

Development, validation and implementation of an in vitro model for the study of metabolic and immune function in normal and inflamed human colonic epithelium

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The 7 original papers are

I. Pedersen G, Saermark T, Giese B, Hansen A, Drag B, Brynskov J. A simple method to establish short-term cultures of normal human colonic epithelial cells from endoscopic biopsy specimens. Comparison of isolation methods, assessment of viability and metabolic activity. *Scand J Gastroenterol* 2000; 35: 772-780.

II. Pedersen G, Saermark T, Bendtzen K, Brynskov J. Cultures of human colonic epithelial cells isolated from endoscopic biopsies from patients with inflammatory bowel disease. Effect of IFN γ , TNF α and IL-1 β on viability, butyrate oxidation and IL-8 secretion. *Autoimmunity* 2000; 32: 255-263.

III. Pedersen G, Saermark T, Brynskov J. Phenol toxicity and conjugation in human colonic epithelial cells. *Scand J Gastroenterol* 2002; 37: 74-79

IV. Pedersen G, Saermark T, Horn T, Giese B, Bendtzen K, Brynskov J. Cytokine-induced impairment of short chain fatty acid oxidation and viability in human colonic epithelial cell. *Cytokine* 2000; 12:1400-1404.

V. Pedersen G, Kierkegaard T, Saermark T, Brynskov J. Spontaneous and cytokine induced expression and activity of matrix metalloproteinases in human colonic epithelium. *Clin Exp Immunol* 2008, 155: 257-265

VI. Pedersen G, Matthiessen MW, Andresen L, Rask-Madsen J, Brynskov J. Expression of toll-like receptor 9 and response to bacterial CpG oligodeoxynucleotides in human intestinal epithelium. *Clin Exp Immunol* 2005; 141: 298-306

VII. Pedersen G, Brynskov J. Topical rosiglitazone treatment improves ulcerative colitis by restoring peroxisome proliferator-activated receptor- γ activity. *Am J Gastroenterol* 2010; 105: 1595-1603

INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD), collectively referred to as inflammatory bowel disease (IBD), are chronic, relapsing immune mediated disorders affecting the gastrointestinal tract. The aetiology of IBD remains an enigma, but increasing evidence suggests that the development of IBD may be triggered by a disturbance in the tightly regulated balance between gut commensal bacteria and host response in the intestinal mucosa in genetically susceptible individual (1-4). Environmental factors including dietary components, antibiotics, tobacco and non-steroidal anti-inflammatory agents may also play a role in initiation and re-activation of UC or CD or both through a regulation of host immune responses, the physiological function of epithelial cells, as well as the composition and function of the commensal microbiota (2,3,5,6).

The epithelium of the human colon is a continuously renewing single layer of cells that forms a primary barrier against microorganisms and toxins present in the intestinal lumen. The classic functions of the epithelium are well known and include absorption and secretion of fluids, electrolytes and nutrients. Yet, it is now increasingly recognized that epithelial cells also have the capacity to secrete and respond to a range of immunological mediators. Consequently, this has led to the suggestion that these cells play a far more prominent role in the pathogenesis of inflammatory and neoplastic colonic diseases than hitherto believed. Hence, a number of abnormalities in intestinal epithelial cell function, including impaired metabolism (fatty acid metabolism), dysregulated cell homeostasis and altered innate immunity, have been implicated in the development of IBD (7-15). The evaluation of possible pathogenetic mechanisms at the epithelial level has, however, for long been hampered by the lack of suitable experimental models of human colonic epithelial cells. Accordingly, most of the available knowledge about the human intestinal epithelium has therefore been obtained using various models based on animal cells, transformed human intestinal cell lines and isolated cells from resected colonic bowel segments (16-26). Species difference, malignant origin and confounders related to the surgical bowel resection obviously make these epithelial cell

models less applicable for patophysiological studies. Consequently, there seems to be a clear need for the development of models of representative intestinal epithelial cells that would allow functional and dynamic studies of the differentiated human epithelium *in vitro*. Theoretical, the use of endoscopically obtained colonic biopsy specimens to establish cultures of human epithelial cells would largely circumvent many of the disadvantages inherent in other models. Such models would provide an opportunity to investigate representative colonic epithelial cells from a broad spectrum of patients in relation to IBD and other intestinal disorders as well as normal colon.

AIM

The primary and foremost purpose of this study was to explore and validate the optimal conditions for establishing a model based on short-term cultures of human colonic epithelial cells obtained from endoscopic biopsies. Secondly, the objective was to explore the ability of this model to describe the interplay between proinflammatory cytokines and normal as well as inflamed colonic epithelium with focus on alterations in viability, butyrate metabolism and secretion of a chemokine and metalloproteinases. Finally, the model was used to characterize expression and activation of receptors like TLR9 and PPARs—known to be important players in regulation of innate and adaptive immune responses in human colonic epithelium—and in addition evaluate possible changes occurring during chronic colonic inflammation.

SHORT-TERM CULTURES OF HUMAN COLONIC EPITHELIAL CELLS

Models of human intestinal epithelium

Surgically removed bowel segment has been widely used as a source for isolation of human colonic epithelial cells and establishment of *in vitro* cultures (18,19,27). By this, a sufficient amount of isolated cells is obtained, but the applicability of the model is narrowed as the material is typically limited to severe end-stage disease. Although recent studies have shown improved survival of the isolated cells through optimized isolation procedures, viability and function of epithelial cells isolated from surgical specimens are affected by a range of confounders like ongoing intensive immunosuppressive treatment, surgical induced ischemia and anaesthesia (18,27,28).

Other widely used models of human intestinal epithelium are based on transformed human cell lines like HT-29, DLD-1, T84 and Caco-2 (13,22-24). Cell lines are easily accessible and have a high viability and metabolic activity, which make them attractive in laboratory work to test hypotheses and obtain fast and large scale results (II, III, IV, V) (29-31). However, these tumour cells exhibit high diversity in the type and state of differentiation, proliferation and metabolic properties. Findings in these models may therefore be less representative of the normal, highly differentiated colonic epithelium (32,33,III,IV). The use of endoscopically obtained colonic biopsy specimens to establish cultures of human epithelial cells would largely circumvent many of the limitations of the mentioned models. At the time this project was initiated, only few previous studies were available and the early attempts illustrated the problems inherent in this technique: notably low cell yield and poor viability in cultures—presumably as a result of detachment-induced apoptosis (27,34,35). Nonetheless, studies in other areas have shown that it is possible to establish cell cultures from even small amount of tissue (e.g. islets of Langerhans and rat pituitary gland), which have been successful for dynamic biochemical studies *in vitro* (36,37). Parallel to these results, we explored a similar approach to establish small dimension cultures of epithelial cells from biopsies specimens.

Colonic epithelial cell cultures; isolation methods, survival and metabolism

Previous experiments in rodents and human colonic resection specimens have shown that chelating agents such as ethylene glycol-bis(β -aminoethyl ether)-N,N,N,N'-tetraacetic acid / ethylenediaminetetraacetic acid (EGTA/EDTA) efficiently isolate epithelial cells from intestinal tissue through Ca²⁺ dependent disruption of cell-matrix attachment (19,38-42). EGTA/EDTA chelation treatment for 30-60 minutes was, however, found to impair cell viability in these studies (43,44). Reducing chelation treatment time to only 10 minutes resulted in a rapid, selective and efficient isolation of whole epithelial cell crypts (Fig.1). Continuous EGTA/EDTA exposure seems to affect the cell-cell contacts in the crypt structure, resulting in isolation of single cells with a marked reduced viability (G. Pedersen, personal observations). Concordant with this, a significantly improved viability was observed in the cell cultures established after short-term EGTA/EDTA treatment (I). Isolation as whole crypt or crypt fragments with sustained cell-cell contact seems essential for regulation of apoptosis and consequently survival of the cells, which currently has been confirmed by others (27,42,45,46). Protolytic enzymes have previously been found to be efficient in the isolation of epithelial cells from rodent intestinal mucosal specimens as well as human colonic resection specimens (18,28,38,42,47,48). Comparison of our short-term EGTA/EDTA based technique with isolation by a collagenase mixture showed that chelation treatment was superior to enzymes in terms of purity of the cell population; whereas no difference was observed in cell yield, viability or proliferation (I). Moreover, a significant fibroblast growth was often observed in the cultures established after enzyme isolation; probably due to dissociation of the connecting tissue leading to increased contamination of non-epithelial cells (Pedersen, G. unpublished data)(42). Nevertheless, purity of the cell cultures is an important issue if the model is to be representative for the colonic epithelium. As a consequence we found that a cell model based on short-term EGTA-EDTA treatment was the most optimal approach to establish pure and viable cultures (I). Viability of the cultures was quantified by a colorimetric assay based on conversion of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoleum bromide (MTT) to formazan by succinate dehydrogenase (49,50). In contrast to other viability tests based on assessment of cell membrane integrity such as trypan blue, the MTT test allows detection of early cellular damage, i.e. impaired mitochondrial function, which occurs before loss of membrane integrity (49-51). Using the MTT test, we found that the isolated cells were viable and metabolically active for at least 24 hours of culture. Consistent with this notion, the finding of normal mitochondrial appearance by electron microscopic examination, confirmed that the epithelial cells had maintained their ultra structural characteristics and viability after 24 hours in culture (I). In addition, cultures were also evaluated by vital staining using membrane-permeable calcein-acetomethylester (calcein AM), which is converted to the fluorescent non-ester form by metabolic active cells visualizing viable cells (52,53). Evaluation by confocal light microscopy confirmed that a majority of the cells were viable after 24 hours and that the viable cells were mainly organized in clusters with close cell-to-cell contact; whereas the dead cells were more likely to be found as single cells (Fig. 2) (I). It is well-known that detachment from the basal membrane activates apoptosis, which is an important limiting factor for viability of isolated colonic epithelial cells *in vitro* (28,46,54).

