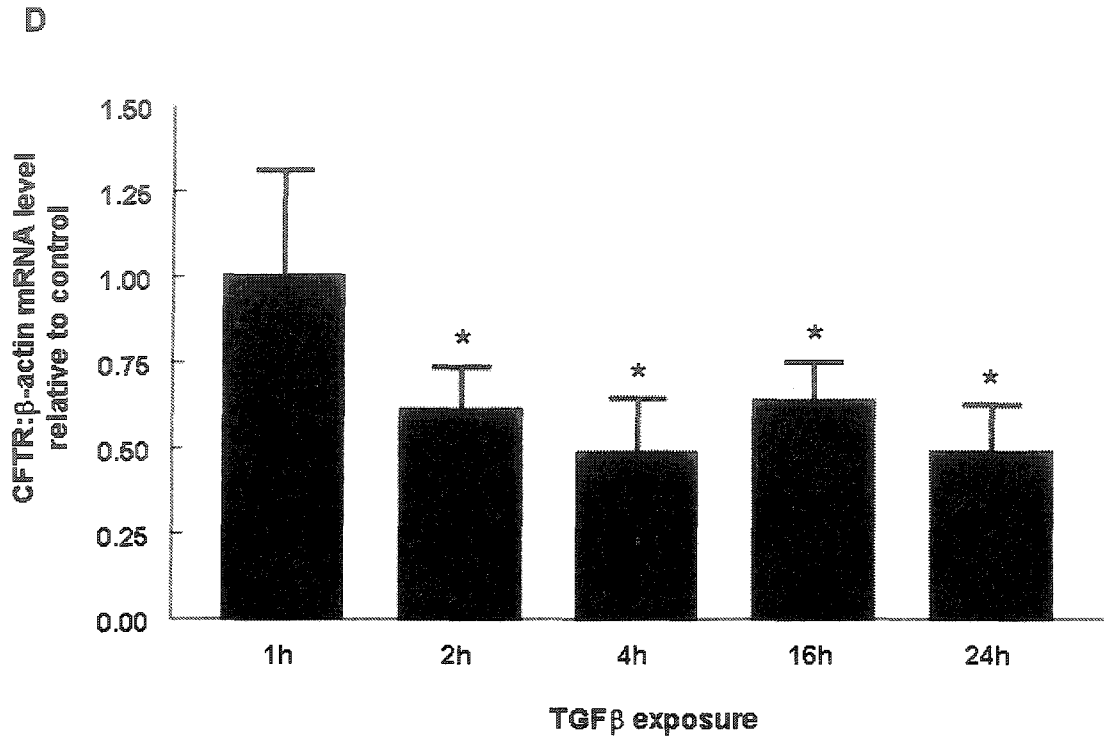
D
CFTR:β-actin mRNA level relative to control
1.50
1.25
1.00
0.75
0.50
0.25
0.00
*
*
*
*
1h
2h
4h
16h
24h
TGFβ exposure

Figure 3.2d

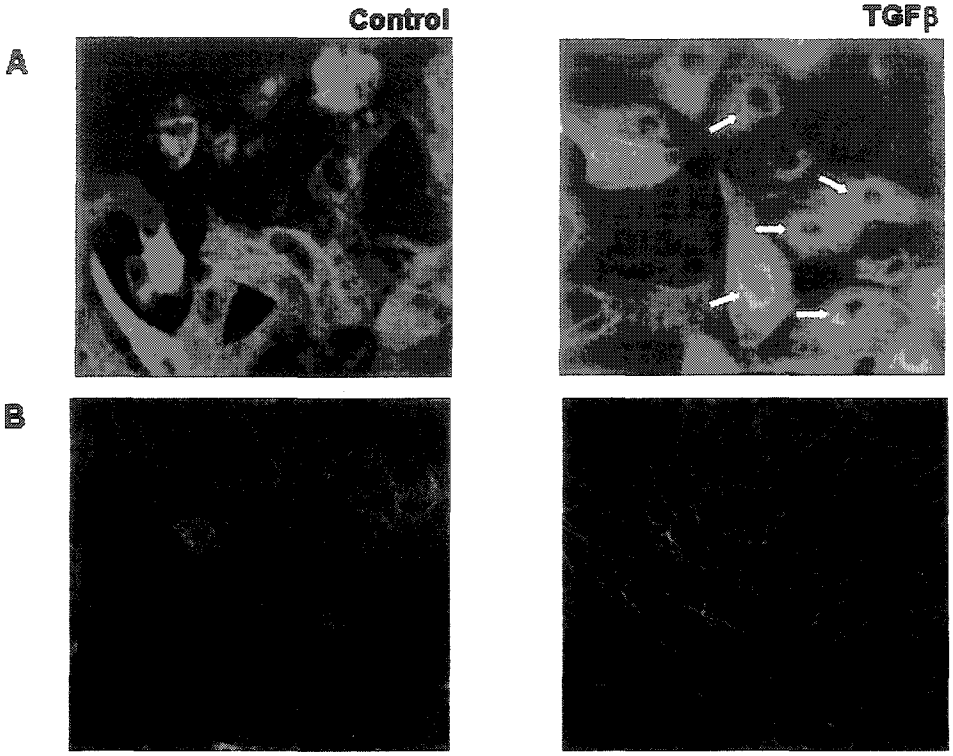

Figure 3.3: **A**: Representative images (630x original magnification) in the XY plane taken from MDCK epithelia stably transfected with GFP-tagged CFTR demonstrate the perinuclear accumulation of CFTR (arrows) in TGFβ-treated cells (50ng/ml, 16h), compared to the diffuse fluorescence emitted from untreated controls (n=6 monolayers). **B**: Representative images in the XY plane showing the reorganization of actin into stress fibres stretching across the cell following exposure to TGFβ (50ng/ml, 16h; n=8 monolayers).

Figure 3.4 (following pages). Representative images of decreased CFTR expression in the apical region of colonic epithelial monolayers treated with TGFβ. Immunostained CFTR is depicted by the green fluorescence, while propidium iodide-stained nuclei appear red. **A**: Serial images (z-series) of a T84 monolayer beginning at the apical surface (0.0μm) and continuing throughout the cell in 1μm increments shows apical expression of CFTR is reduced in TGFβ-treated cells (10ng/ml, 16h; n=9 monolayers). **B**: Compilation and rotation of the z-series by computer software provides a cross-sectional view and similarly demonstrates decreased CFTR expression in the apical region of TGFβ-treated T84 monolayers (n=9 monolayers). **C**: Computer-generated cross-sectional view of HT-29 monolayer shows TGFβ (100ng/ml, 16h) causes a similar reduction in apical CFTR expression (n=4 monolayers). **D**: Line graph showing the decrease in CFTR fluorescence intensity for each 1μm section in the TGFβ-treated T84 monolayer z-series shown in panel A.

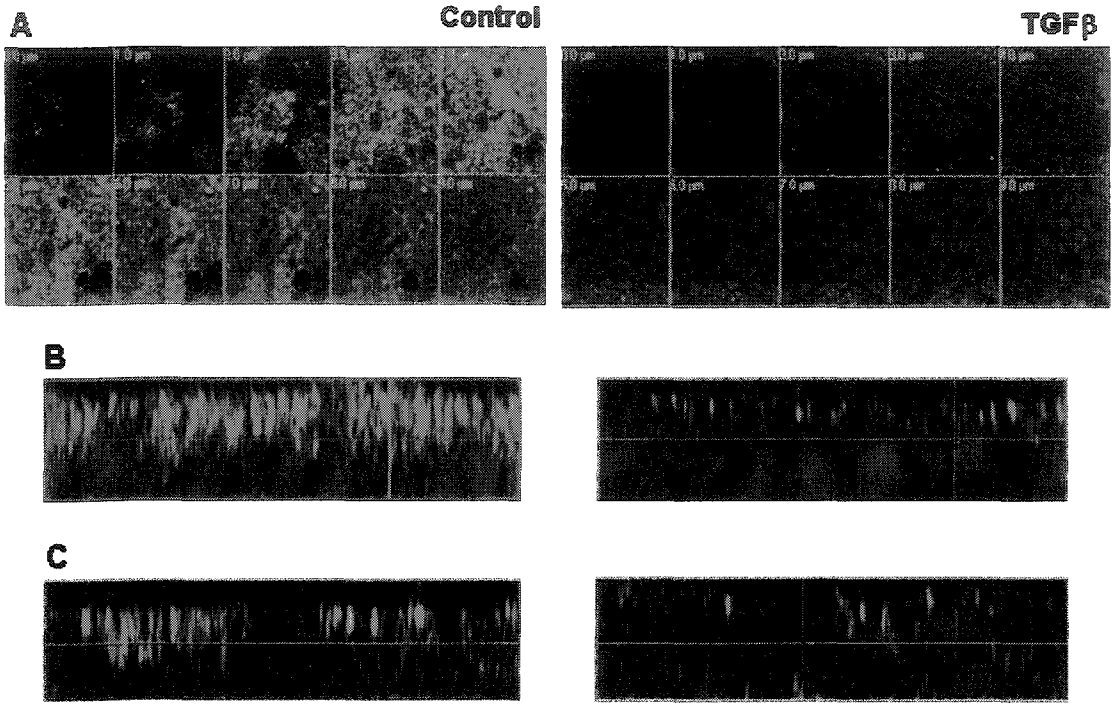

Figure 3.4a,b,c

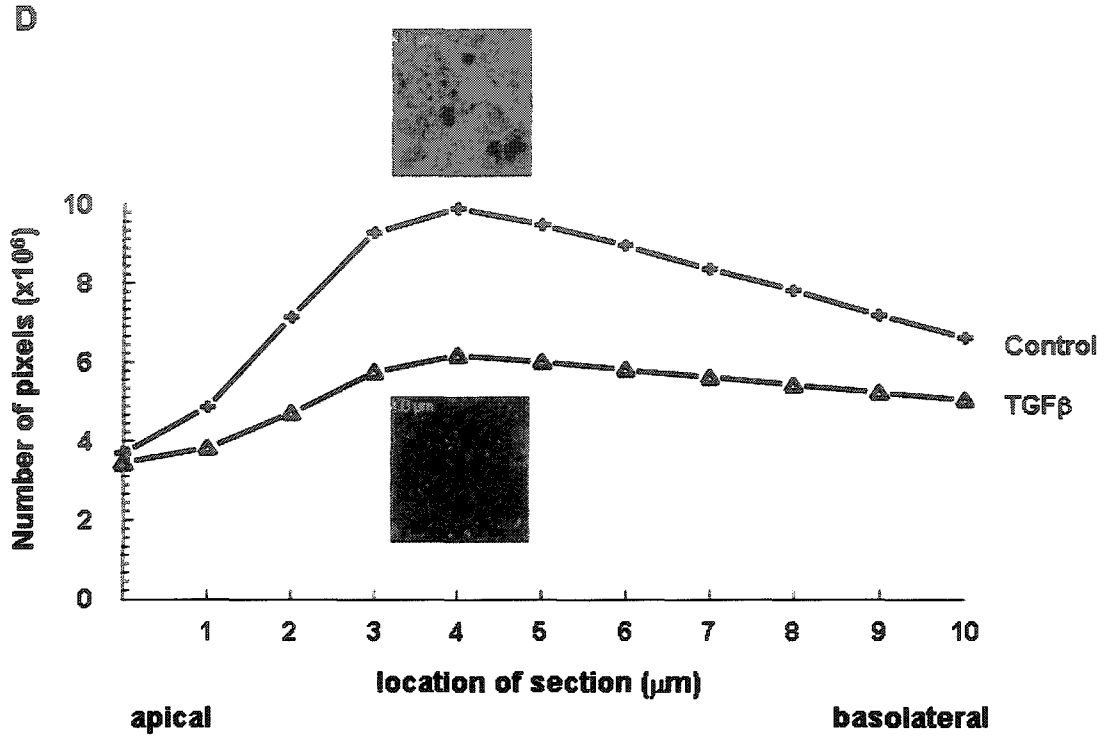
D
Number of pixels (x10⁶)
10
8
6
4
2
0
1
2
3
4
5
6
7
8
9
10
location of section (μm)
apical
basolateral
Control
TGFβ

Figure 3.4d

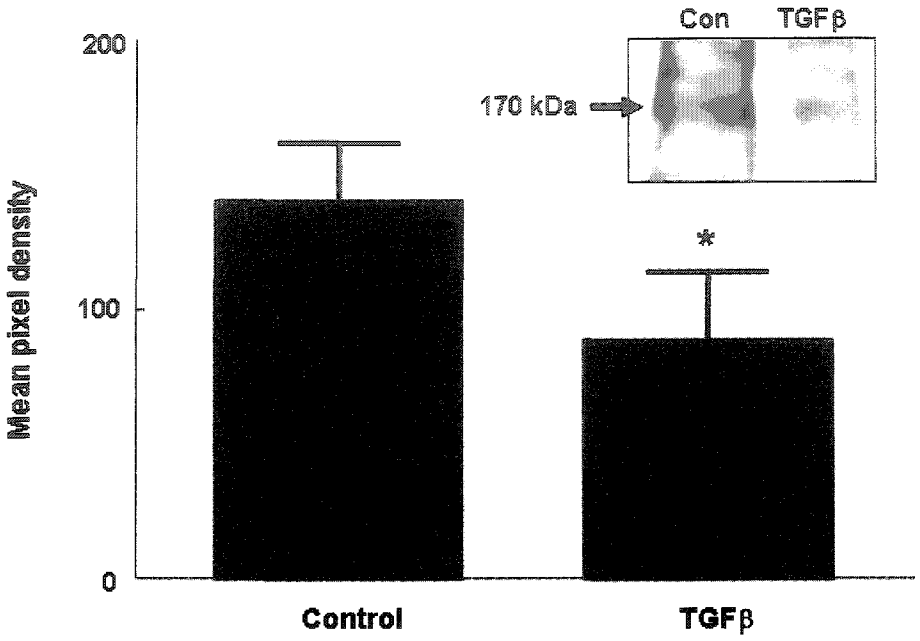

Figure 3.5. Apical membrane CFTR expression during stimulated chloride secretion in T84 colonic epithelia is reduced by TGFβ exposure (10ng/ml, 16h) as determined by densitometric analysis of mean pixel density (*, $p<0.05$ as determined by Student's t-test; n=3 experiments). **Inset**: Representative image from immunoblot of forskolin-stimulated (10^{-5}M) T84 monolayers ± TGFβ (10ng/ml, 16h) probed for CFTR expression (n=3 experiments).