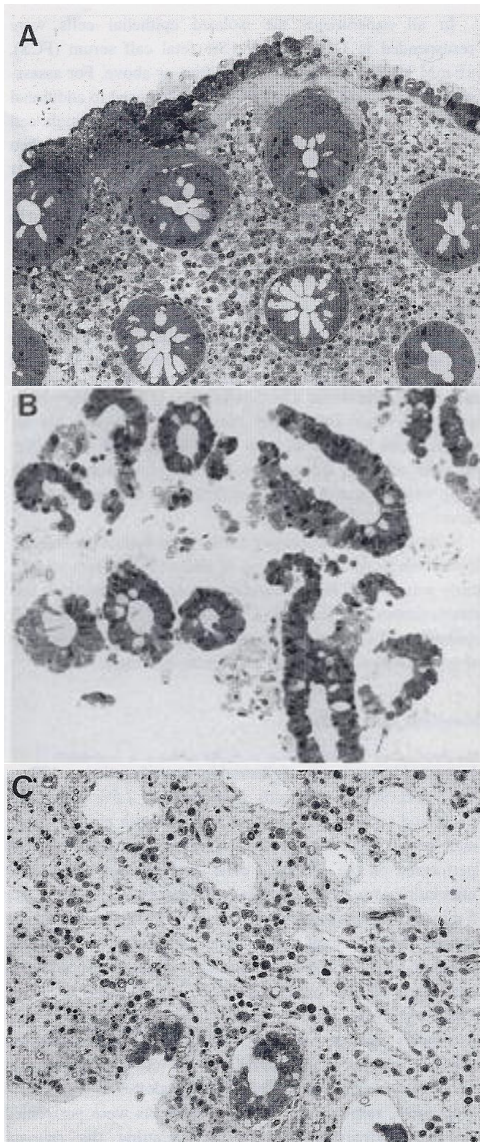


Figure 1 Selective isolation of colonic epithelial crypts from endoscopic biopsies by ultra-short (10 min) chelation treatment using EGTA/EDTA. Panel A shows a biopsy section before EGTA/EDTA treatment and panel B the isolated epithelial cells present mainly as crypts, although single cells and crypt fragments are also present. Panel C shows a biopsy section after EGTA/EDTA treatment illustrating that the majority of the crypts were removed, leaving the biopsy section intact.

Apoptosis is not only controlled by cell-matrix contact, but also by cell-to-cell contacts (46,55,56). This may explain why isolation as whole crypts or crypt fragments is essential for survival in the short-term cultures as also described subsequently by others (42,45,46).

To reduce detachment-induced apoptosis, we seeded isolated crypts immediately in collagen-coated wells, which allowed the crypts quickly to attach to the bottom of the wells. Although, the crypts' structures gradually disappeared, cells were mainly present in clusters with preserved close intercellular contact after 24 hours (Fig. 2) (I). Survival of the cells cultured in coated wells clearly improved, which have also subsequently been reported by others (45,55,57) (I). Overall, our experiments showed that short-time isolation by EGTA /EDTA from endoscopical colonic biopsy specimens allows establishment of viable small scale *in vitro*

cultures of epithelial cell with a minimum of non-epithelial cell contamination. Sustained cell-to-cell contact seems to be essential for the viability of the epithelial cell cultures and thereby one of the most important factors leading to improved survival of the cultures *in vitro*.

Butyrate metabolism

Butyrate is a short-chain fatty acid produced in the colonic lumen by bacterial fermentation of dietary fibers (58-60). It is an essential energy source for colonic epithelial cells, but influences also a wide array of cellular functions affecting colonic health through increased transcription of genes involved in inflammation, carcinogenesis, colonic defence barrier and oxidative stress (61-67). As butyrate oxidation is an important metabolic function in colonic epithelial cells; we developed a miniature collection system to measure butyrate metabolism in these small dimension short-term cultures. Initial studies showed that the cells were able to oxidate butyrate, confirming that this complex metabolic function was preserved in the primary cultures (I). High concentration of butyrate (>10 mM) was found to impair viability in epithelial cells as described by others (30,31,68). More complex concentration dependent experiments revealed that the rate of butyrate oxidation was not described by simple Michaelis-Mentens kinetics in the short-term cultures. When cultures were exposed to butyrate concentration > 0.5 mM, the rate of oxidation increased, suggesting that butyrate metabolism rather follows a substrate-activation pattern, or that two or several processes are involved. Concentration-dependent butyrate oxidation, revealing simple Michaelis-Mentens kinetics, has previously been reported in single cells suspension isolated from surgically resected colonic tissue (59). Low viability of these cells influences membrane integrity, and this may, theoretically, account for the missing membrane transportation dependency, as butyrate may diffuse passively through the cell membrane thereby exhibiting simple kinetics. Our findings are, however, in keeping with recent studies showing that the butyrate oxidation rate in epithelial cells depends on a monocarboxylate transporter receptor on the luminal cell membrane and that the substrate-induced increased oxidation is mediated through up-regulation of the receptor to secure a sufficient butyrate oxidation rate in the colonic epithelial cells irrespective of butyrate availability locally (64,69,70). Conclusively, our results suggest that this transporter system may still be operative in the short-term cultures and may explain the more complex kinetics observed *in vitro*.

Viability and metabolism in the inflamed colonic epithelium

It has been suggested that metabolic stress in the epithelial cells leading to loss of epithelial integrity may be of pathophysiological significance and contribute to the initiation of IBD or the induction of disease relapses (11,63,71). Likewise, it has been suggested that a primary energy deficiency due to inadequate butyrate metabolism is present in the colonic epithelium in patients with active UC (61,72,73). However, others have found normal metabolism in colonic epithelial cells from patients with quiescent disease, which do not support this hypothesis (59,74-76)(II). Some previous studies were performed on whole biopsy specimens (68,73,75,76), whereas others used epithelial cell suspension isolated from surgically resected colonic tissue (59,61). These conflicting findings may reflect different methodologies including variation in isolation procedures, purity and viability of the colonic epithelial cells, and moreover, differences in severity of intestinal inflammation. To avoid possible interference from on-going

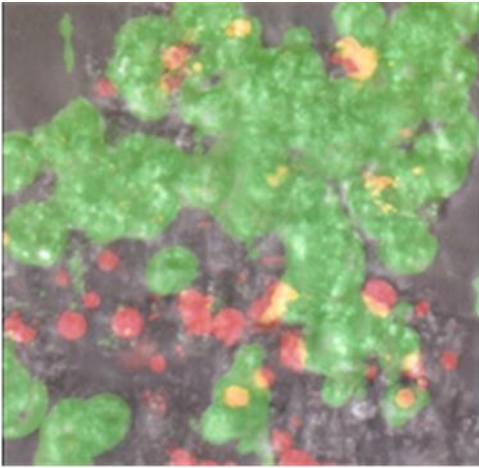


Figure 2 Digitalized confocal laser image of human colonic epithelial cells in primary culture. After culture for 24 hours cells were stained with calcein-acetomethylester (AM) combined with propidium iodide (PI). Viable and metabolically active cells enzymatically convert calcein-AM to the fluorescent non-ester form resulting in a green colour inside the cells. PI visualises dead cells due to loss of membrane integrity (methods section). The micrograph shows that living cells (green) were found mainly in clusters whereas dead cells (red) were found mainly as individual cells indicating that cell-to-cell interaction may be essential for survival in vitro.

inflammation on isolation procedures, viability and metabolic function, all the initial experiments were performed on short-term cultures obtained from patients with normal endoscopic appearance (I).

An important objective of this study was, however, to examine possible metabolic and functional changes occurring in the inflamed colonic epithelium. Accordingly, it was essential to evaluate the applicability of our in vitro model in a range of patients with IBD.

Short-term cultures of colonic epithelial cells were therefore established from a variety of IBD patients with and without ongoing endoscopic inflammation. Cell yields and viability in the cultures obtained from IBD mucosa were in general comparable with those obtained from control patients (Fig. 3, Panel A), (I, II). Were biopsies, however, obtained from severely inflamed IBD mucosa, cell yields as well as survival of the cells were more likely to be low and often not sufficient for establishment of short-term cultures (Pedersen, G, unpublished data). Severe mucosal inflammation is characterized by irregular epithelial crypt formation plus reduced crypt length and shorter life span (77,78). This may, subsequently, result in a relative reduced number of epithelial cells in each biopsy specimens, which affects the final cell yield and survival in vitro.

In addition to the viability studies, we evaluated butyrate metabolism in cell cultures obtained from macroscopically normal colonic mucosa from patients with UC and CD. Our experiments showed that butyrate oxidation in IBD patients were found to be similar to that measured in cultures from control patients (Fig. 3, panel A) (I, II).

Notably, our findings do not accord with the view that butyrate oxidation is extensively impaired in colonic epithelial cells as a primary defect, because metabolism in cultures from quiescent UC patients was identical to findings in non-IBD patients (Fig. 3, panel B) (68, 76, I, II).

Although butyrate oxidation have been reported to be impaired in colonic biopsies in active UC, this defect is secondary to the

inflammation and may therefore play an insignificant role in the early pathogenetic phase of the development of ulcerative colitis (72,75). Nevertheless, it is a well preserved sustained metabolic function in epithelial cell cultures and may therefore represent a useful and biological relevant endpoint in additional dynamic intervention studies in vitro (II).

Detoxification of phenolic compounds in human colonic epithelium

While butyrate production and metabolism seem to have beneficial effects on integrity of the human colonic epithelial cells, the epithelial lining is also constantly exposed to a wide range of potentially harmful luminal agents that may influence the probability of developing IBD or disease relapses (60,63,66,71,79-82). These include endogenous phenols produced by bacterial breakdown of dietary protein in the colonic lumen and exogenous phenols such as the widely used analgetic, paracetamol (71,81-83,85).

The colonic mucosa is equipped to eliminate phenolic compounds by inactivation through glucuronidation and sulfatation processes, after which the metabolites are excreted in the urine. Phenol sulfotransferase (PST) and phenol glucuronidase (PGD) have been demonstrated in colonic mucosa, and it has been suggested that impaired sulphatation capacity in patients with UC may be linked to the onset or perpetuation of the disease (81,86,87). It is unknown, though, whether phenolic compounds actually affect the epithelial cells. Due to the close contact between the epithelial lining and luminal phenols we found it relevant, using the devised model, to examine whether colonic epithelial cells have the capacity to detoxify phenols as a part of a first line defence to these potential harmful components.

Although, it has briefly been stated that sulphatation is the preferred conjugation pathway in colonic epithelial cell suspension isolated from resected colonic tissue, detoxification in viable adherent epithelial cells is unknown. (81,88). The glucuronidated products of the phenols were easily identified in the colonic epithelial cell cultures, whereas the sulphated product of the compounds was undetectable (III). Additional analysis revealed that both phenol sulfotransferase as well as phenol glucuronidase were present in isolated colonic epithelial cells. As a result, the absence of the sulphated phenolic metabolites was not due to lack of active enzyme in the cells. More likely, the results suggest that glucuronidation may be the predominant conjugation pathway in normal human colonic epithelium, as currently found also in a rodent intestinal model (89). It has been reported that concentration of sulfotransferase is increased in the right side of the colon compared to levels in left side and the rectum (90). Our cultures were primarily established from mucosal biopsies obtained from the transverse and left colon; and low expression of PST in the epithelial cells may, therefore, result in minor, undetectable levels of the sulphated products in the cultures. Experiments showed that addition of unconjugated phenolic compounds in physiological relevant concentrations impaired viability equally in the epithelial cell cultures from patients with UC and from control patients (III). Our findings in the differentiated short-term cultures concord with recent studies showing that dinitrophenol (DNP) induces mitochondrial damage and reduced barrier function in T84 and Caco-2 cell lines (79,80). In contrast to these results, HT-29 cells were resistant to the phenolic compounds substantiating the view that the origin and state of differentiation of distinctly transformed cell line models may influence metabolic and immunological functions in vitro (91).

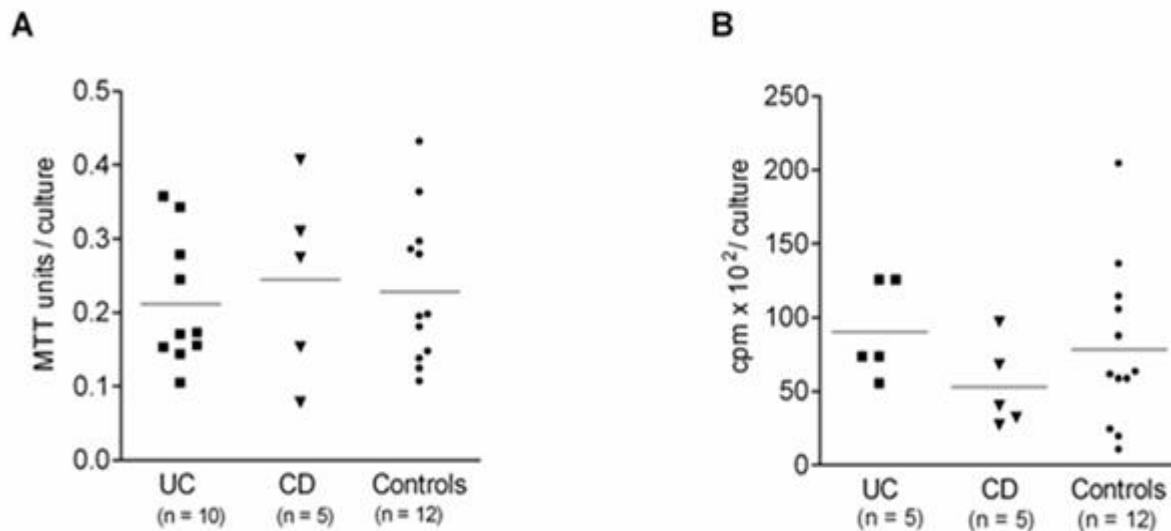


Figure 3 Panel A. Comparison of colonic epithelial cell viability in cultures from ulcerative colitis (UC), Crohn's disease (CD) and control patients. Cell viability was assessed by the conversion of 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) to its formazan after 24 hours of culture.

Panel B. Comparison of butyrate metabolism in cell cultures obtained from UC, CD and control patients. Assessment of butyrate oxidation rate is based on ¹⁴CO₂ liberation after 24 hours in culture. Median values for each group are shown as vertical lines. N-values refer to numbers of individual patients in each group. (P = NS between controls and UC or CD).

Our results, however, showed that these short-term epithelial cell cultures also preserve this highly specialized metabolic function and have the capacity to glucuronidate phenolic compound in vitro. Despite it being unlikely that abnormal sensitivity to luminal phenols play a significant primary pathogenic role in ulcerative colitis, our findings indicates that local excessive amount of un-conjugated phenolic compounds may display a direct adverse effect on colonic epithelium which may modulate chronic inflammatory response.

PROINFLAMMATORY CYTOKINES AND HUMAN COLONIC EPITHELIUM

Proinflammatory cytokines are pleiotropic mediators with a wide range of immuno-inflammatory effects that have emerged as important players in the pathogenesis of IBD (2,3,92-95). This has been supported by the finding of increased levels of TNF- α , IL-6 and IL-10 in inflamed mucosa of IBD patients (96-100). Moreover, the importance of TNF- α has been substantiated by the demonstration that selected patients with active CD and UC respond to treatment with anti-TNF- α antibodies such as infliximab and adalimumab (101-103). The precise mechanisms of cytokine-mediated mucosal injury are largely unknown, but one might be that pro-inflammatory cytokines, such as TNF- α and IFN- γ , directly impair epithelial cell function, perhaps as a part of the early phase in mucosal inflammation, similar to effects seen on distinct target cells in autoimmune rheumatic and endocrine diseases (93,104-113). Consistent with this notion, Deem et al showed that supernatants of activated lamina propria lymphocytes were directly cytotoxic to a human colonic epithelial tumour cell line (HT-29), and that TNF- α and IFN- γ were responsible for this effect (114). A number of studies describe that TNF- α and IFN- γ impairs barrier function in transformed colonic epithelial cell line monolayers (111,115-119). In addition, it has been shown that TNF- α and IFN- γ influence immunological function in transformed cell lines through regulation of the gene expression of various mediators, such as TNF- α converting enzyme, metalloproteinases

(MMP), chemokines, adhesion molecules and heat shock proteins (23,120,121,V).

Cytokine effects on colonic epithelium in vitro

Only few studies have focused on direct effects of proinflammatory cytokines on human colonic epithelium (7,11,34,114). Cell death, as judged by lactate dehydrogenase and Cr⁵¹ release has been a variable finding, and possible cytokine effects on specific metabolic functions of epithelial cells are unknown. Based on our previous demonstration that butyrate oxidation was a well-preserved metabolic function in colonic epithelial cells in vitro, the combined effects of pro-inflammatory cytokines on viability and butyrate oxidation were characterised using a number of relevant in vitro models of human colonic epithelium. As only limited knowledge existed about concentration levels, time-dependency and possible synergistic effects of the combined cytokine exposure, a range of explorative experiments were performed in cultures of the transformed cell lines, HT-29 and DLD-1 using cytokine concentrations of 10⁻¹² to 10⁻⁹ M. Our results revealed that IFN- γ induced a clear dose dependent decrease in viability in both the cell lines models, whereas effect of TNF- α was less pronounced. The combination of IFN- γ and TNF- α induced a concentration- dependent, progressive, synergistic decrease in viability in the cultures, which are in line with subsequent observations by Seidelin et al, showing a similar inhibitory effect in DLD-1 cells (122)(IV). Pre-treatment with IFN- γ rendered HT-29 cells highly sensitive to subsequent TNF- α exposure, resulting in a more pronounced impairment of viability than administration in the reverse sequence or simultaneous addition of both cytokines. Wang et al have shown that IFN- γ treatment primes Caco-2 cells to subsequent TNF- α -mediated epithelial barrier loss through up-regulation of TNF- α receptor 1 and 2 (111). This concurs well with our findings showing that addition of specific antibodies against IFN- γ and IFN- γ receptor reversed the detrimental effect of the combination of TNF- α and IFN- γ on cell viability. Conversely, TNF- α antibodies were less effective in restoring MTT metabo-

lism in the cultures. Cultures exposed to the cytokine mixture showed typical morphological and electrophoretic signs of apoptosis, which agrees with the observation that impaired viability in HT-29 cells was irreversible after removal of the cytokines (G. Pedersen, unpublished data). In contrast, no clear and reproducible signs of apoptosis were observed in cells after IFN- γ treatment alone (IV). These observations are in line with other studies showing that IFN- γ sensitizes HT-29 cells to inducers of apoptosis through a direct or indirect up-regulation of apoptosis-related genes, including TNF- α receptor, Fas receptor or Fas Ligands (122,123).

Although, no primary abnormalities in butyrate metabolism was observed in the epithelium from patient with IBD, it is possible that energy deficiency due to secondary occurring impairment of butyrate oxidation may lead to epithelial cell death and perpetuation of the inflammatory cascade. In line with this hypothesis we found that TNF- α and IFN- γ induced a progressive decrease in butyrate oxidation in the transformed cell line models (IV). The decline in butyrate metabolism was parallel to the reduction in viability in the HT-29 cells, whereas butyrate oxidation in DLD-1 cells decreased early, despite only minor changes in viability after cytokine exposure. Our observation suggests that the importance of butyrate oxidation on cell homeostasis / physiology may differ between distinct epithelial cell models—possibly as a consequence of different origin of the cells. The results corroborate, however, the view that cytokine-induced impairment of energy metabolism through reduced mitochondria function in colonic epithelial cells may precede cell damage (11). Moreover, our results show that IFN- γ is a potent immune mediator and possible key cytokine in the immunological cascade leading to damage of the epithelial barrier function as well as increased permeability — as repeatedly reported (7,111,115)

TNF- α and IFN- γ effects on inflamed colonic epithelium

Our observations and several other studies have shown that TNF- α and IFN- γ have direct effects on barrier function, metabolism and viability in transformed intestinal cell lines (111,115,116,122,IV). It is unknown, though, how, and whether normal differentiated human colonic epithelium respond in similar fashion to these immune mediators. Additional experiments showed that viability in the short-term cultures significantly decreased after cytokine exposure, although the effect was less pronounced than that observed in the transformed cell line models (IV). IFN- γ alone induces a significant inhibition of MTT value, whereas the effect by TNF- α was less pronounced and IL-1 β induced no inhibition. The clear synergistic effect observed in the transformed cell lines was not present in the colonic epithelial cells, and indeed, 10-fold higher cytokine concentrations were required to induce a marked decline in viability (II, IV). These results, however, also suggest that IFN- γ play a pivotal role in cytokine-mediated injury in the primary epithelial cells similar to observation in transformed cell lines (114,122-124,IV).