TABLE 3.2: TGFβ has no effect on [^{3}H]-thymidine incorporation in T84 colonic epithelia

	Control	**1ng/ml TGFβ**	**10ng/ml TGFβ**	**100ng/ml TGFβ**
4 hours	2651 ± 887	3224 ± 754	2570 ± 662	3085 ± 572
16 hours	5990 ± 979	4064 ± 371	5446 ± 728	5790 ± 494

(Mean ± SEM; data are reported as counts per minute; no significant difference between controls and TGFβ-treated cells after 4 or 16h, as determined by one-way ANOVA; n=3-7 monolayers)

DISCUSSION

Epithelial cells have many functions critical to the maintenance of homeostasis, including an immune accessory role, formation of a selectively permeable barrier, and vectorial electrolyte secretion for the regulation of fluid movement. TGFβ is a growth factor with several non-mitogenic properties, including effects on each of the previously listed epithelial functions [14, 21]. Having recently determined that TGFβ diminishes cAMP-driven chloride secretion in colonic epithelia, we sought to delineate the mechanisms behind this event by examining three mechanistic postulates: 1) TGFβ impairs cAMP production, such that activation of the chloride transport pathway is limited; 2) TGFβ regulates the amount and/or localization of the CFTR; and 3) TGFβ promotes epithelial differentiation from a pro-secretory towards a pro-absorptive phenotype. Our data suggest that it is largely regulation of the CFTR by TGFβ, and not a major effect on cAMP production or epithelial differentiation, that accounts for the diminished cAMP-driven chloride secretion following cytokine exposure.

As generation of the second messenger, cAMP, is critical to cAMP-driven chloride secretion, one hypothesis was that interference with cAMP production (e.g. due to an effect on adenylate cyclase activity or its substrate, ATP) could account for the limited secretory responses of TGFβ-treated epithelia. Comparison of basal cAMP levels between control and TGFβ-treated T84 cells revealed no significant difference at 16h post-cytokine exposure for either of the doses tested (10 or 100ng/ml). In contrast, there was a significant reduction in forskolin-stimulated cAMP generation following exposure

to TGFβ (16h) that appeared to be dose-dependent, as it was only observed in cells treated with 100ng/ml. While it is possible that this higher dose of TGFβ inhibits adenylate cyclase activation, forskolin-stimulated chloride secretion is similarly reduced in T84 cells treated with 1, 10, or 100ng/ml TGFβ for 16h (Figure 1a). Thus, whereas diminished cAMP may contribute to the effect of the higher dose of TGFβ (100ng/ml) on reduced secretory responsiveness, it is not responsible for the smaller ΔIsc to forskolin observed in 1 or 10ng/ml TGFβ-treated epithelia. Indeed, the latter assertion is further supported by our previous observation that TGFβ-treated epithelia (10ng/ml, 16h) stimulated with cell-permeant dibutyryl cAMP display significantly reduced secretory responses [14], since this agent bypasses the need for second messenger generation in cAMP-driven chloride secretion. Together, these data suggest diminished chloride secretion in TGFβ-treated colonic epithelia is due to changes that occur downstream of cAMP production.

Regulation of chloride secretion is an energetically expensive, highly co-coordinated process, requiring the co-operation of ATPases, co-transporters, and ion-selective channels in the cell membrane. Although TGFβ may directly inhibit any one of these components, we reasoned that the time-dependent nature of TGFβ-induced diminished ion transport (i.e. required several hours of cytokine exposure) was indicative of altered gene expression. Here again, it was recognized that each component in the secretory pathway was a potential target; however, we began with the hypothesis that TGFβ limits cAMP-driven chloride secretion by down-regulating CFTR gene expression. In T84 colonic epithelia, TGFβ exposure caused a significant decrease in CFTR mRNA at

all doses tested. Kinetic studies determined that CFTR mRNA was first reduced 2h post-treatment, and persisted for 24h (end of experiment). Likewise, HT-29 colonic epithelia displayed a similar time course, with reduced CFTR mRNA evident 2h following TGFβ exposure. In this regard, TGFβ is similar to interferon-γ, interleukin-1β, and tumor necrosis factor-α, which have all been shown to regulate CFTR expression 4-24h post-treatment [22, 23, 24, 25]. It is noteworthy that while the colonic epithelia tested in this study displayed differential sensitivity to TGFβ (not an unexpected result with cell lines), there was consistency within each cell line such that the dose required to significantly decrease stimulated chloride secretion was also effective at reducing both CFTR mRNA and protein expression (e.g. 100ng/ml TGFβ for HT-29 vs. 10ng/ml TGFβ for T84).

The correct folding and insertion of CFTR into the apical membrane of polarized epithelia depends on a series of post-translational modifications in the Golgi apparatus before the protein is packaged in vesicles and trafficked to the cell surface. Although correctly folded CFTR is typically inserted in the apical membrane of polarized epithelia, some studies have shown that the channel is continuously recycled from the cell membrane by an exocytic/endocytic pathway, and thus contributes to the sub-apical pool of newly-arrived CFTR-containing vesicles (reviewed in Kleizen *et. al.* [26]). There is conflicting data regarding the regulation of CFTR during sustained cAMP-driven chloride efflux in secretory epithelia. Some evidence supports insertion of additional CFTR from the sub-apical vesicles into the apical membrane following stimulation by cAMP secretagogues, such as forskolin or vasoactive intestinal polypeptide [27, 28, 29, 30], in addition to inhibiting the endocytic retrieval of CFTR and consequently promoting an

increase in the number of channels available [31, 32]. Other studies have provided contradictory data, indicating CFTR activation by PKA is solely responsible for cAMP-driven chloride secretion [18, 33, 34, 35]. Additionally, the membrane-bound CFTR has been physically linked to its activator, PKA, through the actin cytoskeleton in a process involving E3KARP, ezrin, and ezrin binding protein 50 [36]. Thus, agents capable of rearranging the actin cytoskeleton could interfere with CFTR insertion into the apical membrane, activation by PKA, and lead to reduced chloride secretion.

Using confocal microscopy to address the subcellular distribution of CFTR and the actin cytoskeleton, we found that GFP-CFTR transfected MDCK epithelia treated with TGFβ displayed a perinuclear accumulation of the CFTR. This was temporally associated with a rearrangement of the actin cytoskeleton (i.e. stress fibres), and correlated with diminished cAMP-driven chloride secretion. Interestingly, the altered CFTR localization was dose-dependent, with a higher dose of TGFβ (50ng/ml) resulting in a substantially greater perinuclear CFTR accumulation than that observed in cells treated with 10ng/ml TGFβ (data not shown), perhaps accounting for the greater reduction in secretory responsiveness observed in MDCK epithelia treated with 50ng/ml TGFβ (Figure 1b). Indeed, in terms of chloride secretory function, the consequences of perinuclear CFTR accumulation might be analogous to the mislocalization of CFTR proteins that accounts for defective chloride ion currents in tissues from cystic fibrosis (CF) patients [37, 38]. In this context, it is interesting to note that MDCK cells stably expressing fluorescent ΔF508-CFTR, the most common CFTR mutation in CF patients, demonstrate a perinuclear pattern similar to the one observed in this study, a distribution

that was determined to represent endoplasmic reticulum accumulation/localization [39]. Considering the functional interaction between CFTR and actin that has been associated with cAMP-dependent chloride secretion [40, 41], we speculate that TGFβ-induced actin rearrangement disrupts the necessary actin/actin-binding protein/CFTR organization [42], resulting in a redistribution of the CFTR and/or reduced insertion of the channel into the apical membrane which accounts for, at least in part, the diminished vectorial chloride secretion following stimulation of TGFβ-treated kidney epithelia.

Analysis of colonic epithelial cell lines immunostained for CFTR showed that TGFβ exposure caused a significant reduction in apical CFTR protein expression, data consistent with the observation that CFTR mRNA was also reduced by TGFβ. These studies were then extended to address whether or not apical membrane CFTR protein expression is reduced during stimulated chloride secretion. To this end, biotinylation studies determined that TGFβ treatment, at a time and dose that corresponded to decreased secretory responsiveness to forskolin (i.e. 10ng/ml, 16h), caused a reduction in the quantity of CFTR protein inserted into the apical membrane of T84 colonic epithelia stimulated with forskolin. These data may reflect a decrease in the amount of CFTR protein available for insertion following TGFβ exposure, as opposed to altered trafficking, since apical membrane expression and CFTR immunofluorescence were similarly reduced to approximately 60% of control levels. While it is clear that some different CFTR-regulatory mechanisms exist between kidney and colonic epithelia (e.g. lack of perinuclear rings observed in colonic epithelial z-sections), these data collectively

provide strong evidence that TGFβ directly regulates CFTR gene expression, protein expression, and protein localization, to limit chloride exit from the cell.

Structure and function of the intestinal epithelium is maintained through the balance of proliferation, differentiation, and apoptosis as cells migrate up the crypt towards the lumen: each of these events can be affected by TGFβ [15]. Thus, we tested whether TGFβ effects on epithelial ion transport occurred via the inhibition of proliferation and induction of differentiation of T84 cells from leaky, crypt-like secretory epithelia, into absorptive, electrically "tight" monolayers characteristic of villus or colonic surface epithelia. Indeed, such a transformation in T84 cells has been reported following co-culture with fibroblasts, a cell that can synthesize TGFβ and related growth factors, and was reflected by enhanced barrier function and an increase in brush border enzyme activity [43]. In the present study, we found that TGFβ treatment affected neither thymidine incorporation, a marker of DNA synthesis, nor did it cause an increase in the activity of the brush border enzyme, alkaline phosphatase in T84 colonic epithelia. It is therefore unlikely that the TGFβ-induced changes in epithelial physiology seen here are attributable to inhibition of proliferation or induction of cellular differentiation towards a pro-absorptive phenotype.

In conclusion, we postulated that the TGFβ-induced reduction in epithelial cAMP-driven chloride secretion could be due to inhibition of cAMP generation, differentiation of the cell from a crypt-like chloride secretory cell to an absorptive villus-like cell, or inhibition of CFTR synthesis and/or trafficking to the apical membrane. Data is presented in favour of the latter scenario, such that exposure to TGFβ reduces CFTR

mRNA expression, protein synthesis, and protein accumulation in the apical cell membrane. In defining the mechanism responsible for TGFβ-induced diminished cAMP-driven chloride secretion, we identify TGFβ as a novel regulator of the CFTR. With clear-cut implications for the maintenance of water balance, selective regulation of the CFTR by TGFβ may offer therapeutic potential in secretory enteropathies characterized by diarrhea and severe water loss.

ACKNOWLEDGEMENTS

KH has been a recipient of a Natural Sciences and Engineering Research Council studentship (2001-2003). This work was funded by a CIHR grant (MT-13421) to DMM. We thank Jun Lu for technical assistance.

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Chapter 4

TGFβ Enhances Intestinal Epithelial Barrier and Protects Against EHEC O157:H7 Infection by Differentially Regulating Claudins.

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Manuscript submitted to ***Gastroenterology*** July 2004

ABSTRACT

Enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) is an enteric pathogen which causes potentially fatal symptoms following intimate adhesion, modulation of intestinal epithelial signal transduction, and alteration of epithelial function (e.g. barrier disruption). Although the epithelial barrier is critical to gut homeostasis, TGFβ is one of the few agents known to enhance or protect epithelial barrier function. Therefore, our aim was to delineate the mechanism(s) behind TGFβ-induced barrier enhancement and determine if TGFβ could protect against EHEC-induced barrier disruption. Using human T84 colonic epithelial monolayers, we show TGFβ induces a significant increase in transepithelial electrical resistance (TER; a measure of paracellular permeability) through activation of ERK/MAPK and SMAD signaling pathways, and up-regulation of the tight junction protein, claudin-1. Additionally, epithelial pretreatment with TGFβ completely prevented the decrease in TER caused by infection with EHEC. EHEC infection markedly down-regulated claudin-2 protein expression and disrupted its subcellular localization from the tight junction. TGFβ treatment not only prevented both of these effects, but also inhibited EHEC-induced disruption of the tight junction-associated protein, ZO-1. These studies illustrate mechanisms underlying TGFβ-induced epithelial barrier enhancement and furthermore, identify TGFβ as an agent capable of blocking EHEC-induced increases in epithelial permeability via maintenance of claudin-2 and ZO-1 levels, providing insight into EHEC pathogenesis.

INTRODUCTION

Infection with the intestinal pathogen, enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) causes diarrhea, hemorrhagic colitis, and can lead to hemolytic uremic syndrome in some individuals, the latter being a result of EHEC-derived Shiga toxins (1). Enteropathogenic *E. coli* (EPEC) is a related pathogen that causes much morbidity and mortality by eliciting watery diarrhea (2). Antibiotics although useful in certain cases of EPEC infection, are largely ineffective and may even be harmful during EHEC infection, leaving no effective medical therapy for infected individuals (3). Thus, understanding the interaction(s) between EHEC and the enteric epithelium may be important in defining novel therapeutic targets.

Under normal circumstances the epithelial lining of the gut forms a semi-permeable barrier, the selectivity of which is in large part determined by the intercellular tight junctions (TJ). The TJs consist of inter-digitating homo-dimers of occludin and one or more claudins that are linked to the actin cytoskeleton via a complex of proteins including the zonula occludens (ZO) 1, 2 and 3 (4, 5). Recent evidence suggests that members of the claudin family play a critical role in tight junction formation (6) and permeability characteristics in a variety of tissues including the gut. Claudin-1 in particular is believed to regulate epithelial permeability, as evidenced by the lethality of claudin-1-deficient mice (7), reduced claudin-1 expression after IFNγ-induced barrier defects in thyrocytes (8), increased claudin-1 expression leading to barrier enhancement of airways and kidney epithelia (9, 10), and the significant redistribution of claudin-1 at the onset of rotavirus-induced paracellular permeability in intestinal epithelial monolayers

(11). Investigations with model epithelial cell lines have shown that EHEC forms intimate attaching and effacing lesions on host cells by eliciting and modulating a variety of intracellular signaling pathways to reorganize the cytoskeleton while evoking a significant increase in epithelial permeability. This diminished barrier function is associated with loss of ZO-1 from the TJ region (12), and may be of pathological significance by allowing pathogens and antigens unimpeded access to the mucosa. Indeed, the paracellular pathway may be one way for Shiga toxins to enter the circulation since the receptor for the toxin has not been identified on human gut epithelium (13). Thus, agents capable of enhancing or preserving the epithelial barrier, through preservation of the tight junction or via modulating intracellular signaling pathways that compete with those activated by bacteria, may be of value in reversing the effects of infection with EHEC.

Transforming growth factor β (TGFβ) is a multifunctional cytokine with immunosuppressive properties produced by many cell types including intestinal epithelia, and can enhance epithelial barrier function. For instance, monolayers of human-derived colonic epithelia treated with TGFβ display an increase in transepithelial electrical resistance (TER) (14). Moreover, TGFβ also prevents or reduces epithelial barrier disruption caused by *Cryptosporidium parvum* infection, exposure to IFNγ, or exposure to conditioned medium from activated immune cells (14, 15, 16, 17). However, neither the ability of TGFβ to ameliorate EHEC-induced barrier disruption nor the mechanisms underlying preservation of barrier have been reported. Thus, the aim of the present study was two-fold: 1) to define the mechanism behind TGFβ enhancement of epithelial barrier

function in terms of intracellular signaling pathways and TJ protein expression; and 2) to characterize the effects of EHEC on claudin expression and test the hypothesis that TGFβ would block the EHEC-induced increase in epithelial permeability.