As previously shown, baseline viability and spontaneous butyrate oxidation in short-term cultures established from patients with IBD and controls were identical after 24 hours (Fig.3, panel B) (I, II). Following exposure to TNF- α and IFN- γ , viability decreased in the cultures, but cells from IBD patients were significantly less sensitive (Fig. 4) (II). No differences in cytokine-induced cytotoxicity between cultures from UC and CD patients were encountered. As differences in the baseline survival between cultures from IBD and control patients cannot explain this observation (Fig. 3, panel A), it could reflect that an intrinsic defence mechanism is triggered in IBD cells. Accordingly, it has been reported

that prolonged exposure to low concentration of TNF- α makes HT-29 cells more tolerant to additional cytokine exposure (122). Similarly, a corresponding mechanism may be operative in the human differentiated epithelial cells from IBD patients, as these may be exposed to abnormal TNF- α and IFN- γ levels locally in the intestine in vivo (97-100).

Surprisingly, TNF- α and IFN- γ exposure did not induce inhibition of butyrate oxidation parallel to inhibition of MTT metabolism in the cell cultures, which contrasts the parallel decrease in the transformed cell lines (IV). On the contrary, butyrate oxidation was slightly higher in the cytokine stimulated cultures from both IBD and control patients (Fig. 4) (II). Control experiments —using sonication or detergent to disrupt cell membrane integrity— showed that the relative increase in butyrate oxidation was not explained by enhanced non-limited transport of butyrate over damage cell membranes (69,70).

Our results in the cell lines accord with a recent study showing that TNF- α and IFN- γ stimulation inhibited butyrate oxidation in HT-29 cells through a reduction of the monocarboxylate transporter (MCT) -1 (73). Similarly, impaired mucosal expression of MCT-1 has been reported in active UC, suggesting that the observed impaired butyrate oxidation occur secondary to reduced butyrate transport (75). However, others have found substrate-induced increase in expression and activity in MCT-1, which was followed by an increased rate of membrane transport of butyrate. This mechanism may contribute to optimize intracellular availability of butyrate to obtain normal homeostasis of the epithelium (69,74). It has previous been described that activities of the enzymes in the β -oxidation pathway (i.e. butyryl-CoA and hydroxybutyryl-CoA) are increased in the mucosa from patients with active UC (75).

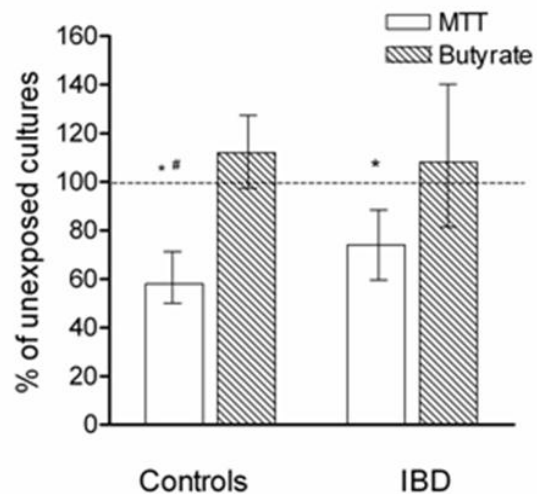


Figure 4 Comparison of TNF- α + IFN- γ (both 10^{-8} M) responses in colonic epithelial cell cultures from control patients and patients with inflammatory bowel disease (IBD). Cell viability was assessed by the conversion of 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) to its formazan and butyrate oxidation rate is measured based on $^{14}\text{CO}_2$ liberation in culture. MTT metabolism and butyrate oxidation was measured in cell cultures after cytokine exposure for 24 hours. (n =10-15 individual patients in each group). * P < 0.01 compared with parallel unexposed cultures. # P < 0.05 compared with MTT values in IBD cell cultures.

It is, therefore, possible that differentiated colonic epithelial cells increase butyrate oxidation by promotion of enzyme synthesis or by activation in combination with increased access to butyrate in vitro in response to cytokine-induced cellular stress (125). Alternatively, impaired mitochondrial function—as measured by MTT—may be an early cellular damage, which could occur before loss of cellular functions related to butyrate metabolism. This theory is further substantiated by the presence of swollen mitochondria with irregular cristae—judged by electron macroscopical examination in epithelial cells from IBD patients indicating a reduced function (126). In comparison, butyrate metabolism is more resistant to cytokine-induced damage than MTT metabolism in differentiated colonic epithelial cells in vitro.

The colonic epithelium has the capacity to express a range of immunological active mediators, which may be involved in the inflammatory cascade occurring in chronic intestinal inflammation (15,23,121,126,127). Transformed intestinal cell lines like HT-29 cells respond to immune stimulation with lipopolysaccharid and TNF- α by secreting IL-8—a chemokine implicated in the pathogenesis of IBD through mediation of chemo-attraction and activation of leucocytes (127-129). It is, nonetheless, unknown whether differentiated colonic epithelial cells also may contribute to leucocyte infiltration by secretion of IL-8 in response to proinflammatory cytokines. We found that cultures obtained from normal human mucosa spontaneously secrete IL-8, and when exposed to the combination of TNF- α and IFN- γ significantly increased IL-8 levels in cell supernatants. In contrast, cytokine exposure only induced a minor increase in IL-8 levels in cultures from IBD patients (II). Whether this is reflecting primary differences in the immunological capacity between IBD and control epithelium or is a consequence of repeatedly or prolonged exposure to cytokines in vivo is currently unsolved, and further studies are required. Overall, our results show that proinflammatory cytokines like TNF- α and IFN- γ induce direct cellular damage to the differentiated human colonic epithelium. This toxic effect on the epithelium may therefore be a contributor to the development of the inflammatory response and tissue destruction present in IBD. Our observations also correlate with the view that INF- γ is a key mediator of the events leading to chronic inflammation. Accordingly, IFN- γ has been considered a potential objective for therapeutic targeting in IBD in line with the widely use of TNF- α antibodies (101,103). However, only a minor clinical effect has been observed of INF- γ neutralizing antibodies in IBD patients (130,131). Currently, further studies evaluating the pathogenetic importance of IFN- γ and optimizing the antibodies are needed.

Matrix metalloproteinase regulation in inflamed colonic epithelium

Increasing evidence suggests that the presence of proinflammatory cytokines locally in the intestine is an important factor in the pathogenesis of IBD. Although, others and we have shown a direct toxic effect by these cytokines on the intestinal epithelium, it is less likely that this alone can lead to the characteristic intestinal tissue injury, notably mucosal ulcerations, fibrosis and stricture, seen in UC and CD. These events may rather occur through disturbance of the tightly regulated extra cellular matrix (ECM). MMPs are potent proteases that appear to control homeostasis of the EMC through regulation of distinct proteins involved in ECM growth, synthesis and degradation (132-139). Knowing that the epithelium actively participates in immunological processes in the mucosa, we hypothesized that these cells contribute to alterations of the ECM through secretion of distinct MMPs due to exposure to proinflammatory cytokines locally. Notably, pivotal

studies in foetal explant cultures and murine IBD models have demonstrated that the presence of distinct MMPs caused extensive tissue injury (140,141). In addition, treatment with MMP inhibitors improves experimental colitis in murine models indicating a role for MMPs in development of intestinal injury (142-144). Interestingly, a recent study has shown alteration of mucosal expression of distinct MMPs that correlates with changes in histological score and faecal calprotectin in CD patients treated with azathioprine or anti-TNF α drugs (133). Increased expression of several MMPs has repeatedly been found in inflamed mucosa from IBD patients (96,133,138,145-148). MMPs have previously been identified in myofibroblasts, smooth muscle cells, activated T-cells and macrophages, but whether human colonic epithelium also have the capability to express functionally active MMPs is less characterized. Accordingly, only a few, descriptive studies have been able to identify distinct MMPs in the colonic epithelium using immunohistochemical analysis (133,138,149,150). Interestingly, studies in other areas, such as gingival or dermal cell models, have consistently shown that epithelial cells strongly express a range of MMPs known to be involved in wound repair, inflammation and chronic ulceration (151,152,153). In addition, studies in murine IBD models also briefly reported increased expression of distinct MMPs localised to the inflamed epithelium, supporting the view that MMPs may both be up-regulated in presence of proinflammatory cytokines and be the final mediator of intestinal tissue injury (142,143,154-156). Our initial experiment showed that HT-29 cells spontaneously express transcripts of a range of MMPs. Furthermore, stimulation

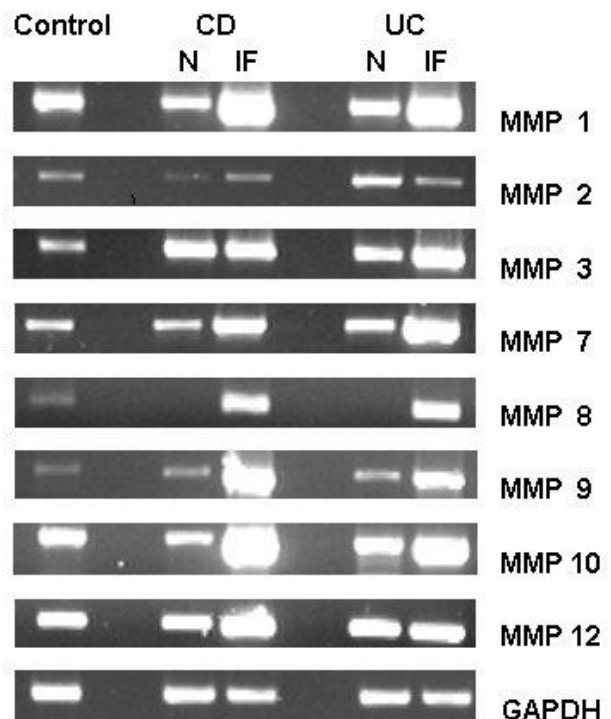


Figure 5 Expression of matrix metalloproteinase (MMP) mRNA in freshly isolated human colonic epithelial cells. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed on extracted mRNA from colonic epithelial cells isolated from control subject (C) and patients with active (I) or inactive (N) ulcerative colitis (UC) and Crohn's disease (CD). Expression of GAPDH is included as a housekeeping gene control. Data from one experiment out of three are presented.

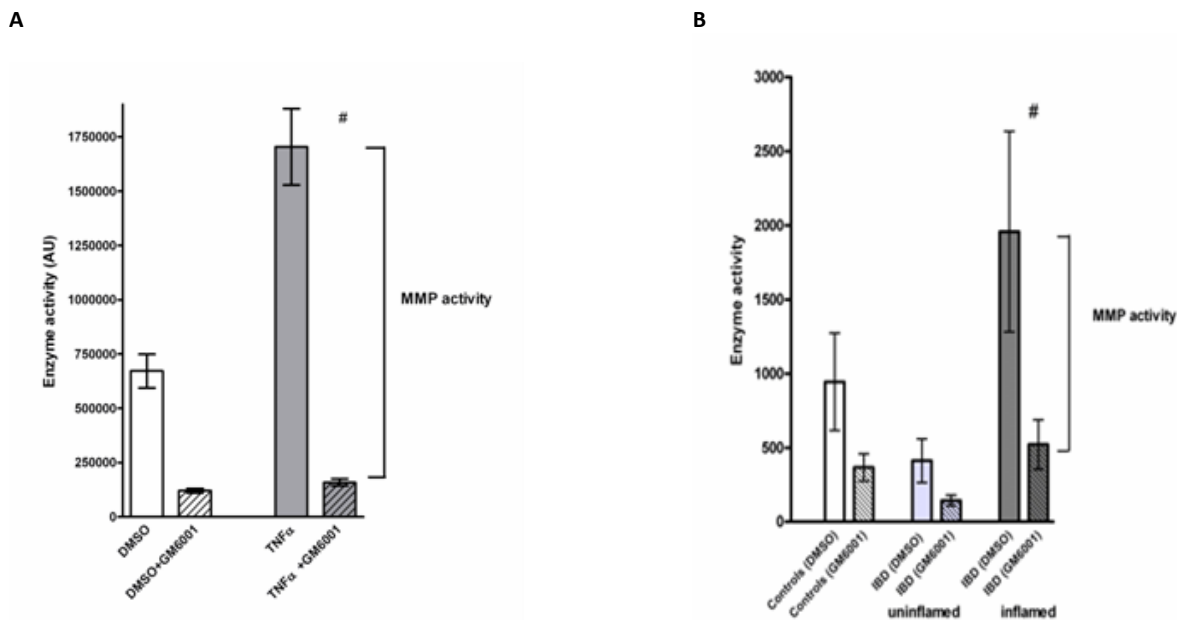


Figure 6

Panel A. Spontaneous and TNF- α -induced matrix metalloproteinase (MMP) enzyme activity in HT-29 cells. After stimulation with TNF- α (10-9 M) for 24 hours, MMP activity in cell supernatant was assessed by measurement of the ability to degrade a fluorescent peptide containing the cleavage sites for MMPs. Incubation with a specific MMP inhibitor GM 6001 was included in all samples, as a control for unspecific degradation of the peptide. The figure shows mean (SEM) values for six independent experiments. # P < 0.01 compared to activity in unstimulated cultures.

Panel B. MMP enzyme activity in human colonic epithelial cells from normal and inflamed colonic mucosa. Epithelial cells were isolated from mucosal biopsies from control patients and from uninflamed or inflamed mucosa from patients with inflammatory bowel disease (IBD). MMP activity in cell supernatant was assessed by measurement of the ability to degrade a fluorescent peptide containing the cleavage sites for MMPs. Incubation with a specific MMP inhibitor GM 6001 was included in all samples as a control for unspecific degradation of the peptide. The figure represent results from 5-7 patients, and bar represent means (SEM). # P < 0.01 compared to activity in unstimulated cultures.

with TNF- α and IFN- γ , which mimics an inflammatory stimulus, significantly increased mRNA levels of MMP-3, -10 and 13 in HT-29 cells after 6 hours (V). These findings are in line with observations in a wound model of the skin, showing a significantly upregulation of MMP10 and MMP13 in the epithelial cells after TNF- α exposure (152,157). Likewise, a similar regulatory effect of proinflammatory cytokines on MMP activities has been reported in gingival epithelial cells in vitro (157,158).

To extend the initial exploratory studies in cell lines, human colonic epithelial cells were next isolated from mucosal biopsies from control patients and patients with IBD using the devised technique. The cells clearly expressed transcripts for MMP-1, MMP-3, MMP-7, MMP-10 and MMP-12 (Fig. 5)(V), whereas the other MMPs tested were weaker, inconsistently expressed or undetectable. Moreover, MMP expression patterns were identical in epithelial cells isolated from UC and CD mucosa samples. These results are in line with a few studies reporting roughly identical expression of MMP 7, 10 and 13 in epithelial cells from UC and CD patients—as judged by immunohistochemical analysis. Finally, semi quantitative PCR analysis revealed that mRNA levels for MMP-1, MMP-3, MMP-7, MMP-9 and MMP-10 were significantly increased in epithelial cells isolated from inflamed mucosa compared to expression in cells from non-inflamed areas from the same IBD patient (V).

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Our detection of enhanced MMP 1-3, -7, -9 and -10 transcript levels in the epithelial cells from inflamed IBD mucosa expands the studies showing increased levels of these distinct MMPs in colonic mucosa from UC patients (136,138,149,159-162). MMP levels in the epithelium were not evaluated specifically in these studies, but it is possible that the increased MMP expression in these cells may have contributed the enhanced total mucosal

levels. Hence the detection of MMP-1 in differentiated colonic epithelial cells, as well in transformed cell lines and increased levels during inflammation, are actually in agreement with two previous studies in humans reporting MMP-1 expression in epithelial cells during wound healing and regeneration in necrotizing colitis and ulcerative colitis in children (163,164). MMP-8 was only detected in the colonic epithelial cells from inflamed mucosa in our setting. This observation is in line with the detection of MMP-8 in oral and skin epithelial cells mainly in areas of chronic inflammation (151,165,166), as well as the finding of increased levels of MMP-8 in inflamed epithelium in a murine colitis model (167). MMP-9 mRNA expression was up-regulated in epithelial cells from inflamed IBD mucosa; whereas this gelatinase could not be detected in the transformed colonic epithelial cell lines (V). Findings in murine colitis models suggest that increased levels of MMP-9 may play a crucial role in the development of intestinal inflammation (154,168). In addition, it has been suggested that regulated MMP-9 expression in epithelial goblet cells might play a role in maintaining normal mucosal defence (156). The presence of MMP-9 could therefore be linked to the highly differentiated colonic epithelial cells, which may explain why this particular MMP was undetectable in undifferentiated HT-29 and DLD-1 cells.

MMPs are secreted as pro-enzymes, which must undergo proteolytic processing to become fully active. The presence of several MMP RNAs in both HT-29 cells and isolated epithelial cells from IBD patients raised the question as to whether the expressed enzymes actually have functional proteolytic activity in vitro. Substrate gel electrophoresis has been widely used for qualitative identification of the active forms of MMPs based on gelatinase or casein degradation. This method is, however, rather insensitive and detects mainly MMP-2, MMP-3 and MMP-9 activity (155, 156). Using a specially designed cleavage assay based on degradation of a synthetic peptide mimicking the cleavage site for MMPs (Mca-Pro-Leu-Gly-Leu-Dap(Dnp)-Ala-Arg-NH₂), functionally active MMPs were easily detected in culture supernatants from HT-29 cells (Fig. 6, panel A)(V). TNF- α exposure increased MMP enzyme activity in these cells after 24 hours, which is consistent with the increased MMP transcript levels. Importantly, the levels of MMP activity were strongly inhibited by addition of the inhibitor GM 6001, known to inhibit MMP enzyme activity, thus confirming presence of MMP activity in the samples (Fig. 6, panel A) (V). Interestingly, functional MMP activity was also detected in cell supernatants from epithelial cell cultures from controls and IBD patients. Enzyme activity was identical in cultures from controls and non-inflamed IBD mucosa. In contrast, the level of functional MMP activity was significantly increased in cell supernatants from inflamed IBD mucosa compared with activity in cells from non-inflamed IBD mucosa from the same patient (Fig. 6, panel B)(V). The unspecific degradation of the peptide due to other protease activities was minimal, and the enzyme activity was again clearly inhibited by GM 6001.

In conclusion, our results show that differentiated human colonic epithelial cells have the capacity to express a range of proteolytic active MMPs and that expression and functional activity are increased in inflamed mucosa. This suggests that epithelial cells, like myofibroblasts, T-cells and macrophages, may contribute to local intestinal mucosal damage, through secretion of proteolytic active MMPs. Further studies are required to show if modulation of MMP expression and activity may be an adjunct to other therapeutic approaches aiming to control intestinal mucosal injury in IBD.