METHODS

Cell and bacterial culture. T84 cells were grown and maintained in a 1:1 (vol/vol) mixture of Dulbecco Modified Eagle medium and Ham's F-12 medium, supplemented with 10% fetal calf serum, 1.5% HEPES, and 2% penicillin-streptomycin (all from Life Technologies, Grand Island, NY, USA) at 37°C, 5% CO_2. EHEC O157:H7 (strain CL-56) (12) and EPEC O127:H6 (strain E2348/69) (18) were maintained on 5% sheep blood agar plates (PML Microbiologicals, Mississauga, ON, Canada) at 4°C, cultured overnight in Luria-Bertani (LB) broth at 37°C with continuous agitation, diluted in LB broth, and then grown for 4-6 h (to mid-logarithmic growth phase) prior to T84 monolayer infection. The bacterial suspension was pelleted (2500 rpm, 20 min), resuspended in antibiotic-free T84 cell culture medium, and 10^8 CFU were added to the apical surface of epithelial monolayers (100 moi; i.e. 100 bacteria: 1 epithelial cell). A laboratory strain of non-pathogenic *E. coli*, HB101, was used as a control and was cultured as described above (19).

Measurement of epithelial barrier. T84 cells were seeded onto filter supports (10^6 cells/well, surface area 1 cm^2; Costar Inc., New York, NY, USA) and grown to confluence (≥ 6 days), at which point transepithelial electrical resistance (TER) was monitored by a voltmeter and chopstick electrodes (Millicell-ERS, Millipore, Bedford, MA, USA). To assess the effects of TGFβ, cytokine was added to the basolateral surface of T84 monolayers (human recombinant $TGF\beta_1$, 10 ng/ml; R&D Systems, Minneapolis, MN, USA) and TER was recorded at 4, 8, 16, 24, 48, and 72 h post-treatment. Based on

previous studies showing TGFβ has barrier-enhancing effects at doses of 1-100 ng/ml (14, 20), we selected a dose of TGFβ at 10 ng/ml for each study. The acute effects of TGFβ were assessed by "wash out" experiments, where monolayers were exposed to TGFβ for 1 h, rinsed twice with culture media, and maintained daily in fresh culture media with TER recorded at 24 h intervals. For pharmacological inhibition of ERK MAPK, T84 monolayers were pretreated with the MEK1 inhibitor PD98059 (apical and basolateral, 1 h, 25 μM; Calbiochem, San Diego, CA, USA (20)), exposed to TGFβ (10 ng/ml, 1 h), rinsed and maintained with fresh culture media for 72 h, and TER recorded. In bacterial co-culture experiments, T84 monolayers were treated with TGFβ (10 ng/ml) 45 min prior to infection, with TGFβ left in for the duration of infection, and TER recorded 16 h later, a time point at which EHEC infection has been shown to reduce epithelial barrier function (12).

Adenoviral infection. T84 cells were seeded as described above and, at 4 h post-seeding, infected with replication-deficient adenovirus contructs encoding SMAD7 for 16 h (Ad-SMAD7, 50 moi; a generous gift from Dr. ten Dijke, Netherlands Cancer Institute) (21, 22). Cells were rinsed, grown for 3 days, and then treated with TGFβ (10 ng/ml, 24 h) and TER recorded 72 h post-cytokine exposure.

Immunoprecipitation for SMAD2/3. T84 monolayers were seeded onto filter supports ($3x10^6$ cells/well, surface area 4.7 cm^2; Costar Inc.), grown to confluence, serum-starved for 24 h, and treated with TGFβ (10 ng/ml; 5-60 min). Whole cell protein

extracts were prepared, protein concentrations determined (23), and samples (1 mg/ml in PBS) incubated with gentle agitation at room temperature for 2 h with mouse anti-human SMAD2/3 antibody (2 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by centrifugation and incubation with protein A-conjugated beads (Easy-view, Sigma-Aldrich, St. Louis, MO, USA). Immunoprecipitated SMAD2/3 was recovered following the manufacturer's protocol and loaded immediately onto a 10% SDS-PAGE gel.

Immunoblotting. Samples were prepared from T84 monolayers as described above ± TGFβ (10 ng/ml) ± bacteria (100 moi). Proteins (40 μg/sample) were electroblotted to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA) and blocked in 5% BSA in Tris-buffered saline (containing 0.1% Tween-20, TBST) or 5% milk diluted in TBST, according to the manufacturer's directions. *Phospho-SMAD2/3, SMAD2/3 and SMAD7*: Blots were probed with mouse anti-human phospho-serine antibody (phospho-SMAD2/3), rabbit anti-human SMAD2/3, or goat anti-human SMAD7 diluted in BSA-TBST (1:1000, 1:1000, and 1:750, respectively; 1 h room temperature; Abcam, Cambridge, UK), washed in TBST, and exposed to the appropriate HRP-conjugated secondary antibodies (1:3000; Santa Cruz Biotechnology). *FLAG*: Blots were probed with mouse anti-FLAG diluted in milk-TBST for 1 h at room temperature (1:1000; Sigma-Aldrich), washed in TBST, and exposed to HRP-conjugated rabbit anti-mouse IgG (1:3000; 1 h room temperature; Santa Cruz Biotechnology). *Phospho-ERK1/2 and tight junction proteins*: Blots were either probed for 1 h at room temperature (mouse

anti-human phospho-ERK1/2, 1:800; rabbit anti-human ERK1/2, 1:3000: Santa Cruz Biotechnology) or overnight at 4°C (rabbit anti-human claudin-1, 0.5 μg/ml; rabbit anti-human claudin-2, 1.0 μg/ml; mouse anti-human claudin-4, 1.5 μg/ml; rabbit anti-human occludin, 0.25 μg/ml, rabbit anti-human ZO-1, 1.5 μg/ml: Zymed Laboratories, San Francisco, CA, USA), washed in TBST (x5) and then exposed to the appropriate HRP-conjugated secondary antibodies (1:2000–1:15000; 1 h room temperature; Santa Cruz Biotechnology). Immunoreactive proteins were visualized using chemiluminescence (ECL, Amersham Pharmacia, Piscataway, NJ, USA) and exposure to Kodak XBL film (Eastman Kodak Company, Rochester, NY, USA). Tight junction protein immunoblots were stripped and re-probed for β-actin (goat anti-human actin, 1:200; bovine anti-goat 1:400; Santa Cruz Biotechnology) to assess total protein loading.

Confocal microscopy. T84 cells were seeded onto filter supports (10^5 cells/well, surface area 0.33 cm^2) and either treated with TGFβ for 72 h (10 ng/ml) or infected with EHEC O157:H7 for 16 h (100 moi) ± TGFβ pretreatment for 45 min (10 ng/ml) (as described above). Monolayers were rinsed in PBS, fixed in 4% formalin, permeabilized with 0.1% TritonX-100 (Mallinckrodt Inc., Paris, KY), and blocked for 30 min in blocking solution (5% BSA/5%goat serum (Sigma-Aldrich) in 0.1% Triton X-100). Primary and secondary antibodies were diluted in blocking solution (rabbit anti-human claudin-1, 15 μg/ml, mouse anti-human claudin-2, 2 μg/ml, or rabbit anti-human ZO-1, 2 μg/ml, Zymed Laboratories; Alexa Fluor® 488 goat anti-rabbit IgG or Alexa Fluor® 488 goat anti-mouse IgG, 1.5 μg/ml; Molecular Probes, Eugene, OR, USA) and exposed to monolayers for 1 h at room temperature. After extensive washing, filters were excised

from the polystyrene filter supports, and mounted on glass slides in Gel/Mount anti-fade reagent (Biomeda Corporation, Foster City, CA). Images were acquired using an inverted Zeiss laser scanning microscope (LSM 510, Axiovert 100M; Oberkochen, Germany) equipped with argon (450-514 nm). Immunoreactive claudin and ZO-1 was excited using the 488 nm laser line and collected using a standard fluorescein isothiocyanate (FITC) filter set. For each experiment, image acquisition (i.e. confocal microscope settings) and processing was identical between controls and TGFβ, EHEC, or TGFβ+EHEC treated cells.

[^{3}H]-thymidine incorporation. T84 cells were seeded onto 96-well plates ($4x10^4$ cells/well) and grown for 4 days. [^{3}H]-thymidine (1μCi/well; DuPont-New England Nuclear, Wilmington, DE) was added for the duration of TGFβ treatment (10 ng/ml, 72 h). Cells were harvested onto glass-fiber filter discs and assayed in 5 ml aqueous counting scintillant for beta-ray emission and counts per minute determined in a scintillation counter (Becton Dickinson, Mississauga, ON, Canada). Results are expressed as mean cpm ± SEM.

Bacterial growth. EHEC was cultured to mid-logarithmic growth phase (described in *cell and bacterial culture*), resuspended in antibiotic-free T84 cell culture media ± TGFβ (10 ng/ml), and incubated at 37°C with continuous agitation for 16 h. Bacterial growth was determined by spectrophotometric analysis of each sample (OD_{600}: $0.1=10^9$ CFU). In similar experiments, supernatants from naïve T84 monolayers or

TGFβ-treated T84 cells were added to EHEC in mid-logarithmic growth phase, cultured as above, and OD_{600} measured.

IL-8 ELISA. T84 cells were seeded onto filter supports (10^6 cells/well, surface area 1 cm^2), grown to confluence and infected with EHEC ± TGFβ pretreatment (as described above) for 16 h. Subsequently, culture medium samples were collected from the basolateral wells and frozen at -20°C until the concentration of IL-8 was determined by commercial ELISA (R&D Systems). Detection limit of the assay was 16 pg/ml.

Statistical Analysis. Data are presented as means ± standard error of the mean (SEM). Data were analyzed using one-way ANOVA followed by post-hoc comparisons using Tukey's test, or Student's t-test, with $p<0.05$ accepted as the level of statistical significance.

RESULTS

TGFβ-induced increase in epithelial barrier function is mediated by ERK MAPK and SMAD signaling pathways. Figure 1 shows that TGFβ (10 ng/ml) evokes a time-dependent increase in TER, that is significant at 16 h post-treatment and more pronounced at 72 h when there is a two-fold increase above pretreatment values. Epithelial TER is similarly increased 72 h after a single 1 h pulse with TGFβ, indicating that acute exposure to this cytokine has long-term effects (Figure 1, bar labeled $72h^{\Delta}$). Indeed, our earlier publication that 1 h TGFβ treatment causes alterations in epithelial ion transport 16 h later supports this observation (20).

TGFβ is known to activate the ERK MAPK pathway and mobilize SMAD proteins in multiple cell types (24). Figure 2a is an immunoblot showing ERK 1/2 activation (i.e. increased phosphorylation) in whole cell protein extracts of T84 cells after 15 and 30 min of TGFβ exposure, while figure 2b shows SMAD2/3 activation was notably increased 30-60 min post-cytokine treatment. The naturally-occurring inhibitor of the SMAD signaling pathway, SMAD7, is typically induced following TGFβ activation and provides a negative feedback mechanism for TGFβ signaling (24). In TGFβ-treated T84 epithelia, SMAD7 expression was increased 4 h after cytokine exposure and remained elevated at 24 h (end of experiment, Figure 2c). Inhibition of ERK MAPK and SMAD signaling via the pharmacological inhibitor PD98059 and Ad-SMAD7, respectively, completely prevented the TGFβ-induced increase in epithelial TER (Figure 3).

TGFβ stimulates increased expression of the tight junction protein claudin-1. To determine the structural mechanism behind epithelial barrier enhancement, we compared tight junction protein expression between TGFβ-treated T84 monolayers and untreated controls. TGFβ exposure (10, 50, or 100 ng/ml, 72 h) resulted in specific up-regulation of claudin-1, but not claudin-2 or claudin-4 protein expression, demonstrated by a 2- to 3-fold increase above control levels (Figure 4a and b). Neither occludin nor ZO-1 expression was affected by TGFβ treatment (data not shown), indicating the effect is specific to claudin-1. Confocal microscopy confirmed the increase in claudin-1 expression following TGFβ treatment, and also demonstrated increased protein localization to the tight junction (Figure 4c). This effect was not due to increased proliferation, as [^{3}H] thymidine incorporation was actually reduced after 72 h of TGFβ exposure (31,477 ± 4,861 vs. 5,294 ± 612 cpm, control vs. TGFβ respectively, n=12; $p<0.001$).

TGFβ prevents epithelial barrier dysfunction caused by EHEC. TGFβ protects T84 monolayers from barrier dysfunction caused by infection with *Cryptosporidium parvum* (15). Thus, we tested whether TGFβ (10 ng/ml) could prevent the barrier dysfunction caused by infection with pathogenic bacteria by challenging TGFβ-treated T84 monolayers with EHEC O157:H7 (100 moi, 16 h). Figure 5 shows that treatment with TGFβ for 45 min prior to bacterial infection completely prevented the decrease in epithelial TER.

Infection with the related enteric pathogen, EPEC, is also known to cause epithelial barrier disruption (18). Similar to EHEC infection, TGFβ pretreatment protected T84 monolayers against barrier dysfunction induced by EPEC (103.1 ± 1.7 vs. 57.2 ± 3.3* % pretreatment TER, respectively; * $p<0.01$ compared to control pretreatment values (95.2 ± 3.3 %); n=3 monolayers).

TGFβ-induced barrier protection is independent of an effect on bacterial growth. Cytokines can possess antimicrobial activity (25). Thus we tested if TGFβ itself, or epithelial factors induced by TGFβ (e.g. antimicrobials), could impair EHEC growth. TGFβ did not directly affect EHEC viability after 16h of co-incubation (OD_{600}: EHEC 1.3 ± 0.1 vs. TGFβ + EHEC 1.3 ± 0.1; n=2). Bacterial growth was similarly unaffected by supernatants from TGFβ-treated T84 monolayers (data not shown), suggesting that an epithelial-derived factor was also not involved.

EHEC infection causes severe down-regulation of claudin-2 expression, which can be inhibited by TGFβ. Infection with pathogenic bacteria, such as EHEC, EPEC, and *Shigella flexneri*, decreases expression of tight junction (TJ) and TJ-associated proteins (12, 26, 27, 28). However, little is known about their effects on claudin expression. Figure 6a shows that EHEC infection causes a dramatic decrease in claudin-2 expression, but not claudin-1 or claudin-4 in whole cell protein extracts of T84 cells. Similarly, claudin-2 protein expression was dramatically reduced by EPEC in identical experiments (data not shown). In marked contrast, claudin-2 expression is preserved in EHEC-

infected T84 monolayers pretreated with TGFβ (Figure 6b). Furthermore, as assessed by confocal microscopy (Figure 6c), claudin-2 expression in T84 monolayers is significantly disrupted after EHEC infection compared to control cells. TGFβ stimulation markedly preserved the subcellular distribution of claudin-2 in the face of EHEC infection (Figure 6c, right panel).

TGFβ prevents EHEC-induced ZO-1 removal from the tight junction. Philpott et al. showed that T84 monolayers infected with EHEC display disrupted ZO-1 staining at the cell periphery (12). Using confocal miscroscopy we show that epithelial monolayers treated with TGFβ alone display staining patterns comparable to controls (Figure 7, top panels). In contrast, EHEC infected T84 cells (100 moi, 16 h) have strikingly reduced ZO-1 protein expression, with a significant lack at the cell periphery (Figure 7, bottom left panel). However, treating with TGFβ 45 min (10 ng/ml) prior to EHEC infection resulted in a ZO-1 distribution resembling that of control monolayers (Figure 7, bottom right panel).

TGFβ does not prevent EHEC induction of IL-8 production by intestinal epithelia. EHEC infection induces IL-8 production from intestinal epithelia (29). Given the protective effects of TGFβ on barrier function, we also tested whether pretreatment with TGFβ would prevent EHEC-induced IL-8 production from T84 monolayers. In comparison to untreated control monolayers or TGFβ-treated epithelia which produced 108.4 ± 18.4 and 130.4 ± 20.4 pg/ml IL-8, respectively, EHEC-infected T84 cells

produced significantly greater amounts of IL-8 that was not prevented by TGFβ pretreatment (192.9 ± 27.1* and 186.4 ± 26.7* pg/ml, respectively; * $p<0.05$ compared to control and TGFβ only; n=5-6 monolayers).

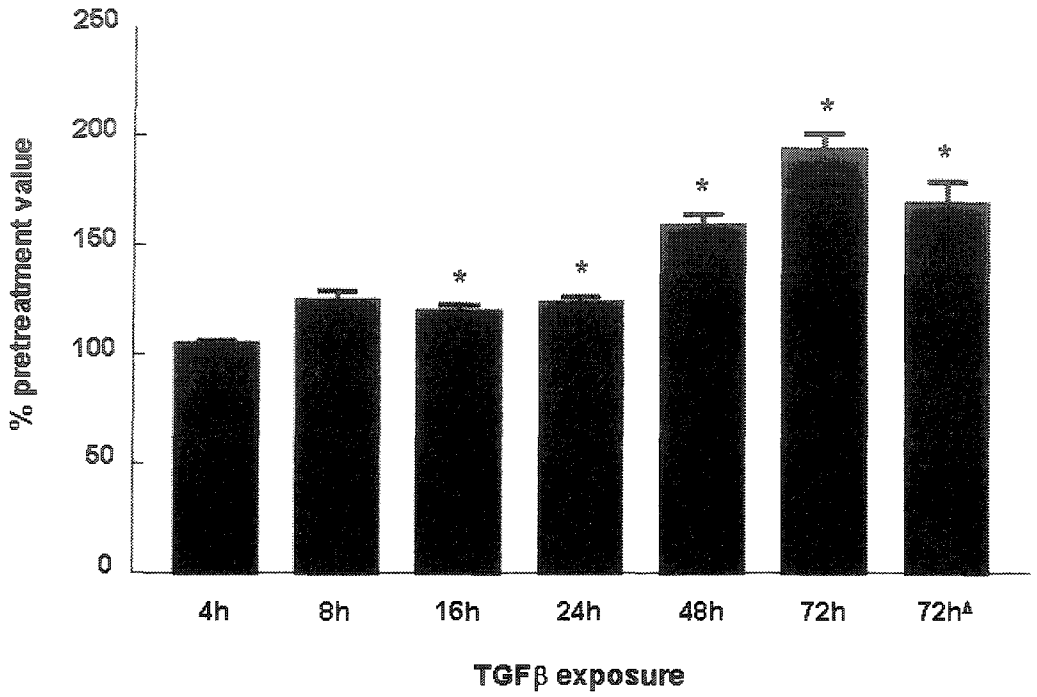

Figure 4.1. Bar chart showing increased TER in T84 monolayers after continuous TGFβ treatment (10 ng/ml, 4-72 h) or following a 1 h pulse with cytokine (denoted as $72h^{\Delta}$). Epithelial TER values were recorded prior to cytokine treatment for comparison against TER values at the end of each timepoint (indicated as % pretreatment value), and these were compared against naïve, time-matched controls (mean ± SEM, $*P<0.05$, n=3-10 monolayers). Pretreatment TER values were 609 ± 189 and 607 ± 158 Ω/cm^2 for naïve controls and pre-TGFβ, respectively (mean ± SEM, n=10 monolayers).

Figure 4.2 (following pages). TGFβ activates ERK/MAPK and SMAD signaling pathways in colonic epithelia. (**a**) Immunoblot of serum-starved TGFβ-treated (10 ng/ml) T84 monolayers probed for phosphorylated ERK1/2 (p-ERK) and total ERK1/2. (**b**) SMAD2/3 immunoprecipitates from TGFβ-treated (10 ng/ml) T84 monolayers probed for phosphorylated serine (denoted as p-SMAD2/3), stripped and re-probed for total SMAD2/3. (**c**) Immunoblot of serum-starved, TGFβ-treated (10 ng/ml) monolayers probed for the inhibitory SMAD7. All three panels depict representative images from three separate experiments (arrows: lanes where protein expression is visibly different from untreated controls).

A

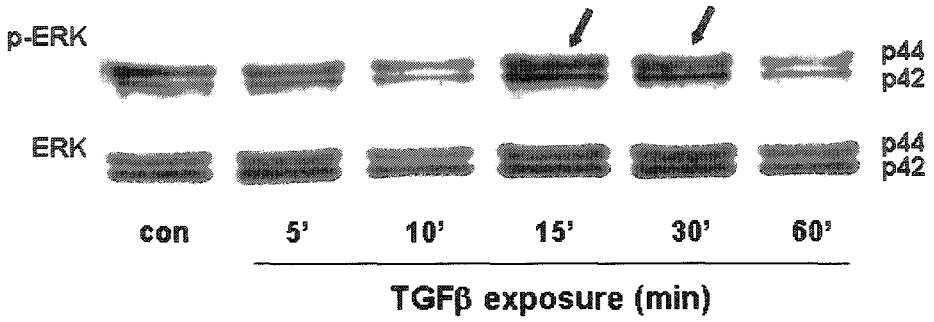

Figure 4.2a

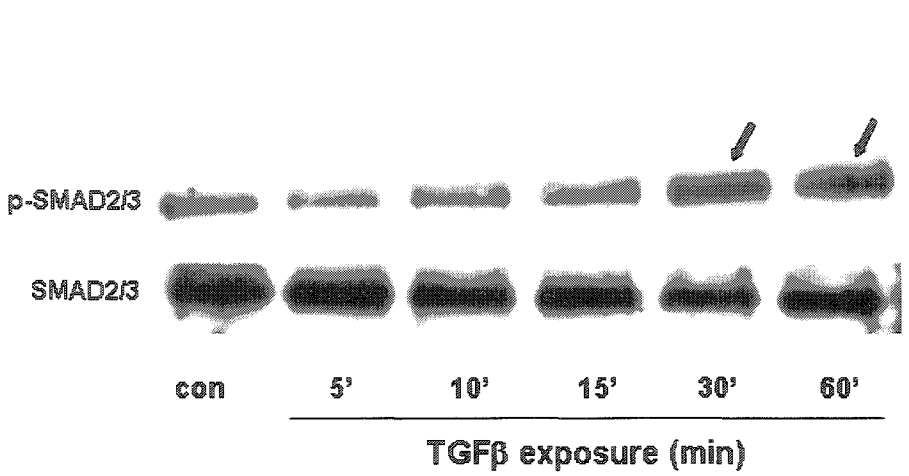

Figure 4.2b

C

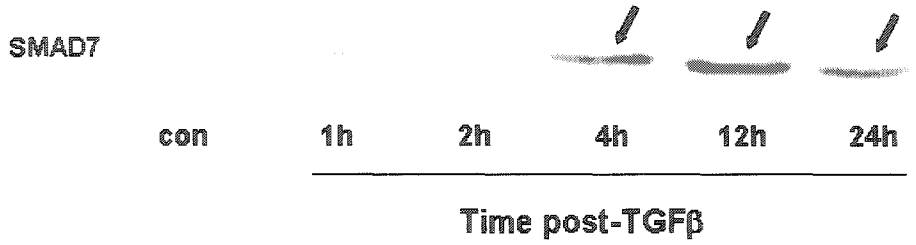

Figure 4.2c

Figure 4.3 (following pages). Inhibition of ERK/MAPK and SMAD signaling pathways prevents the TGFβ-induced increase in TER. T84 monolayers were pretreated with an inhibitor of ERK/MAPK signaling (PD98059, 25 μM) ± TGFβ (10 ng/ml) (**a**) or were infected with an adenovirus encoding inhibitory SMAD7 (Ad-SMAD7, 50 moi) ± TGFβ (10 ng/ml) (**b**) and TER values (% change from pretreatment) were compared against naïve controls (means ± SEM, $*P<0.05$, n=9-12 monolayers; average control TER values were 784 ± 251 Ω/cm^2). Inset: T84 whole cell extracts probed for FLAG-tagged adenoviral SMAD7 protein expression, confirming adenoviral infection.

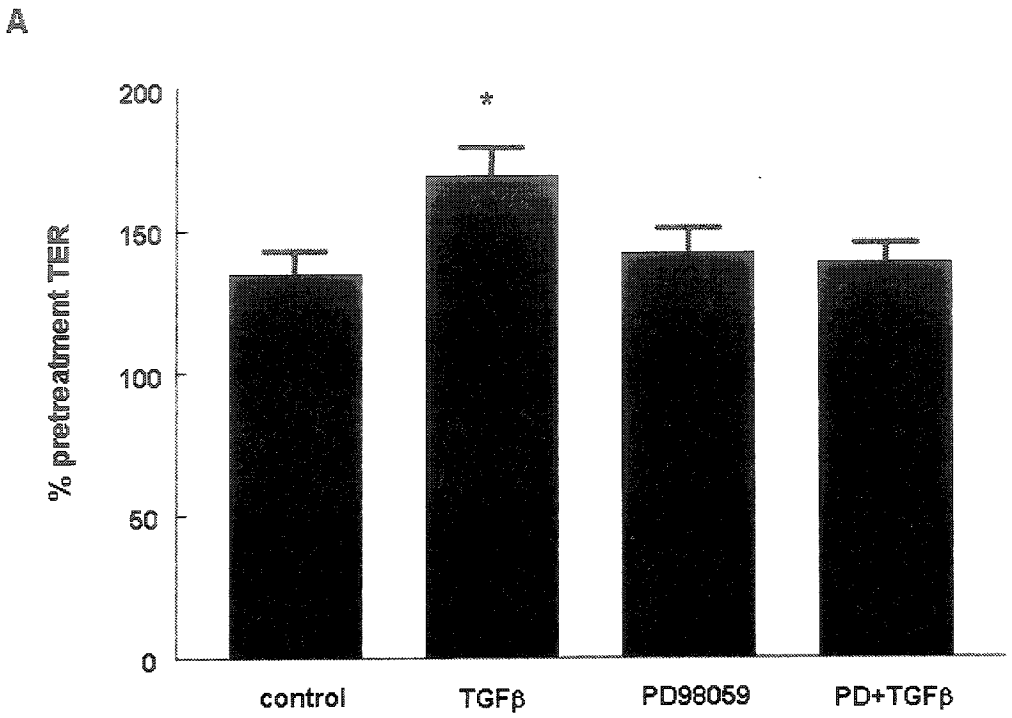
A
200
150
100
50
0
% pretreatment TER
*
control
TGFβ
PD98059
PD+TGFβ

Figure 4.3a

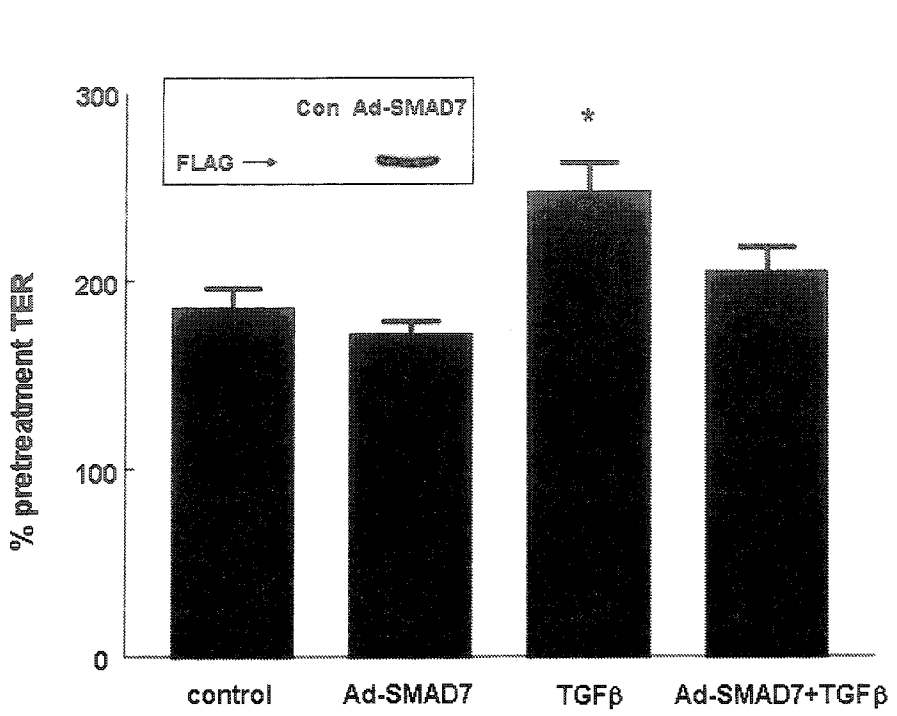
B
300
200
100
0
% pretreatment TER
Con Ad-SMAD7
FLAG
*
control
Ad-SMAD7
TGFβ
Ad-SMAD7+TGFβ

Figure 4.3b

Figure 4.4 (following pages). TGFβ increases T84 epithelial claudin-1 expression. (**a**) Immunoblots of TGFβ-treated (72 h) T84 monolayers probed for claudin-1, claudin-2, or claudin-4 were stripped and re-probed for β-actin as a control. Representative images of three separate experiments (arrows: lanes where claudin-1 expression is visibly increased compared to untreated controls). (**b**) Densitometric analysis of claudin-1 immunoblot depicted above (Kodak 1D Imagining Software, Guelph, ON, Canada). For each sample, the integrated pixel intensity of claudin-1 was compared to that of the internal control for protein loading, β-actin, and is expressed as a ratio of claudin-1 to β-actin. (**c**) Representative images of T84 monolayers ± TGFβ (10 ng/ml, 72 h) immunostained for claudin-1 taken in the x-y plane using confocal microscopy. Claudin-1 expression is localized to the cell periphery, the region where tight junction formation occurs (n=6 monolayers).