INNATE AND ADAPTIVE IMMUNITY AND THE COLONIC EPITHELIUM: THE ROLE OF TOLL LIKE RECEPTOR (TLR) 9

Our observations have shown, in line with others, that the intestinal epithelium not only acts as a physical barrier to commensal bacterial and foreign antigens, but also exhibits functional immunological activity involved in cytokine mediated mucosal damage and immune cell regulation (II,IV,V). Recognition of distinct microbial features and discrimination of potential harmful pathogens from commensals is an integral part of the innate and adaptive immune system, and disturbances in this tightly regulated immune function has increasingly been conceived as implicated in IBD. One pathophysiological explanation may be that commensal luminal bacteria which we normally tolerate, are mistakenly recognized as possible pathogens leading to chronic inflammation in genetically susceptible individuals (2,7,15,129,169-171). Some of the most important susceptibility genes described for IBD comprises NOD2 / CARD15 and toll like receptors (TLR). Accordingly, it has been suggested that polymorphism in distinct TLR genes could lead to innate/adaptive immune hypo- or hyper reactivity, which may contribute to the development of chronic intestinal inflammation and mucosal damage (7,14,170,172-174). Innate immune responses are orchestrated by a variety of pattern-recognition receptors, notably the TLRs in humans (169,175-178). The family of TLRs comprises at least eleven members, which recognise distinct pathogen-associated molecular patterns (PAMPs), including lipoproteins (TLR2), dsRNA (TLR3), LPS (TLR4), flagellin (TLR5) (171,177-179). One important is TLR9, which recognises specific bacterial DNA sequences characterised by a high content of unmethylated cytidine-phosphate-guanosine (CpG) motifs, that are prevalent in bacterial, but not in mammalian genomic DNA (169,180,181). Several studies from murine IBD models and intestinal cell line models strongly suggest that TLR9 plays a role in innate and adaptive immunity and development of chronic inflammation through recognition and response to bacterial products in the gut, but the experimental results are surprisingly conflicting (182-186). Some studies have found that stimulation with bacterial DNA or synthetic CpG-oligonucleotides induces a robust TLR9 mediated, proinflammatory cytokine response in cell lines and aggravates chemically induced colitis in rodents (186-189). In contrast, others have shown that early administration of CpG-oligonucleotides or probiotic bacterial DNA actually reduces the severity of experimental colitis (29,183,190-192). As the epithelial lining is considered to be a key player in first line defence against the luminal components and therefore likely to be involved in the regulation of adaptive immune responses in the intestine, we aimed to explore the role of TLR9 in the human colonic epithelium in relation with IBD.

TLR9 expression in human colonic mucosa

Only limited knowledge exists about the role of TLR9 in the human intestine and firstly, we therefore explored whether this receptor was present in the human colonic mucosa. Our initial experiments identified TLR9 mRNA in whole mucosal biopsies from both control subjects and patients with IBD. The expression was, however, variable and in some of the samples undetectable. Additional, semi-quantitative analysis showed no significant differences in TLR9 expression between control subjects and IBD patients (VI). Conversely, when a subset of patients served as their own control, the levels of TLR9 expression was significantly lower in samples from inflamed mucosa compared with those obtained in parallel from an endoscopically un-inflamed area (Fig. 7).

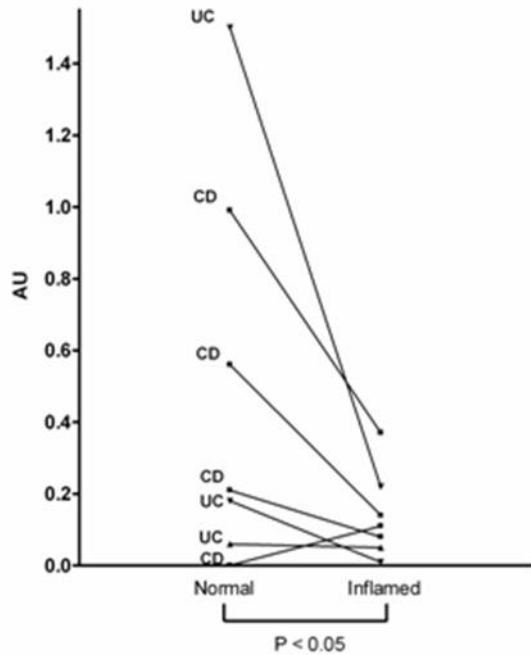


Figure 7 Expression of Toll-like receptor (TLR) 9 mRNA in paired biopsies obtained from inflamed and uninfamed colonic mucosa in patients with inflammatory bowel disease. Reverse transcriptase (RT)-PCR analysis was performed on RNA extracted from colonic biopsy specimens. The identity of the TLR9 PCR products was confirmed by sequencing. Expression of GAPDH is included as house-keeping gene control. Figure presents data from 3 ulcerative colitis and 4 Crohn's disease patients. # $p < 0.05$ compared to expression in uninfamed biopsies.

Previous studies of other TLRs in human IBD have shown highly variable results in relation to inflammation ranging from increased TLR4 expression to reduced or unchanged levels of TLR3 and TLR2 respectively (170,172,193-195). Mucosal biopsies consist of several cell types including mononuclear cells and epithelial cells. These variable results could be a consequent of inflammation-related alterations in TLR levels in the different cells in the colonic mucosa (172,194-196). Additional PCR analysis performed on freshly isolated colonic epithelial cells showed that TLR9 mRNA was widely expressed in these cells both from IBD patients and controls (VI). TLR9 mRNA was also detected in lamina propria mononuclear cells isolated in parallel from the mucosal biopsies. This suggests that several cells contribute to the total mucosal TLR9 level and TLR expression patterns may thus reflect a need for adjusted TLR ligand recognition at distinct locations in the mucosa. Our findings, however, extend a previous study showing presence of TLR9 transcript in normal colonic epithelium and are in line with the observations that intestinal epithelial cells express several other TLRs including TLR2, TLR4, TLR5 and TLR8 (12,170,179,197,198).

TLR9 ligand mediated immune activation in colonic epithelial cells

The presence of TLR9 in human colonic epithelium raised the question as to whether these cells also have the capacity to recognize and respond to specific TLR9 ligand stimulation in vitro.

Previously, several synthetic immunostimulatory CpG-ODNs (TLR9 agonists) have been presented in a variety of in vitro studies. Consequently, we used the CpG oligonucleotide ODN 2006 due to its earlier described strong TLR9 mediated immunostimulatory activity in human B cells (181,199-201). As demonstrated earlier, colonic epithelial cell models respond to immune stimulation by LPS and TNF- α by secretion of IL-8 and this chemokine was selected as a marker for possible TLR9 ligand-mediated effects (15,127,II,IV). Initial experiments were performed in HT-29 cells, which spontaneously also expresses TLR9 (29,VI). Stimulation with ODN 2006 induced a significantly increased IL-8 secretion in these cells, whereas only a minor, non-significant increase was observed in cultures exposed to the inverted (non-CpG) version and the phosphorothioate backbone oligonucleotide as expected (Fig. 8, panel A). None of the tested oligonucleotides impaired viability in the HT-29 cells, and the increased IL-8 secretion was, therefore, not a secondary event due to cell membrane damage or low viability in the cultures.

Although IL-8 is considered a NF- κ B responsive gene, no detectable NF- κ B or I κ B activation in the ODN 2006 treated HT-29 cells was observed, despite a clear NF- κ B activation of the oligonucleotide in TLR9 positive mononuclear cells (180,202-204,VI). The oligonucleotide induced levels of IL-8 in the HT-29 cells were considerably lower than in TNF- α stimulated control cultures and it is possible that the immune stimulatory effect of ODN 2006 is too small to induce a measurable NF- κ B activity. Another explanation might be that alternative downstream signalling pathways like activator protein-1, protein kinase C and MAPK are involved in TLR9 activation in human epithelial cells as suggested previously (13,171,189,205).

Next, we tested whether short-term cultures of primary human colonic epithelial cells would respond similarly to TLR9 ligand stimulation in vitro. However, ODN 2006 exposure induced no change in IL-8 secretion in these cultures, despite a clear response was observed following cytokine stimulation as expected (Fig. 8, panel B). TLR9 mRNA was present in freshly colonic epithelial cells, but TLR9 protein was only weakly expressed and seemed to be present in two isoforms as judged by Western blot analysis (VI). At least 4 different isoforms of TLR9 have been described, but it is currently unknown whether CpG responsiveness is correlated to the presence of these isoforms of the protein. These findings have, however, led to the hypothesis that differential expression of TLR9 isoforms during inflammation may contribute to variation in inflammatory responses in distinct cell types (206,207). Moreover, the immune stimulatory potential of various CpG oligonucleotides is determined by structural properties, such as frequency and placement of CpG motifs, composition of flanking nucleotides and length of the oligonucleotide (192,201,203,206,208,209).

The ODN 2006 tested in our study was selected due to its proven ability to activate mononuclear cells. It is possible, though, that other CpG oligonucleotides may exhibit stronger immune stimulatory potential in primary colonic epithelial cells. A recent study has shown that apical prestimulation with oligonucleotides in a TLR9 positive intestinal cell line rendered the cells insensitive to basolaterally TLR9 ligand re-stimulation (210). Furthermore, it has been reported that spontaneous increased levels of TNF- α , IL-1 β and IL-8 found in organ cultures of colonic biopsies from inflamed UC mucosa are reduced after stimulation with distinct oligonucleotides in vitro and that this effect is mediated by triggering of TLR9 (192). Our results are in line with these findings thus indicating that primary human colonic epithelial cells may obtain tolerance to TLR9 mediated CpG immune stimulation through pre-