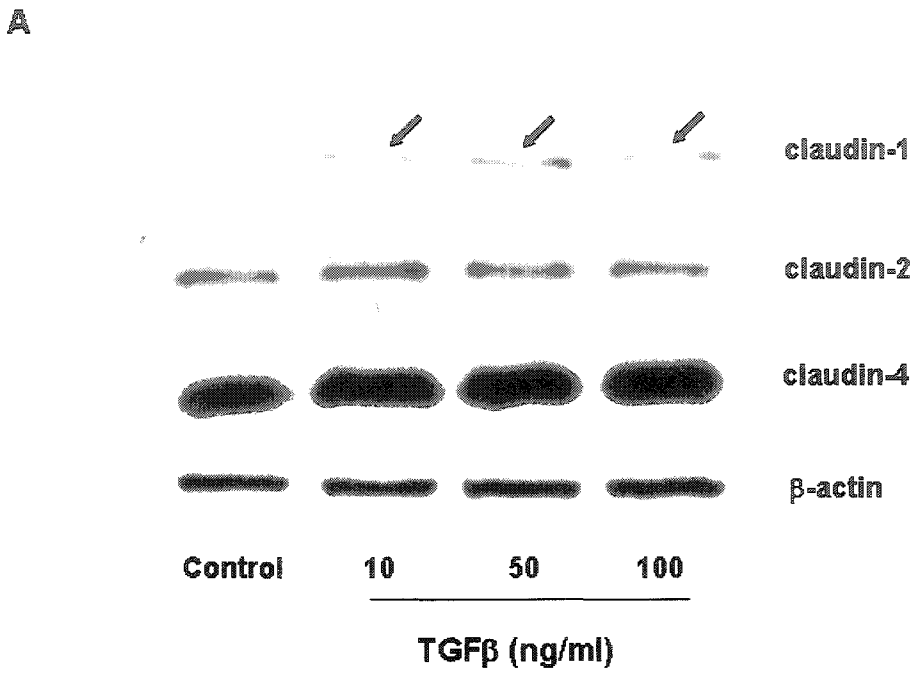
A
claudin-1
claudin-2
claudin-4
β-actin
Control
10
50
100
TGFβ (ng/ml)

Figure 4.4a

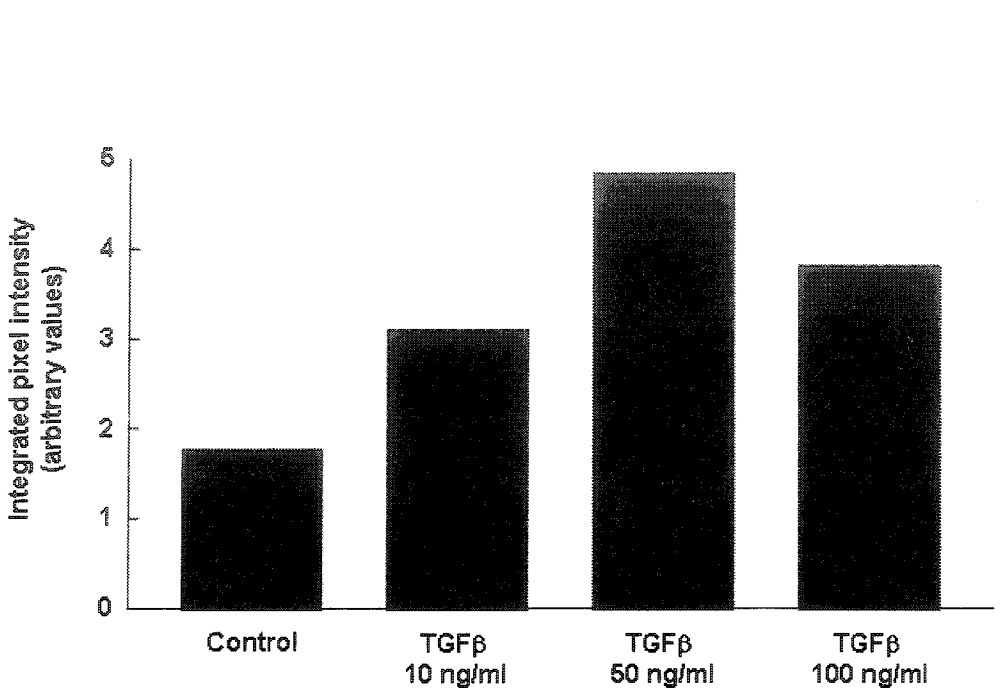
B
Integrated pixel intensity (arbitrary values)
0
1
2
3
4
5
Control
TGFβ 10 ng/ml
TGFβ 50 ng/ml
TGFβ 100 ng/ml

Figure 4.4b

C

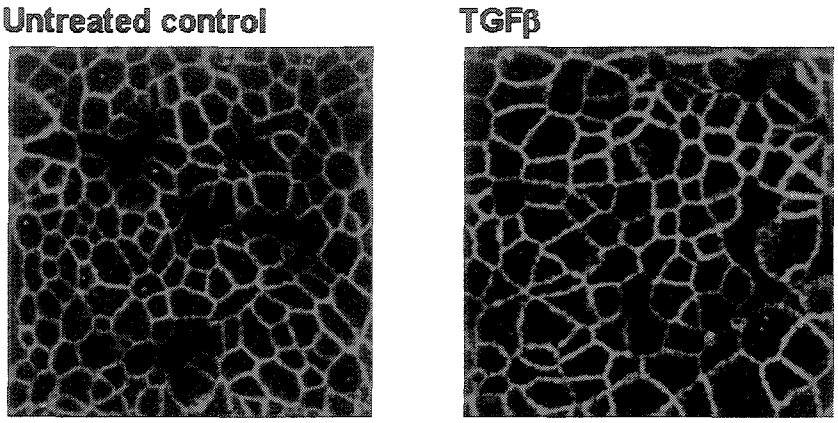

Figure 4.4c

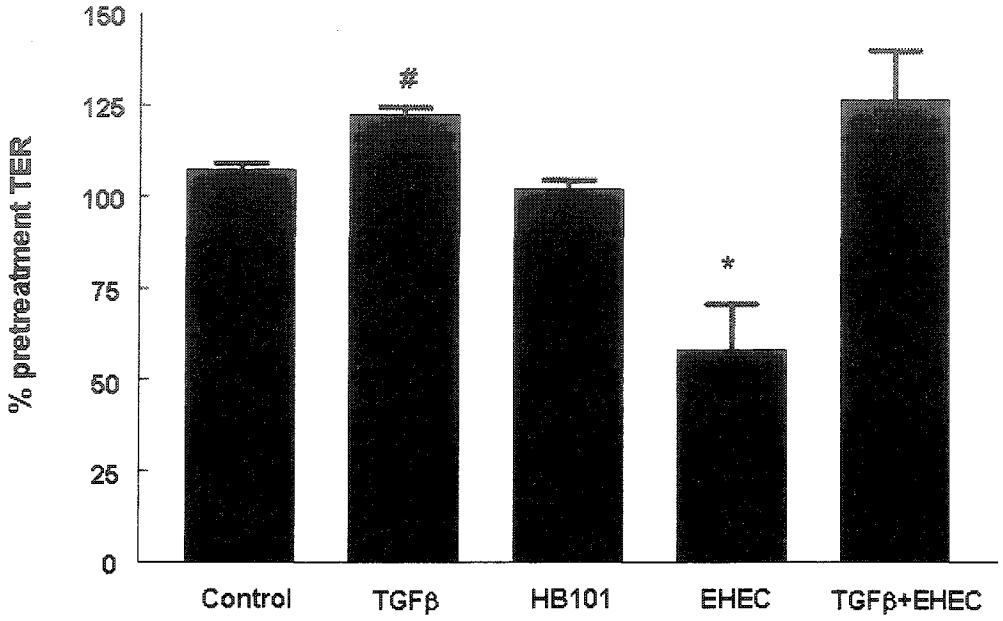

Figure 4.5. Bar chart showing T84 monolayers pretreated with TGFβ (45 min, 10 ng/ml) prevents the decrease in TER caused by EHEC (16 h post-infection). Controls consisted of untreated monolayers or infection with the non-pathogenic *E. coli*, HB101. Data is presented as the percent change in TER compared to pretreatment values (means ± SEM, $^{*}P<0.05$ compared to untreated controls and HB101; n=6 monolayers). Note that TGFβ treatment alone causes a small, but significant increase in TER after 16 h ($^{\#}P<0.05$ compared to control), consistent with the timecourse data presented in Figure 1. Average pretreatment TER values ranged between 432-931 Ω/cm^2.

Figure 4.6 (following pages). The effects of EHEC on claudin-2 expression are prevented by TGFβ. (**a**) Immunoblots of EHEC-treated T84 monolayers probed for claudin-1, claudin-2, or claudin-4 were stripped and re-probed for β-actin as a control. Representative images from three separate experiments (arrow: lane where claudin-2 expression is visibly decreased compared to uninfected controls). (**b**) Claudin-2 expression in EHEC-infected monolayers pretreated with TGFβ. Representative images from two separate experiments (arrow: lane where claudin-2 expression is visibly reduced). (**c**) Representative images of T84 monolayers immunostained for claudin-2 taken in the x-y plane using confocal microscopy showing claudin-2 disruption by EHEC infection and the structural preservation by pretreatment with TGFβ (n=3 monolayers).

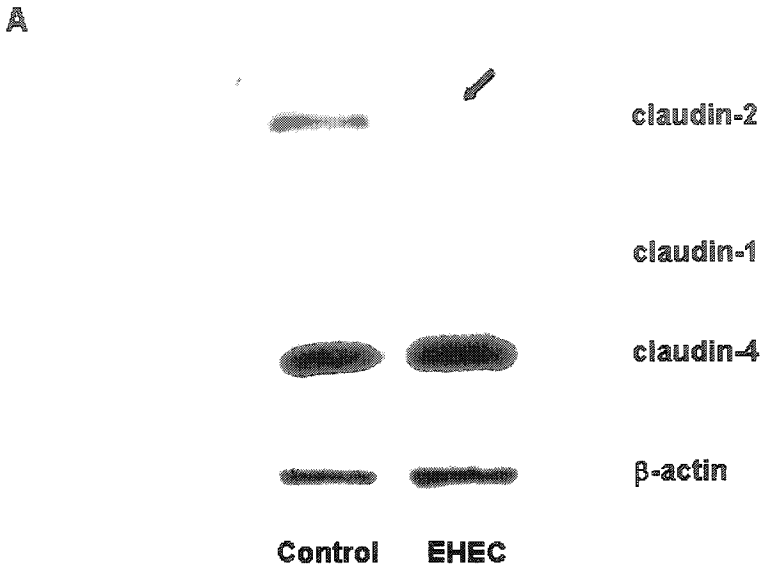

Figure 4.6a

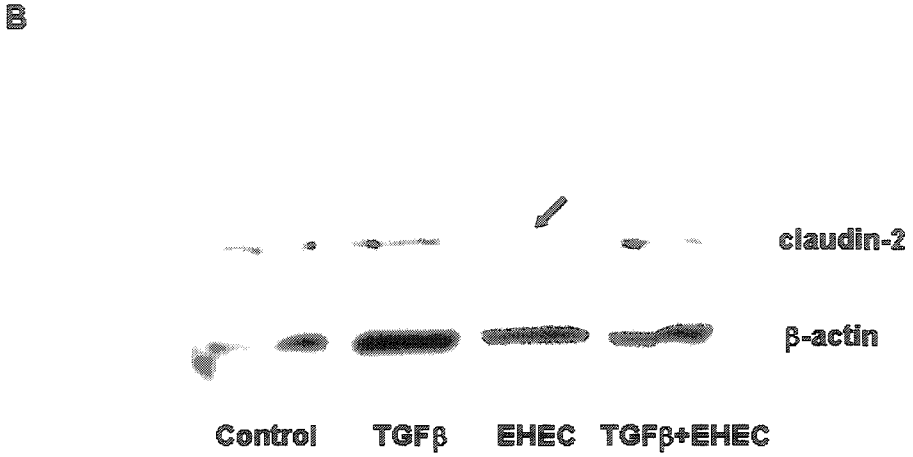

Figure 4.6b

C

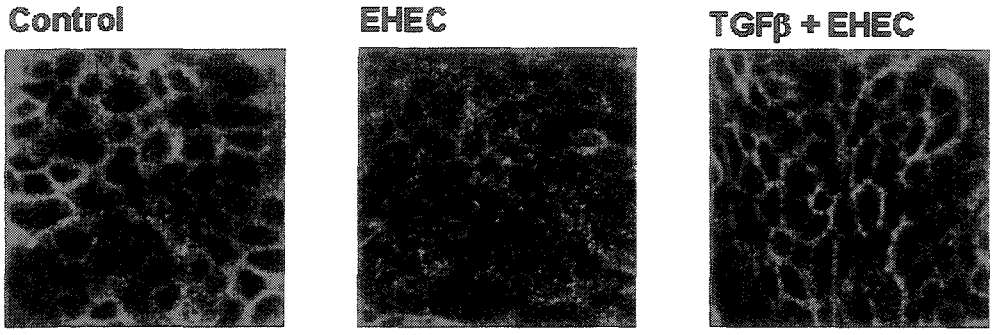

Figure 4.6c

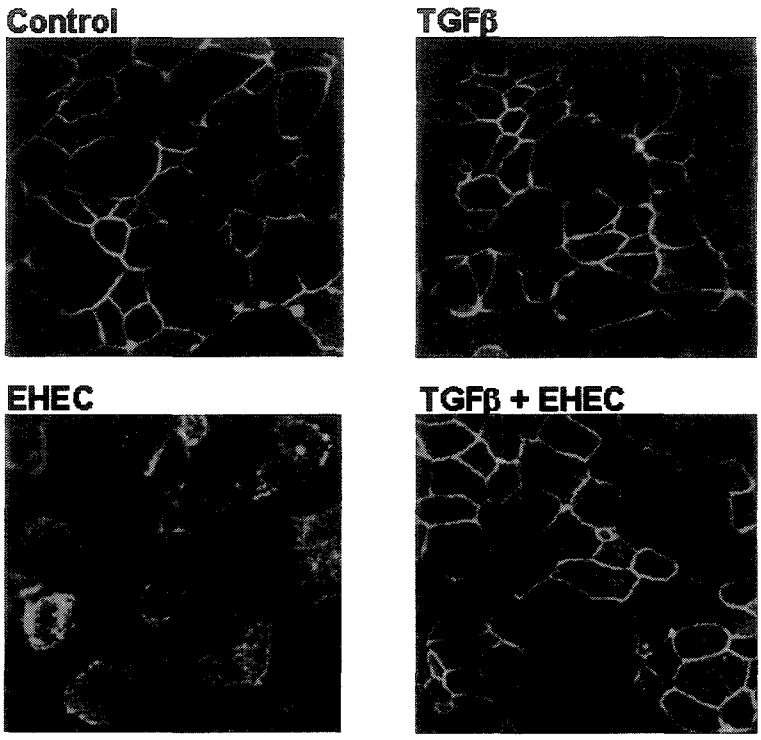

Figure 4.7. TGFβ prevents EHEC-induced altered ZO-1 distribution. Representative images of T84 monolayers immunostained for ZO-1 taken in the x-y plane using confocal microscopy (n=4-5 monolayers). Top panels show ZO-1 distribution at the tight junction in control and TGFβ-treated (10 ng/ml, 16 h) monolayers. In contrast, EHEC infection (100 moi, 16 h) causes a marked reduction in ZO-1 staining overall, with a noticeable redistribution to the centre of the cell (bottom left panel). Pretreatment with TGFβ (45 min) prior to EHEC infection preserves ZO-1 protein expression and retains it at the tight junction.

DISCUSSION

Intestinal epithelia serve multiple physiological roles, including the provision of a defensive barrier to protect against potentially harmful luminal contents such as pathogenic bacteria. The importance of maintaining an intact barrier is underscored by recent reports stating increased intestinal permeability contributes to the pathogenesis of several intestinal disorders, including inflammatory bowel disease (30). Thus, our aim was to define the mechanism behind TGFβ-induced barrier enhancement and determine if this cytokine could prevent epithelial barrier dysfunction caused by infection with pathogenic bacteria, such as EHEC. Here we showed that TGFβ enhances the epithelial barrier via ERK MAPK and SMAD signaling pathways, in addition to specifically up-regulating claudin-1 protein expression. Moreover, we determined that TGFβ preserves the epithelial barrier during EHEC infection, and prevents EHEC-induced removal of claudin-2 and ZO-1 from the tight junction.

Our studies provide mechanistic insight into TGFβ signaling pathways governing the epithelial barrier. Here we showed that TGFβ activates SMAD2/3 and ERK MAPK, and that inhibition of either pathway prevented the TGFβ-induced increase in epithelial barrier. These data suggest that cross-talk may exist between ERK MAPK and SMAD signaling cascades, such as that observed in TGFβ-treated human mesangial cells where ERK MAPK activation enhances SMAD2/3 activation (i.e. phosphorylation) (31). We cannot, however, discount the possibility that the MAPK and SMAD signaling pathways independently affect TJ function. Furthermore, the observation that SMAD7 was increased following TGFβ exposure is consistent with the paradigm of TGFβ signaling,

where SMAD2/3 activation up-regulates expression of the inhibitory SMAD7 to provide a negative-feedback loop during TGFβ-mediated signaling (24). The long-lasting effects of a 1 h TGFβ pulse on epithelial barrier shown here may reflect downstream effects of transiently-induced SMAD-dependent gene transcription rather than constant SMAD signaling. This lasting effect of TGFβ on epithelial barrier enhancement is unique in comparison to other cytokines, for example IFNγ, where the cytokine-induced barrier defect is not observed with exposure duration of less than 6 h (personal observation).

In addition to TGFβ-mediated signaling events, we determined structural effects of TGFβ on the tight junction. TGFβ exposure led to increased claudin-1, but not claudin-2, claudin-4, occludin, or ZO-1 protein expression, an effect that was independent of dose. By also demonstrating this increase in claudin-1 protein expression is localized to the tight junction, these data delineate the cellular changes and signaling pathways responsible for mediating TGFβ-induced intestinal epithelial barrier enhancement. Our observations are supported by the work of Kinugasa *et al.*, who demonstrated that IL-17 enhances development of the paracellular barrier in immature T84 epithelial monolayers by activation of ERK MAPK and up-regulation of claudin-1 and claudin-2 mRNA and protein expression (32). However, unlike the effects of IL-17, TGFβ does not increase claudin-2 protein expression. This difference indicates cytokine-specific effects on TJ proteins and suggests distinct roles for claudins during epithelial monolayer development versus barrier enhancement of an established monolayer. Indeed, a protein kinase C agonist, bryostatin-1, enhances the barrier function of confluent T84 monolayers by recruiting claudin-1, ZO-2, and occludin to the tight junctional complex (33). While the

contribution of other tight junction proteins is less clear, up-regulation of claudin-1 appears to be a common mechanism by which colonic epithelial barrier function can be established and/or enhanced.

In addition to barrier enhancement, TGFβ preserves intestinal epithelial barrier function following exposure to agents known to cause barrier disruption, such as IFNγ, supernatants from superantigen-stimulated immune cells, and infection with *Cryptosporidium parvuum* (14, 15, 17). Here we showed that TGFβ protects against intestinal epithelial barrier dysfunction caused by infection with enterohemorrhagic *Escherichia coli*. This occurred in a manner that was independent of direct effects on EHEC growth or viability. Our observation that TGFβ also preserved epithelial barrier function during infection with another enteric pathogen, enteropathogenic *E. coli*, perhaps suggests TGFβ has broader protective effects that span a spectrum of enteric pathogens.

Pathogenic bacteria, including EHEC and EPEC, have been shown to cause removal of tight junction proteins from epithelial monolayers following infection. While EPEC infection causes dephosphorylation of occludin and its subsequent dissociation from the tight junction (26), EHEC infection disrupts the TJ-associated protein, ZO-1 (12). Here we observed that pretreatment with TGFβ prevents the EHEC-induced disruption of subcellular ZO-1 distribution in T84 monolayers. Also, considering the important role of claudin proteins in tight junction formation (34), and that another pathogenic bacterium, *Clostridium perfringens*, causes epithelial barrier disruption by removing claudin-4 from the tight junction (35), we examined the effects of EHEC infection on claudin expression. Our data show that EHEC specifically affects claudin-2,

but not claudin-1 or claudin-4, as exhibited by the significant reduction in claudin-2 protein expression and association with the tight junction. This effect was completely reversed by pretreating T84 monolayers with TGFβ, and suggests claudin-2 is important in TJ barrier regulation. However, at present, the exact role of claudin-2 in TJ barrier function is unclear. In a study by Furuse *et al.*, introduction of claudin-2 into high-resistance Madin Darby Canine Kidney (MDCK) epithelia caused decreased TER, and led to the concept that claudin-2 forms conductive pores within TJ strands (36). Indeed, claudin-2 has since been shown to induce cation-selective channels in tight junctions of epithelial cells (37). In contrast to Furuse *et al.*, induction of claudin-2 expression in low-resistance MDCK cells led to a 20% increase in TER (38), suggesting the role of this tight junction protein may differ between cell lines. Furthermore, using a model of T84 monolayer barrier enhancement, Nishiyama *et al.* determined that increased TER is associated with an up-regulation of claudin-2 protein expression at the TJ (39). In light of the latter two studies, our data suggest that the structural basis for barrier preservation by TGFβ lies in preventing EHEC-mediated down-regulation and removal of claudin-2 and also retention of ZO-1 at the region of the tight junction. As TGFβ does not increase claudin-2 expression, it may ameliorate the EHEC-mediated decrease in claudin-2 via inhibition of a bacteria-induced epithelial signaling pathway or by stabilization of claudin-2 mRNA.

One epithelial signaling pathway induced by EHEC infection is myosin light chain (MLC) kinase activation and its subsequent phosphorylation of MLC_{20}, which has been postulated to cause contraction of the perijunctional actomyosin ring (PAMR) and

thereby increase tight junction permeability (12, 40). We speculate that TGFβ may prevent EHEC activation of epithelial MLCK to effectively inhibit the PAMR contraction, and this may be physically linked with TJ protein removal given the nature of their association with the actin cytoskeleton. In this regard, it is noteworthy that another agent capable of protecting T84 epithelial barrier during EHEC infection, *Saccharomyces boulardii*, does so by preventing EHEC-induced MLC_{20} phosphorylation (41). In terms of other effects on host cell function by EHEC, Dahan *et al.* determined *S. boulardii* significantly decreased epithelial IL-8 secretion during EHEC infection by inhibiting the MAPK and NF-κB signaling pathways responsible for this pro-inflammatory response (41). Given the anti-inflammatory properties of TGFβ and the barrier-protective effects demonstrated in this study, the ability of TGFβ to prevent epithelial IL-8 secretion during EHEC infection was tested. TGFβ did not prevent EHEC-induced epithelial IL-8 secretion, indicating the effect of TGFβ was specific for protecting against EHEC-mediated disruption of barrier function. However, we cannot rule out the possibility that TGFβ may regulate other aspects of an EHEC-induced pro-inflammatory response. For example, TGFβ exerts anti-inflammatory properties against *Bacteroides vulgatus* and lipopolysaccharide-mediated NF-κB activation and subsequent IL-6 secretion in intestinal epithelia (42). As activation of NF-κB regulates not only IL-8 gene expression but also several other pro-inflammatory mediators such as TNFα and IL-6 (43, 44), TGFβ may exert anti-inflammatory properties against EHEC-induced expression of such genes. Finally, EHEC-derived Shiga toxin and its translocation across the epithelium pose a significant biological threat to renal function that may culminate in hemolytic uremic

syndrome (HUS). It is therefore remarkable that elevated levels of TGFβ are associated with reduced incidence of HUS in children infected with EHEC O157:H7 (45), making it feasible that increased TGFβ enhanced epithelial barrier function and limited toxin entry into the body.

In conclusion, TGFβ is a multifunctional cytokine that is involved in many aspects of intestinal epithelial homeostasis. Understanding the mechanisms by which TGFβ enhances the epithelial barrier provides insight into the regulation of barrier function and may identify new therapeutic targets for use in enteropathies associated with increased permeability. Moreover, the observation that TGFβ prevents barrier dysfunction caused by EHEC infection by retaining claudin-2 and ZO-1 expression at the tight junction, suggests specific targets exist for pathogen-mediated barrier disruption. The significance of TGFβ preserving claudin-2 expression in the face of EHEC infection, in addition to increased claudin-1 following TGFβ-mediated barrier enhancement, is underscored by the work of Furuse *et al.*, who showed claudin-1 and claudin-2 reconstitute tight junction strands in fibroblast cells lacking tight junctions (34). Overall, the data presented here offer compelling evidence that differential regulation of claudin expression by TGFβ provides beneficial effects capable of enhancing the epithelial barrier and protecting it against severe insult by pathogenic bacteria.

ACKNOWLEDGEMENTS

KH has been a recipient of a Natural Sciences and Engineering Research Council studentship (2001-2003) and Canadian Institutes of Health Research (CIHR)/Canadian Digestive Health Foundation partnership award (2003-2004). This work was funded by a CIHR grant (MT-13421) to DMM. We thank Jun Lu and Arthur Wang for technical assistance.

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Chapter 5

TGFβ Regulation of Intestinal Epithelial Function

The intestinal epithelium is uniquely positioned to serve as an interface between underlying tissue compartments and the external environment. Its role in homeostasis is underscored by the fact that normal functioning of this interface is critical for human health, and even slight alterations in epithelial function may lead to diarrhea, constipation, malnutrition, dehydration, infectious disease or chronic GI inflammatory diseases such as Crohn's disease and ulcerative colitis (Gewirtz *et al.*, 2002).

One factor that affects epithelial function is TGFβ. Data from in *vitro studies* suggests that in the intestine, TGFβ may have barrier-enhancing properties (Planchon et al., 1999). However, the mechanism behind TGFβ barrier enhancement was undefined and its effects on epithelial secretory function unknown. The work contained herein provides novel data regarding TGFβ-mediated effects on intestinal epithelia. First, it was determined that TGFβ inhibits cAMP-driven Cl^- secretion in a p38 MAPK-dependent manner (Howe, Gauldie, and McKay, 2002). Second, TGFβ was shown to regulate CFTR gene and protein expression, an effect that was not due to TGFβ effects on epithelial proliferation or differentiation (Howe *et al.*, In Press). Third, the mechanism behind TGF-mediated barrier enhancement was defined, both in terms of signal transduction pathways and regulation of tight junction proteins. A final and important aspect to this latter study was the observation that TGFβ protected against EHEC O157:H7-induced barrier defects by preventing tight junction protein dysregulation.

TGFβ REGULATION OF EPITHELIAL ION TRANSPORT

Water is important for several GI processes including maintaining fluidity of the intestinal contents and facilitating nutrient absorption, making water balance a critical element of GI homeostasis. Given that water movement is largely a passive process, there must be tight regulation of the active process responsible for directing water. This occurs at the level of intestinal epithelial ion transport. In chronic inflammatory bowel disease where epithelial function is often altered, there is increased expression of several cytokines. Thus, research has focused on determining the direct effects of these molecules on epithelial physiology and defining the mechanisms by which altered epithelial function occurs.

TGFβ Regulation of Short-Circuit Current (Isc) Responses

Although the effects of pro-inflammatory cytokines on epithelial ion transport have been documented since 1989 (Holmgren, Fryklund, and Larsson), there were no data concerning the effects of the anti-inflammatory cytokine, TGFβ, on intestinal epithelial ion transport at the outset of this thesis. Using human T84 and HT-29 colonic epithelia, it was determined that addition of recombinant $TGF\beta_1$ to the basolateral surface of the monolayer diminished subsequent stimulated Isc responses to the cAMP secretagogue forskolin. The kinetics of this effect were found to be concentration-dependent, as a threshold dose of 1 or 100 ng/ml was necessary for T84 and HT-29 respectively, and time-dependent, as decreased Isc responses only occurred 16 h post-treatment at the threshold dose. These data are not unlike the time- and dose-dependency effects

described for other cytokines. For example, IFNγ progressively diminishes T84 forskolin-responsiveness beginning 12 h post-treatment and peaking after 48 h, at which time a threshold dose of 10 ng/ml was recorded (Colgan *et al.*, 1994a). Similarly, in T84 monolayers treated with IL-4 at varying concentrations, stimulated Cl^- secretion is dose-dependently inhibited (Colgan *et al.*, 1994b). This time-dependent observation may indicate that cytokines modulate gene expression rather than directly manipulating cellular processes to exert their functional effects. Furthermore, in contrast to IFNγ and IL-4 where continuous stimulation is required for inducing effects on intestinal epithelia (Holmgren, Fryklund, and Larsson, 1989, Ceponis and McKay, unpublished observations), exposure to TGFβ for as little as 15 min was sufficient to cause diminished T84 forskolin responses when tested 16 h later. This latter observation illustrates the unique actions of various cytokines on intestinal epithelial function, and that the specific activities of each must be determined separately.

To more fully define the effects of TGFβ on cAMP-driven Cl^- secretion in T84 monolayers, Isc was examined in response to other secretagogues known to elevate intracellular cAMP. VIP and cholera toxin are both biologically relevant peptides that, unlike forskolin, induce Cl^- secretion by receptor-mediated pathways (Barrett, 1993). Thus, the observation that Isc responses to VIP and cholera toxin were similarly reduced by 16 h TGFβ stimulation suggested that adenylate cyclase activation or expression may have been inhibited by TGFβ, effectively limiting the production of cAMP required for sustained Cl^- secretion. However, when TGFβ-treated T84 monolayers were stimulated with cell-permeant cAMP, Isc responses were still reduced by the same magnitude as that

recorded for VIP, cholera toxin, and forskolin (i.e. 30%). Furthermore, direct measurement of cAMP indicated TGFβ-treated cells (16 h, 10 ng/ml) exhibited control-level intracellular cAMP concentrations both before and after stimulation with forskolin. These data are comparable to the effects of IFNγ on VIP-, cholera toxin-, and cell-permeant cAMP-stimulated Cl^- secretion in T84 monolayers, and indicate that the effects of these cytokines rest down-stream from cAMP generation (Colgan *et al.*, 1994a).