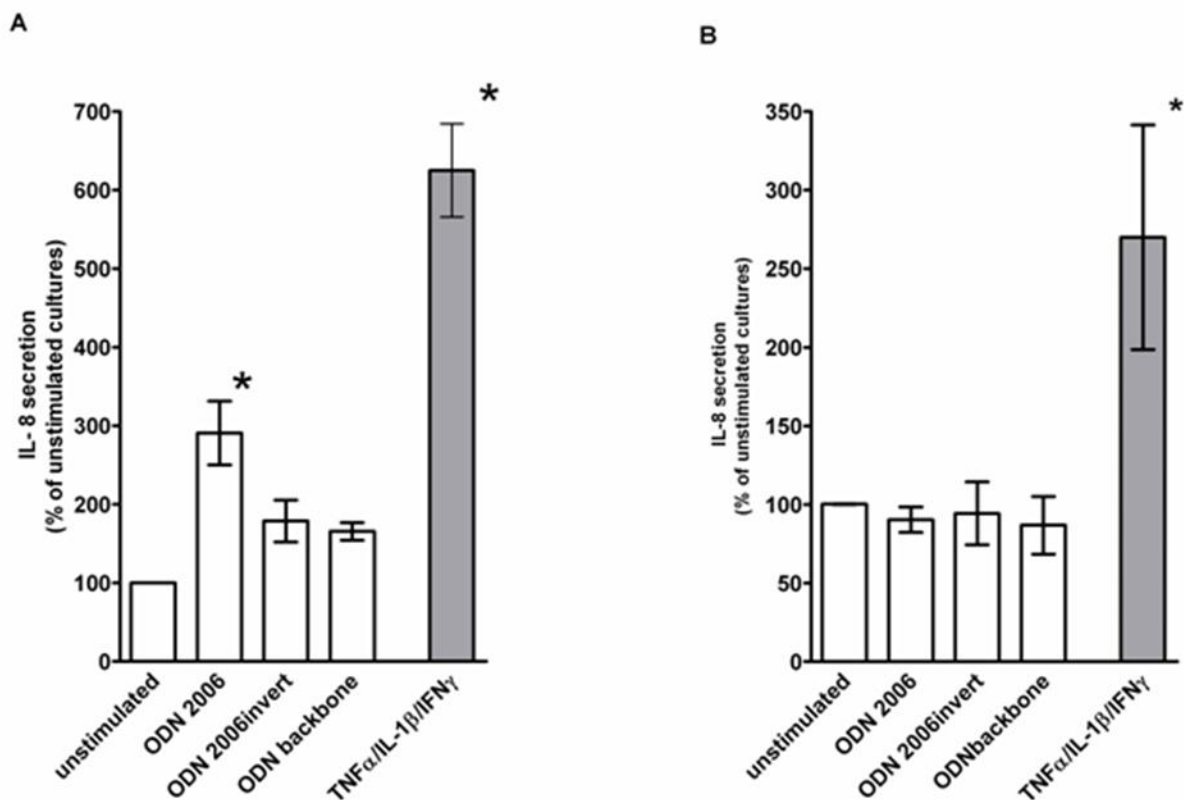


Figure 8 Effects of oligonucleotides (ODN) on IL-8 secretion in HT-29 cells (Panel A) and in primary human colonic epithelial cells (Panel B). IL-8 protein secretion in cell supernatants was assessed by ELISA after stimulation with CpG ODN 2006, non-CpG ODN 2006 (invert version) or ODN phosphorothioate backbone (all 10.0 μ g/ml) for 5 hours. For comparison, the response to mixed TNF α , IL-1 β and INF γ (10 \cdot 8 M) stimulation is shown. IL-8 values in stimulated cultures are expressed relative to values in unstimulated cultures. Data represents mean (SEM) values of 6 - 8 independent experiments. * $p < 0.05$ compared to unstimulated cultures.

exposure to luminal bacterial products and theoretically, play an important role in maintaining intestinal homeostasis and a balanced immune response to luminal content in vivo (169,188,210). Alternatively, other studies have shown Tollip, a toll inhibitory protein to be involved in TLR2 and TLR4 mediated hypo-responsiveness to repeated ligand stimulation in epithelial cells (12,205,209). We have recently described that differentiated colonic epithelial cells also express Tollip (211). Therefore, a similar inhibitory mechanism may play a role in the observed lack of TLR9 mediated response to ODN 2006 in these cells. This needs, however, to be further evaluated. Our findings show that epithelial cells have the capacity to express TLR9, which are in line with the view that these cells are involved in regulation of the innate and adaptive immune system. Alterations in TLR9 expression and signalling may result in dysregulation of the adaptive immune system, which could be an important event in the development of tissue damage and progressive inflammation in IBD. Further studies are, however, required to explore the importance of TLR ligands in intestinal homeostasis and chronic inflammation, but also to elucidate whether regulation of TLR signalling could be a new therapeutic target in IBD (29,169,186,202).

PPAR γ ACTIVITY IN HUMAN COLONIC EPITHELIUM: A POSSIBLE THERAPEUTIC TARGET IN ULCERATIVE COLITIS?

PPAR α , PPAR β and PPAR γ , are members of the nuclear hormone receptor group of transcription factors. They share a relatively high degree of structural and functional similarities, and are best known for their role in regulating of number of genes participating in lipid and carbohydrate metabolism (212-214). In addition, they are implicated in the regulation of proliferation, signal transduction and cellular motility in the intestinal mucosa (104,215-220). PPAR γ is also playing an important role in immune responses, through its ability to down-regulate the expression of pro-inflammatory cytokines and to direct immune cell differentiation towards an anti-inflammatory phenotype (213,219-225). Studies in rodent models have revealed that PPAR γ is expressed throughout the gastrointestinal tract from the duodenum to rectum and interestingly with the highest levels in the epithelium of the distal colon (104,222,226). Activation of PPAR γ with natural or synthetic PPAR γ ligands, such as rosiglitazone, has been shown to attenuate the severity of inflammatory lesions, weight loss and expression of inflammatory mediators in experimental rodent models of colitis (223,227-229). Accordingly, genetic ablation of PPAR γ was shown to increase susceptibility to experimental colitis (216,222,223,230). Observation in these models suggests that adequate epithelial PPAR γ expression and activity are important

to maintain mucosal homeostasis and prevent colitis (215-217,222,226,231,232).

It has recently been reported that a mutation in the PPAR γ gene in a subset of UC patients, which results in impaired mucosal PPAR γ mRNA expression, leads to increased expression of distinct TLRs and pro-inflammatory cytokines (233). Consequently, we aimed to characterize expression and activity of PPARs in human colonic epithelium and, additionally, to evaluate the functional significance of a possible imbalanced PPAR γ regulation at the cellular level in relation to inflammation.

PPARs and the inflamed human colonic epithelium

Our initial experiments confirmed that the PPARs are present in human colonic mucosa and that PPAR γ mRNA expression is decreased in mucosal biopsies from patients with active UC as reported briefly previously (234). These findings have been further substantiated by a recent study reporting that PPAR γ gene expression is negatively correlated with severity of endoscopic appearance in UC patients (235). Importantly, PPAR α , β and γ mRNA were consistently detected in epithelial cells isolated from colonic mucosal biopsies (VII). The PPARs were also identified in isolated lamina propria mononuclear cells, but at a clearly lower level than in the epithelial cells. Our results are consistent with repeated findings in rodent colitis models and extend a few previous studies detecting PPAR γ by western blot analysis in epithelial cells isolated from colonic tissue obtained in patients undergoing surgically resection for colorectal cancer (216,222,226,236-238). In addition, studies in human colorectal adenomas and cancers have confirmed that epithelial cells are the primary cellular component of PPAR γ signalling in human colon (239,240). PPAR γ mRNA expression was significantly reduced in epithelial cells from patients with active UC compared to controls, while expression of PPAR α and δ was unaltered. This indicates that impaired PPAR γ expression is a unique feature rather than an epiphenomenon due to overall inhibition of PPAR signalling in inflamed human mucosa (VII). It is currently unknown which factors are responsible for the impaired PPAR γ expression in inflamed UC epithelium. Decreased PPAR γ expression has been described during the course of intestinal inflammation in rodent models, suggesting that immune mediators may be involved in down regulation of the receptor (231,241). Hence, proinflammatory cytokines such as TNF- α , and IL-1 β have been shown to decrease PPAR γ expression in adipocytes and liver cells leading to altered differentiation and dysregulation of cellular metabolism (219,242-244). As levels of pro-inflammatory cytokines are increased in actively inflamed ulcerative colitis mucosa, a similar cytokine mediated negative feedback may explain the reduced PPAR γ levels in the inflamed epithelium (Fig. 9, panel A) (97-99, 245,VII). It has been suggested that PPAR γ polymorphism leading to reduced PPAR γ expression and TLR mediated intolerance to commensal bacteria, may perpetuate to the development of chronic intestinal inflammation (233,247). These theories accord with observations showing that mice with targeted disruption of the PPAR γ gene in intestinal epithelial cells have an enhanced susceptibility to TNBS-induced colitis compared to wild type littermates (222,248). In case impaired PPAR levels were explained by a genetic mutation, expectations would be however, that this feature is also identified in uninfamed epithelium from the same patient. Our findings showing a decreased expression only in the inflamed areas therefore contradict such theories (Fig. 9, panel A)(VII).

The finding of selectively impairment of PPAR γ expression in the inflamed UC epithelium prompted us to evaluate whether this has

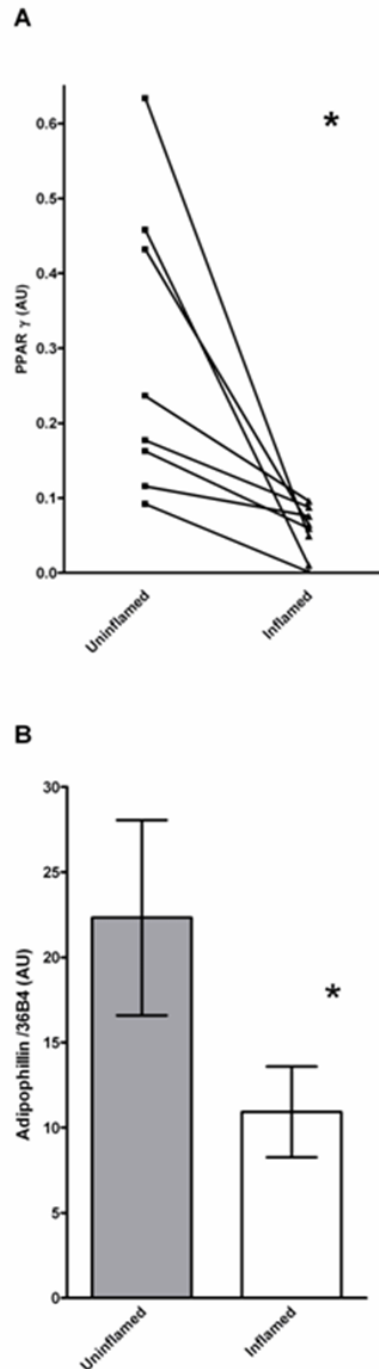


Figure 9

Panel A. Expression of PPAR γ mRNA in paired samples of epithelial cells isolated from endoscopically normal and inflamed mucosa from the same patient with active ulcerative colitis and measured by reverse transcriptase-polymerase chain reaction (RT-PCR). Mean (SEM) values from 8 patients are presented.

* $p < 0.01$ compared to cells from un-inflamed mucosa.