TGFβ Regulation of the CFTR

Cytokines that regulate intestinal epithelial ΔIsc can mediate their effects by modulating the expression of key ion pumps, channels, or co-transporters required for ion secretion. For example, studies using radio-labeled ions determined apical Cl^- channels and basolateral $Na^+/K^+/2Cl^-$ cotransporters, K^+ channels, and Na^+/K^+/ATPase were all functionally down-regulated in intestinal epithelia treated with IFNγ (Colgan *et al.*, 1994a). It has since been shown that protein expression of NKCC1 and the Na^+/K^+/ATPase α-subunit are down-regulated by IFNγ, and these data have been corroborated *in vivo* using IFNγ-treated mice bearing human fetal small intestinal xenografts (Sugi *et al.*, 2001, Bertelsen, Eckmann, and Barrett, 2004). In addition to these mechanisms, Cl^- secretion is also regulated by the level of CFTR expression, the main apical chloride channel (discussed below). Thus, the effects of TGFβ on CFTR mRNA expression were tested. At concentrations of 1, 10, or 100 ng/ml, TGFβ (16 h) similarly reduced T84 cell CFTR mRNA expression, and this is in good agreement with the diminished ΔIsc observed in this cell line under identical conditions. Furthermore, in

T84 and HT-29 cells, TGFβ significantly reduced CFTR mRNA expression after 2 h of exposure. In comparison to TGFβ, IFNγ and TNFα down-regulate colonic epithelial CFTR gene expression through de-stabilizing mRNA transcripts, while IL-1β stimulates airways epithelial CFTR mRNA production through activation of NF-κB (Besancon *et al.*, 1994, Nakamura *et al.*, 1992, Brouillard *et al.*, 2001). While the mechanisms differ, the kinetics of TGFβ effects on CFTR mRNA is similar to IFNγ, TNFα, and IL-1β, where regulation of CFTR gene expression occurred 4-24 h post-treatment.

Regulation of CFTR gene expression does not always correlate with altered protein levels. For example, despite the fact that both IFNγ and TNFα affect CFTR mRNA transcripts, only IFNγ or IFNγ+TNFα, but not TNFα alone, reduces CFTR protein expression to 47% of control T84 levels (Fish, Proujansky, and Reenstra, 1999). This finding seems peculiar given the synergistic effects of IFNγ and TNFα in diminishing stimulated Cl^- secretion, and highlights the importance of examining both CFTR mRNA and protein expression when defining the effects of a cytokine on ion transport channels. Thus, the effects of TGFβ on CFTR protein expression were determined in both T84 and HT-29 monolayers. As detected by confocal microscopy, CFTR protein expression in the apical region of colonic epithelial monolayers was significantly reduced by TGFβ treatment at a time and dose corresponding to altered ΔIsc and CFTR mRNA expression (e.g. 16 h; 10 ng/ml for T84, 100 ng/ml for HT-29). While there is still some debate over CFTR recruitment to the apical membrane from subapical pools during cAMP-driven Cl^- secretion versus PKA activation (previously discussed in Chapter 3), the number of channels available for Cl^- efflux constitutes a limiting determinant. Thus, to complement

the confocal data, CFTR expression in the apical membrane was also quantified after forskolin stimulation in TGFβ-treated T84 cells using biotinylation and immunoblotting techniques. Relative to untreated controls, apical membrane CFTR expression was decreased by 40% in TGFβ-treated cells. This decrease appeared consistent with the ~30-40% decrease in forskolin-stimulated Isc typically observed in T84 monolayers. Furthermore, the magnitude of the decrease in forskolin-stimulated Cl^- secretion caused by IFNγ and IL-4 also correlates with the degree to which these cytokines down-regulate CFTR protein expression (Fish, Proujansky, and Reenstra, 1999, Zund *et al.*, 1996).

In addition to colonic epithelial monolayers, these studies also examined the effect of TGFβ on polarized Madin-Darby canine kidney (MDCK) epithelia. These cells had been stably transfected with green fluorescence protein (GFP)-tagged to the CFTR amino-terminus, and previously tested using whole-cell patch clamp studies to verify GFP does not alter CFTR function (Moyer *et al.*, 1998). Similar to colonic epithelia, cAMP-driven Cl^- secretion was significantly reduced by TGFβ in MDCK monolayers. While the magnitude of Isc responses to secretagogues was less overall in MDCK cells compared to colonic epithelia, the range of TGFβ doses that exerted an effect was comparable to T84 and HT-29 monolayers (i.e. 10-100 ng/ml). Notably, TGFβ treatment caused a greater percentage decrease in MDCK ΔIsc compared to controls than that induced in colonic epithelia. Therefore, given the unique opportunity for examining GFP-CFTR subcellular localization in these cells, the effect of TGFβ on CFTR was defined using confocal microscopy. Interestingly, TGFβ treatment caused a redistribution of CFTR into perinuclear rings that, following quantification, were identified in 70% of the cells

examined. This perinuclear accumulation was previously observed in MDCK cells stably transfected with GFP-tagged mutant CFTR, and was determined to represent defective protein trafficking (Loffing-Cueni *et al.*, 2001). Thus, it is possible TGFβ affects CFTR trafficking to the apical membrane. However, with the exception of the a report that EGF activates Na^+/H^+ exchange (Gekle *et al.*, 2002), few studies have examined the effect of cytokines on MDCK ion transport, and none have determined their regulation of the CFTR.

cAMP-dependent Cl^- secretion is dependent on interactions between CFTR and the actin cytoskeleton (Cantiello, 2001). Consequently, agents that perturb the normal assembly of F-actin may alter CFTR distribution and/or function. Therefore, the structure of the F-actin cytoskeleton was assessed in TGFβ-treated MDCK cells. At doses and times that corresponded to altered Isc responses and CFTR distribution, TGFβ caused a reorganization of F-actin into stress fibres throughout the cell. This observation is supported by Humes *et al.* (1993) who showed $TGF\beta_1$ induced a dramatic rearrangement of actin microfilaments into stress fibres in MDCK epithelia. However, unlike MDCK cells, T84 colonic epithelia did not display altered F-actin organization after TGFβ treatment. This is in contrast to IFNγ stimulation, where F-actin accumulation is observed at the perijunctional ring (Colgan *et al.*, 1994a). It is possible this IFNγ-induced rearrangement contributes to its barrier-disruptive effects as opposed to its regulation of epithelial ion transport. Nevertheless, these data show cytokines can regulate the actin cytoskeleton in a cell-type specific manner. In the case of TGFβ, this may represent a

mechanism by which CFTR localization is altered and concomitantly, epithelial Cl^- secretion is reduced.

Finally, it is also possible that TGFβ regulates one or more of the other components of the secretory apparatus in addition to the CFTR. While Cl^- efflux from an apical membrane channel is the end product of stimulated chloride secretion in colonic epithelia, Na^+/K^+/ATPase pump activity and $Na^+/K^+/2Cl^-$ cotransporter expression is also necessary. As the activity and/or expression of these proteins were not tested in TGFβ-treated epithelia, it is not known if the diminished cAMP-driven Cl^- secretion is also due to a reduced driving force or decreased Cl^- uptake. Furthermore, it has been shown that intestinal epithelial cells can also express an apical chloride channel called ClC-2 (Mohammad-Panah *et al.*, 2001), and that chloride currents from these channels can be inhibited by treatment with TGFα (Bali *et al.*, 2001). In light of these data, future studies should determine the role of ClC-2 currents in TGFβ-treated epithelia. Nonetheless, given the significant decrease in CFTR protein expression (colonic epithelia) and the dramatic perinuclear accumulation (MDCK cells), it seems unlikely that regulation of other channels contributes substantially to the 30-40% decrease in Isc.

Consideration of TGFβ as a Growth Factor

T84 colonic epithelia are crypt-like secretory-type cells that are not generally induced to differentiate in culture, unlike HT-29 or Caco-2 cells (Rousset, 1986). However, the growth factor properties of TGFβ on epithelia are typically to inhibit proliferation and promote differentiation (Roberts and Sporn, 1988). Thus, it was

possible the decrease in secretory responsiveness and increase in TER reflected a phenotype switch in T84 monolayers from crypt-like secretory cells to villus-like absorptive cells. Yet under conditions where TGFβ induces altered ion transport, cytokine treatment had no effect on T84 cell proliferation. Similarly, alkaline phosphatase expression, a marker of differentiation, was unchanged by TGFβ-treatment of T84 monolayers. In comparison, when HT-29 and Caco-2 intestinal epithelia are induced to differentiate by either glucose deprivation or growing them past confluence, alkaline phosphatase expression is increased (Pinto *et al.*, 1982, Pinto *et al.*, 1983). Interestingly, differentiation of these cell lines is accompanied by a decrease in CFTR protein expression (Sood *et al.*, 1992). Therefore, it was concluded that the effects of TGFβ on epithelial CFTR expression was specific and not an indirect result of differentiation.

The non-mitogenic effects of other growth factors have also been documented in a cell-type specific manner (Uribe and Barrett, 1997). Epidermal growth factor (EGF), for example, is a potent inhibitor of Ca^{2+}-mediated Cl^- secretion in T84 monolayers (Uribe *et al.*, 1996a). It was therefore tested whether 16 h EGF treatment of T84 monolayers would cause diminished responses to cAMP-mediated epithelial ion transport, similar to TGFβ. Neither 10 nor 100 ng/ml EGF stimulation resulted in altered epithelial Isc responses to forskolin. Likewise, TGFβ did not have the short-term (i.e. 30 min) effects on ΔIsc previously observed with the growth factors EGF or IGF-1, further emphasizing that differences exist between growth factor regulation of epithelial ion transport (Uribe *et al.*, 1996a, Chang *et al.*, 2001).

TGFβ Modulation of Signal Transduction

As discussed earlier, several signal transduction pathways have been implicated in the regulation of Cl^- secretion. In the MAPK signaling family, ERK1/2 is typically activated by mitogens, while p38 MAPK and JNK signaling can be induced by stress stimuli and cytokines (English *et al.*, 1999, and Kyriakis and Avruch, 1996). Although TGFβ has been shown to activate ERK1/2, p38 MAPK, and JNK in many cell types (Mulder, 2000), no data was available regarding its effects on these signaling pathways in enteric epithelia. Using pharmacological inhibitors of these three signal transduction cascades, it was shown that the TGFβ-induced diminished Cl^- secretion was mediated specifically by p38 MAPK in both T84 and HT-29 colonic epithelia. This observation is supported by work from Keely and Barrett (2003) who showed that activation of p38 MAPK by the muscarinic agonist carbachol inhibited Ca^{2+}-dependent Cl^- secretion in T84 cells. While they also demonstrated a role for ERK1/2 in limiting ion transport, inhibition of this pathway did not restore Cl^- secretion in TGFβ-treated T84 cells. Similarly, in contrast to EGF and insulin which inhibit Cl^- secretory responses via stimulation of a PI-3K-dependent signaling pathway (Uribe *et al.*, 1996b, Chang *et al.*, 2001), TGFβ-induced diminished forskolin responses were not prevented by inhibition of this pathway.

Although p38 MAPK mediates inhibition of Cl^- secretion in colonic epithelia after both TGFβ and carbachol, the mechanisms may differ. The decrease in Ca^{2+}-driven Cl^- secretion in T84 cells occurs in an acute manner after exposure to agents that activate the EGF receptor (EGF, ACh agonists) and it is a transient event. In contrast, TGFβ-mediated diminished ΔIsc occurred only after several hours exposure. Also, although p38

MAPK activation (i.e. phosphorylation) by TGFβ could not be definitively detected, a reasonable prediction would be that TGFβ activation of p38 MAPK decreases CFTR gene transcription and protein expression to regulate Cl^- secretion. Indeed, decreased CFTR gene transcription observed in HT-29 epithelia exposed to hyperosmolar conditions was regulated by p38 MAPK (Baudouin-Legros *et al.*, 2000). Together, these data make it tempting to speculate that inhibition of p38 MAPK with SB203580 would prevent the TGFβ-induced decrease in CFTR gene transcription and protein expression that was observed by RT-PCR and confocal/biotinylation, respectively. Future studies should address this possibility.

Chronic Effects of TGFβ on Ion Transport

The effect of active TGFβ secreted by T84 cells expressing adenovirally-derived TGFβ on ΔIsc was determined 6 days following infection with Ad-TGFβ in order to examine the longer-term effects of TGFβ on epithelial function. Similar to colonic epithelial monolayers exposed to recombinant TGFβ, Ad-TGFβ infected cells exhibited Isc responses to forskolin that were diminished by ~30-40%. Infection with the empty vector or adenovirus encoding latent TGFβ controls did not affect ΔIsc, and this was consistent with their use as controls in a model of Ad-TGFβ-induced lung fibrosis (Sime *et al.*, 1997). Furthermore, this prolonged TGFβ exposure dramatically increased T84 barrier function, as determined by the statistically significant increase in T84 TER at 72 h, and three-fold increase observed 6 days post-infection. These data show that long-term exposure of TGFβ causes sustained effects on both epithelial ion transport and barrier

function. This is noteworthy since the functional effects of exposure to other growth factors/cytokines can be limited by down-regulation of receptor expression (e.g. EGF) (Dikic, 2003) and/or induction of negative-feedback signaling cascades (e.g. IFNγ induces Suppressors Of Cytokine Signaling, SOCS) (Krebs and Hilton, 2000). In the context of TGFβ, there is evidence to support both events occur following TGFβ stimulation, as TGFβ induction of the inhibitory SMAD, SMAD7, has been found to complex with the nuclear protein Smurf2 which then binds TGFβRI and targets it for degradation via a proteasome-dependent pathway (Kavsak *et al.*, 2000, Ebisawa *et al.*, 2001).

Given that acute exposure to TGFβ causes diminished ΔIsc several hours later and that TGFβ receptors may be degraded after cytokine stimulation, it appears that continual activation of TGFβ receptors is unnecessary for mediating altered epithelial ion transport. Combined with the evidence that TGFβ treatment causes a down-regulation of CFTR gene and protein expression, it is likely the sustained effects of TGFβ on epithelial ion transport and barrier are initiated by early signaling events that culminate in modulation of gene transcription. Overall, this results in altered expression of ion transport and barrier proteins critical for epithelial function. Indeed, in chronic GI inflammatory situations such as IBD where barrier function is perturbed and water movement is dysregulated, it is possible that in addition to its immunosuppressive role, TGFβ could help to restore the epithelial barrier and serve as a braking mechanism for excess Cl^- secretion, reducing the loss of fluid and electrolytes.

In the past decade, there has been a significant interest in using cytokines in the management of GI disease. For example, anti-TNFα therapy is effective at reducing symptoms of disease in patients with Crohn's disease (Abuzakouk, Feighery, and Jackson, 2002). Administration of the immunosuppressive cytokine IL-10 showed initial promise in the treatment of Crohn's disease (Fedorak *et al.*, 2000), but other clinical trials determined it was ineffective (Schreiber et al., 2000), for reasons not completely understood (Lindsay and Hodgson, 2001). It is possible that TGFβ may have therapeutic value in certain cases of IBD, given its ability to limit secretory responses and beneficial effects on epithelial barrier. However, this would have to be considered in the context of TGFβ's ability to induce fibrosis, the cellular targets (i.e. TGFβ receptors on enteric epithelium, signaling cascades), and the duration of cytokine exposure.

FROM *in vitro* TO *in vivo*

In vitro cell culture models with intestinal epithelia have many useful purposes, and have allowed researchers to address the direct action of cytokines on epithelial function under experimentally controlled conditions. By growing epithelial cells on semipermeable supports, the development of a polarized monolayer is facilitated (Dharmsathaphorn *et al.*, 1984) and this allows distinct access to apical and basolateral surfaces, which can then be treated with biological mediators (e.g. cytokines, bacteria) to test their effects on epithelial function. Alternatively, cytokine production can be derived from the epithelium itself or neighbouring cells grown in co-culture. For example, bacterial infection frequently induces epithelial-derived IL-8 that is essential for the

recruitment of neutrophils and generation of the host immune response (Hecht and Savkovic, 1997). Indeed, co-culture models are an extension of the epithelial monolayer design, and are useful for studying the filter-grown epithelia in the proximity of other cell types. This methodology has been used to assess intestinal epithelial function in the presence of fibroblasts, immune cells, and microbes (McKay, Philpott, and Perdue, 1997). Together, such studies have not only improved understanding of cytokine interplay and cross-regulation between cells in the gut, but also greatly enhanced knowledge of bacterial-host interactions at the enteric epithelium. To this end, it is felt that the TGFβ studies discussed in this thesis improve understanding of epithelial cell biology.

There are, however, disadvantages to *in vitro* cell culture. For example, co-culture models do not precisely mimic the gut environment *in vivo* since enteric epithelial cells are typically in close proximity to nerves, immune cells, smooth muscle, and fibroblasts, all of which are in communication with each other. Therefore, the effect of a cytokine on epithelial function observed *in vitro* may not be the same as *in vivo*. Another concern is whether or not transformed intestinal epithelial cell lines, such as T84, HT-29, and Caco-2, accurately represent intestinal epithelia found *in vivo*. However, since primary human intestinal epithelial cell culture is difficult to obtain and dies quickly, one way to overcome this concern is to employ multiple transformed cell lines and to compare their function (McKay, Philpott, and Perdue, 1997). Indeed, the effect of TGFβ on epithelial ion transport was tested in multiple cell lines, and found to be consistent.

A follow-up approach towards a deeper understanding TGFβ regulation of epithelial ion transport and barrier function might be to assess its effects on human or

animal intestinal tissue mounted in Ussing chambers. This could be accomplished via recombinant cytokine addition to the tissue preparation, or prior systemic TGFβ delivery, such as was successful using Ad-TGFβ in the mouse (Sime *et al.*, 1997). Moreover, the therapeutic potential of this cytokine may be tested in animal models of enteric disease that attempt to mimic the situation observed in humans. For example, in a murine model of dextran sulfate sodium (DSS)-induced colitis, animals exhibit watery stool (i.e. excess fluid movement) and increased epithelial permeability (Cooper *et al.*, 1993). Based on *in vitro* observations, it is plausible that adenoviral delivery of TGFβ prior to DSS administration could ameliorate disease by preserving epithelial function. This *in vivo* situation would be substantially more complicated than that found *in vitro* and the data would have to be interpreted cautiously, in the context of route of administration and other cell types responsive to TGFβ.

TGFβ REGULATION OF THE EPITHELIAL BARRIER

Inflammatory bowel diseases, such as Crohn's disease, are associated with increased intestinal permeability, and this is hypothesized to play a role in disease pathogenesis (Meddings, 2000). However, it is currently unknown if increased permeability is the primary defect responsible for initiating disease, or if altered permeability occurs as a secondary consequence of chronic inflammation. In the meantime, a substantial body of literature has developed showing that numerous biological factors can regulate the intestinal barrier (e.g. cytokines and bacteria), and that the majority of these factors cause significant impairment of this epithelial function. Due

to the discovery of the molecular components of epithelial tight junctions, it is now understood that cytokines such as IFNγ and enteropathogenic bacteria cause such barrier defects by disrupting TJ protein expression (Bruewer *et al.*, 2003, Philpott *et al.*, 1996). It is remarkable that very little data was available concerning the effects of TGFβ on TJ regulation, as it belongs to a select group of factors known to increase epithelial barrier function.

TGFβ Regulation of Tight Junction Proteins and Signal Transduction

In Chapter 4, it was determined that TGFβ-mediated intestinal epithelial barrier enhancement is associated with an increase in TJ protein expression. Specifically, TGFβ exposure causes up-regulation of claudin-1 protein expression in human colonic epithelia, but not claudin-2, claudin-4, occludin, or the TJ-associated protein ZO-1. These data point to the importance of claudin-1 in the development of an electrically "tight" barrier, and are supported by studies showing that claudin-1 removal from the tight junction by *Shigella flexneri* or IFNγ stimulation causes increased permeability in human intestinal epithelial monolayers (Sakaguchi *et al.*, 2002, Bruewer *et al.*, 2003). Furthermore, in a model of intestinal epithelial barrier enhancement, T84 monolayer exposure to the PKC agonist bryostatin-1 resulted in increased TER and recruitment of claudin-1, occludin, and ZO-1 to the TJ (Yoo *et al.*, 2003). It is possible that claudin-1 insertion into the TJ is regulated through induction of gene transcription or mRNA stabilization, and this would certainly be supported by the observation that increased TER occurs several hours following TGFβ treatment. However, in the bryostatin-1 model, claudin-1 mobilization

to the TJ is evident after 4h, suggesting that the protein may also be recruited from intracellular pools.

In addition to protein expression and subcellular localization, regulation of phosphorylation status is a mechanism for retaining occludin and ZO-1 at or near the TJ (Wong, 1997, Atkinson and Rao, 2001). Following the identification of a putative MAPK phosphorylation site on claudin-1, further studies using transfected cell lines determined threonine phosphorylation causes claudin-1 localization to the TJ and is required to enhance epithelial barrier function, and that both of these events could be prevented by pretreating the cells with the MEK inhibitor, PD98059 (Fujibi *et al.*, 2004). Based on this study, it could be postulated TGFβ induction of ERK/MAPK signaling in T84 monolayers leads to claudin-1 phosphorylation, increased recruitment to the TJ, and ultimately results in increased TER. Indeed, similar to Fujibe *et al.* (2003), inhibition of ERK/MAPK signaling with the MEK inhibitor PD98059 prevented the TGFβ-induced increase in epithelial barrier function. As increasing expression of the negative regulator of SMAD activity, SMAD7, also inhibited the increase in TER caused by TGFβ exposure, it is possible that SMAD3 signaling acts on claudin-1 expression or recruitment to the TJ. At the present time, there is no literature to support SMAD regulation of TJ proteins. Alternatively, given the role of SMAD3-ERK/MAPK cross-talk in enhancing TGFβ-induced collagen synthesis in human mesangial cells (Hayashida *et al.*, 2003), it may be that cross-talk whereby ERK1/2 phosphorylates SMAD3 is involved in the model of TGFβ-mediated barrier enhancement presented here. Taken together, these data suggest that claudin-1 expression and its phosphorylation status may serve as a

therapeutic target in the treatment of GI diseases where intestinal permeability is increased such as IBD.

Barrier-Protective Effects of TGFβ

Intestinal epithelial permeability defects occur not only during IBD, but also following infection with enteric pathogens. For example, children suffering rotavirus or enteropathogenic *E. coli* (EPEC) infection display increased intestinal permeability, as measured by the lactulose/mannitol ratio (Roy *et al.*, 1992). Furthermore, cell culture models have determined these enteropathogens and others act directly on the intestinal epithelium to induce barrier disruption (Dickman *et al.*, 2000, Philpott *et al.*, 1996), allowing the permeability defect to be modeled and studied *in vitro*. It is interesting that in addition to having barrier-enhancing effects, TGFβ is capable of preventing colonic epithelial barrier dysfunction *in vitro* caused by infection with *Cryptosporidium parvuum* oocysts (Roche *et al.*, 2000). Thus, the next goal was to determine if TGFβ could prevent the barrier disruption induced by T84 infection with the human pathogen enterohemorrhagic *E. coli* (EHEC) O157:H7. TGFβ pretreatment not only completely prevented the EHEC-induced barrier defect 16h post-infection, but also protected T84 monolayers from barrier defects caused by the related pathogen, EPEC. Given that T84 cells produce bactericidal factors in response to stimulation with the Gram-negative outer membrane component, lipopolysaccharide (Ogle *et al.*, 2002), and that chemokines with antimicrobial activity are inducible by IFNγ (Cole *et al.*, 2001), it was tested whether TGFβ itself, or epithelial factors induced by TGFβ could inhibit EHEC growth. Neither

addition of TGFβ directly to EHEC cultures, nor incubation of EHEC with supernatants from TGFβ-treated monolayers affected bacterial growth. This result is consistent with the *C. parvuum* study, as TGFβ had no direct effects on the barrier-disruptive properties of the organism, suggesting that TGFβ may either inhibit EHEC-induced signal transduction or compensate for EHEC effects on the TJ.

EHEC-induced barrier dysfunction is associated with removal of ZO-1 from the TJ (Philpott 1996), and here it was determined TGFβ prevented this event. However, despite the recognition that claudins are a critical element of the TJ, there is no literature examining the effect of EHEC on claudin expression. As such, this is the first study to determine that EHEC specifically down-regulates claudin-2, and that TGFβ can prevent this event. The differential regulation of claudin-1 and claudin-2 in governing T84 epithelial barrier function suggests that both of these claudins are important in TJ function. Indeed, this is supported by enhanced membrane association of claudin-1 and claudin-2 in a model of barrier enhancement by IL-15 (Nishiyama *et al.*, 2001). It is noteworthy that in this study, claudin-1 was recruited to the apical pole of cell-cell contacts, whereas claudin-2 was observed in a larger lateral membrane compartment, suggesting these proteins may have distinct functions in the regulation of TJs. Comparative functional studies will be needed to address the relative contributions of each claudin to TJ function.

Finally, the observation that TGFβ did not prevent EHEC induction of an epithelial pro-inflammatory response (e.g. IL-8 secretion) suggested that its effects may be specific for preserving epithelial barrier function. However, the precise potential

mechanisms underlying TGFβ modulation of EHEC-induced barrier function remain to be fully characterized. While it is clear EHEC growth was unaffected by TGFβ, it is possible pretreatment with cytokine prevents the bacteria-induced signal transduction cascades regulating barrier disruption. One such epithelial signaling cascade involved in EHEC-mediated disruption of barrier function is activation of myosin light chain kinase (MLCK) and its subsequent phosphorylation of myosin light chain (MLC), since pharmacological inhibition of this signaling pathway prevents the bacteria-induced decrease in epithelial TER (Philpott *et al.*, 1998). However, MLCK is postulated to regulate barrier through contracting the perijunctional actin-myosin ring. To date, there is no evidence to date that MLCK regulates claudin expression or its localization to the tight junction. It is interesting that *Saccharomyces boulardii*, a probiotic yeast and another agent capable of preventing EHEC-induced barrier disruption, prevents MLC_{20} phosphorylation and decreased epithelial TER (Dahan *et al.*, 2003). Whether TGFβ does the same remains to be determined, as does whether activation of MLCK signaling regulates TJ protein expression or subcellular localization.

Thus, the delineation of factors and signaling pathways that improve intestinal epithelial barrier function is anticipated to provide a solution for one key aspect of IBD pathogenesis, that of a "leaky gut". This could be important since increased intestinal permeability allows pathogens and luminal antigens unimpeded access to the underlying mucosa where they can perpetuate or initiate an inflammatory episode. Many cytokines significantly damage epithelial barrier function, and may thereby potentiate bacterial attachment. It is promising that anti-TNFα therapy has been shown to restore the gut

barrier in Crohn's disease (Suenaert *et al.*, 2002). However, studies show that stress, whether physical (Ando *et al.*, 2000) or psychological (Soderholm *et al.*, 2002), also induces intestinal epithelial permeability and facilitates bacterial translocation. Moreover, a study by Nazli *et al.* (2004) has recently shown epithelia under metabolic stress perceive commensal bacteria as a threat, resulting in reduced barrier function, bacterial translocation, and increased IL-8 production. In a murine model of *Salmonella* infection, TGFβ treatment reduces bacterial translocation to the liver and spleen, possibly reflecting improved intestinal barrier function (Galdiero *et al.*, 1999). Indeed, the probiotic mixture VSL #3, containing several strains of putatively beneficial bacteria, improves epithelial barrier function *in vitro* (Otte and Podolsky, 2004) and shows clinical benefits in pouchitis (Mimura *et al.*, 2004). Overall, these multiple lines of evidence lend support to the argument that TGFβ may have therapeutic value in enteropathies *in vivo*.

FUTURE DIRECTIONS

Throughout this discussion and individual chapter 'Discussions', I have made suggestions for future directions. Therefore, I have chosen to focus the following section on a new avenue of research that further explores the protective effects of TGFβ against EHEC O157:H7 infection.

In addition to barrier disruption, EHEC O157:H7 infection induces a pro-inflammatory response from the intestinal epithelium. One event downstream of EHEC infection of intestinal epithelial cells is activation of the NF-κB signaling pathway and subsequent induction of epithelial IL-8 secretion, a potent neutrophil chemoattractant

(Berin *et al.*, 2002). Several other biological mediators are regulated by NF-κB (Li and Verma, 2002), including the pro-inflammatory cytokines TNFα and IL-6 (Azzolina, Bongiovanni, and Lampiasi, 2003). Recent evidence showed that TGFβ inhibits induction of NF-κB signaling by non-pathogenic bacteria in enteric epithelial cells (Haller *et al.*, 2003). Thus, it should be determined whether TGFβ can also prevent EHEC O157:H7-induced NF-κB activation and induction of epithelial pro-inflammatory cytokine production.

In vivo models of EHEC O157:H7 infection are limited by the inability of EHEC to cause disease in many laboratory animal species (Melton-Celsa *et al.*, 2003). However, the related murine pathogen *Citrobacter rodentium* carries the LEE pathogenicity island, colonizes the colon, and elicits mucosal inflammation and colonic hyperplasia in mice. Hence, it is considered a surrogate *in vivo* model of EHEC infection (Higgins *et al.*, 1999, Vallance *et al.*, 2003). Since TGFβ appears to be protective against EHEC infection *in vitro*, and since it is protective against *S. typhimurium* infection of mice (Galdiero *et al.*, 1999), it is possible TGFβ could ameliorate infection with *C. rodentium*. Thus, the aims of such studies would be to examine whether TGFβ improves the disease score, prevents *C. rodentium*-induced colonic hyperplasia, prevents bacterial attachment to the epithelium and/or translocation to mesenteric lymph nodes, or modulates the inflammatory response to *Citrobacter* infection (e.g. IFNγ production).

Concluding Statement

This thesis represents the culmination of multiple experiments designed with the overall objective of determining the effects of TGFβ on intestinal epithelial function and the mechanisms by which these occur. Here, I have provided evidence that TGFβ limits stimulated chloride secretion and enhances epithelial barrier function through regulating key proteins of the secretory apparatus and tight junction, respectively. Moreover, I have detailed the signal transduction pathways employed by TGFβ in the regulation of these events. While the modulation of intestinal epithelial function by pro-inflammatory cytokines (e.g. IFNγ, IL-4) has been the focus of ongoing research for several years, very little was known about the effects of TGFβ. This was surprising given that TGFβ is one of the few agents known to enhance intestinal epithelial barrier function. As a consequence of the studies reported herein, we now have a molecular understanding of the mechanisms behind TGFβ barrier enhancement, and these may represent new therapeutic targets that can be modulated in the treatment of enteropathies characterized by permeability defects. Indeed, the additional observation that TGFβ is protective against barrier disruption caused by infection with EHEC O157:H7, further emphasizes the importance of this cytokine in regulating epithelial function. Thus, with the success of bench-to-bedside research exemplified by the therapeutic value of anti-TNFα therapy in Crohn's disease, it is possible that new insight provided here may contribute to the development of novel therapies for GI diseases.

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