Panel B. Expression of adipophilin mRNA in paired samples of epithelial cells isolated from endoscopically normal and inflamed mucosa from the same patient with active ulcerative colitis and measured by reverse transcriptase-polymerase chain reaction (RT-PCR). Mean (SEM) values from 6 patients are presented. * $p < 0.02$ compared to cells from un-inflamed mucosa.

any functional applications for PPAR γ mediated signalling. Additional experiments showed that the low receptor expression in the epithelial cells was followed by a down regulation of adipophilin expression, a gene that is transcriptionally activated by PPAR γ ligands suggesting that PPAR γ signalling was significantly impaired in the inflamed epithelium (Fig. 9, panel A and B) (239,249,VII). Interestingly, stimulation of isolated colonic epithelial cells with rosiglitazone (a synthetic PPAR γ ligand) showed a clear concentration dependent increase in adipophilin expression, indicating an in vitro activation of the receptor. A five-fold increase of adipophilin expression was observed in epithelial cells isolated from inflamed UC patients, suggesting that PPAR γ signalling can be restored by the synthetic agonist in spite of low expression levels of the receptor itself (VII). It has been suggested that PPAR γ ligands/agonists may have a positive feedback effect on receptor levels, but in our setting no increase of PPAR γ mRNA expression in the epithelial cells was observed after rosiglitazone exposure (250). Previously, it has been reported that the beneficial effect of PPAR γ ligands in rodent colitis models may to some extent be independent of functional PPAR γ in the epithelium (216,228). In line with these observations, PPAR ligand-mediated down regulation of pro-inflammatory cytokines appears to be independent of PPAR γ expression at high ligand concentration levels in immune cells (221,225,251). A high local concentration of the PPAR γ ligand may be consequently be able to activate the PPAR γ response elements despite low receptor levels, thus explaining our observations in the epithelial cell cultures in vitro (216,228,VII).

From bench to bedside: effects of PPAR γ ligand enema in active ulcerative colitis

The beneficial effect of treatment with synthetic PPAR γ ligands in rodent colitis models, and the outcome of two recently published clinical studies suggesting therapeutic efficacy of rosiglitazone in patients with UC, have raised interest in PPAR γ as a potential target for anti-inflammatory therapy (252,253). However, there is growing evidence that systemic treatment of type 2 diabetes with PPAR γ ligands like rosiglitazone and other drugs belonging to the thiazolidinediones class of anti-diabetic drugs are associated with increased risk of severe cardiac complication (212,254). Systemic treatments of UC with these drugs are therefore less promising (252,254-256). The functional studies in the devised cell model showed that rosiglitazone has the capacity to activate epithelial cells in vitro despite low PPAR γ receptor levels (VII). This prompted us to evaluate whether stimulation with rosiglitazone directly at the colonic mucosa surface similarly could activate PPAR γ signalling and lead to a clinical beneficial effect. The topical approach allows application of ligand directly on the PPAR γ positive epithelium, and at the same time reduces the risk of possible systemic side effects by the drug.

We designed a small, randomised pilot study comparing clinical effect of rosiglitazone enema treatment with traditional topical mesalazine treatment in patients with moderate to severe distal ulcerative colitis. Patients with endoscopically and clinically active disease were randomised to treatment with either rosiglitazone enemas (4 mg) or mesalazine enemas (1 g) for 2 weeks. Interestingly, treatment with rosiglitazone enemas induced a significant clinical improvement after 14 days, similar to the effect of mesalazine as judged by the reduction in Mayo Score (Fig.10) (257,VII). All patients obtained clinical and endoscopical improvement, defined as at least a 3-point reduction in the Mayo Score. In the more severe cases of proctitis or proctosigmoiditis, topical treatment with mesalazine often needs to be extended to a minimum

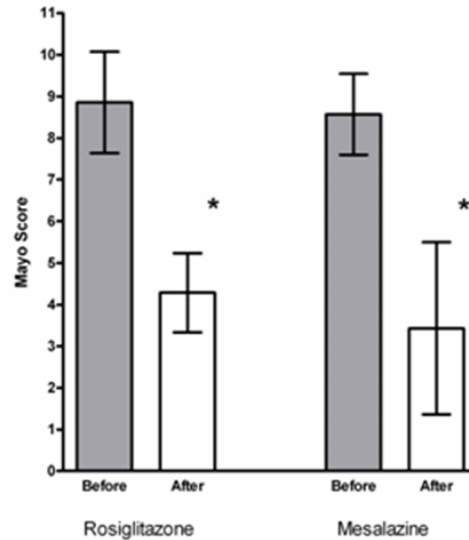


Figure 10 Clinical effect of rosiglitazone and mesalazine enema treatment in patients with active distal ulcerative colitis. Patients were treated with rosiglitazone (84.5 μ mol) or mesalazine (1 gram) enemas once daily at bedtime for 14 days. Mayo Score was assessed at inclusion and after 14 day treatment. Mean (SD) values from 7 patients are presented. * $p < 0.01$ between Mayo score before and after 14 days treatment.

of 4 weeks to obtain clinical remission and mucosal healing (258,259). Most of the included patients had previously experienced slow or incomplete effect of topical treatment and an element of chronic activity may explain why only a single patient obtained full clinical remission defined as a Mayo Score of zero. Extension of the treatment time may therefore lead to a higher rate of remission (258,259). The concentration of rosiglitazone (84.5 μ mol/l) in the enemas was chosen on the basis of our in vitro experiments in colonic epithelial cells. Importantly, this concentration induced a clear activation of the PPAR γ receptor in colonic epithelial cell cultures in vitro without affecting cell viability (VII). The biological effect of applying rosiglitazone locally in the colonic mucosa was completely unknown; believing it safe, we used a total rectal dose of 4 mg in the trial, which is half the recommended oral dose of rosiglitazone in the treatment of diabetes and also the dose used in the previous mentioned clinical trials in ulcerative colitis (212,214,252,260). Accordingly, the rosiglitazone enemas were well tolerated and no side effects were reported. In addition, no systemic effect was observed on liver or kidney function judged by analysis of blood samples. Consequently, it is possible that administration of higher doses or prolonged treatment time of rosiglitazone may be even more effective, but this has to be evaluated by further studies. Treatment with topical application of rosiglitazone induced a significant increase of adipophilin expression in isolated epithelial cells from the patients already after the first installation of an enema (VII). These results indicate that the beneficial therapeutic effect of rosiglitazone enema treatment was linked to activation of PPAR γ signalling pathways (VII). Furthermore, a minor non-significant increase of adipophilin expression was observed in the epithelium after installation of mesalazine enemas. Increasing recent evidence suggests the anti-inflammatory effect of 5-aminosalicylic acids like mesalazine is mediated through PPAR γ signalling (223,250,256,258). The observed adipophilin activation

from patients treated with 5-ASA was less pronounced compared with the effect of rosiglitazone, which may reflect that the synthetic PPAR γ ligand is a more potent PPAR γ activator than traditional 5-ASA components (256,258).

In conclusion, our results support the view that PPAR γ signalling may be a new potential therapeutic target in the treatment of IBD. Further studies are necessary to evaluate and optimise the potential effect of topical PPAR γ ligands in distal ulcerative colitis, alone and in combination with other anti-inflammatory treatment. Moreover, to minimise systemic side effect, it may even be of interest to develop new oral PPAR γ stimulating drugs, which are released and perform their anti-inflammatory effect locally in the distal part of the intestine similar to 5-ASA components.

CONCLUSION AND PERSPECTIVE

This work has shown that it is possible to establish short-term cultures of representative, viable human colonic epithelial cells from routine endoscopic mucosal biopsies of patients with inflammatory bowel disease (IBD) and control patients. The use of biopsy specimens was a new approach that overcomes the various limitations inherent in commonly used models based on animal cells, transformed human intestinal cell lines and isolated cells from resected colonic bowel segments. Initial studies showed that it is crucial to preserve the isolated epithelial cells in crypt-like structures and by optimising isolation procedure and culture conditions we succeeded to establish cultures that survived with intact metabolic function for at least 24 hours in vitro. The devised biopsy-based model was applicable for evaluation of a number of important cellular metabolic and immunological functions in human colonic epithelium. These include the ability to oxidate butyrate, detoxify phenol compounds, secrete the chemokine interleukin (IL)-8 and express metalloproteinases (MMP), toll like receptor (TLR) 9 as well as peroxisome activated proliferators (PPAR) in vitro. Although cell yield and life span of the cultures is limited, the model has also shown its usefulness in a number of other experimental settings in our laboratory and other groups have later presented similar techniques. The increasing data available on the homeostasis of the intestinal epithelium may facilitate further optimization of the isolation procedures and culture conditions and thus improve survival and applicability of models based on biopsy specimens in the future. Tumour necrosis factor (TNF)- α and interferon (IFN)- γ are key pro-inflammatory cytokines, which are present in increased amounts in colonic mucosa with active IBD. Using the devised model, both cytokines were found directly to impair the viability of human differentiated colonic epithelial cells and to induce secretion of IL-8 and distinct MMPs in vitro. Interestingly, colonic epithelial cells from inflamed mucosa were less sensitive to cytokine-induced damage, which suggests that an intrinsic defence mechanism is triggered in these cells, perhaps as a result of exposure to toxic luminal factors or high local cytokine levels in vivo. It is well established that TNF- α and IFN- γ play important roles in the pathogenesis in IBD, but the precise interaction with the intestinal epithelial cell is not well characterized. Our findings show that the cytokines affect viability and immune signalling in human colonic epithelial cells which is consistent with effects on distinct target cells seen in a number of other chronic immune-inflammatory diseases. Accordingly, our data extend the emerging view that alterations of homeostasis of the intestinal epithelium may be one of the early events in the pathogenesis of IBD. Systemic treatment with antibodies against TNF α is now widely used in IBD and the growing evidence for an important role of the intestinal epithelium raises the interest for developing drugs with

a similar action that are suitable for topical application in selected patients in the future.

IBD is characterized by chronic mucosal inflammation, which in severe cases may lead to intestinal ulcerations and in CD eventually development of fibrosis. Although the data presented here suggest that direct TNF- α and IFN- γ mediated cytotoxicity may be an initial event, these cytokines are known to have other effects, including activation of MMPs. Using the model, and a specifically devised functional peptide cleavage assay it was shown that these proteolytic enzymes are secreted by colonic epithelial cells in response to TNF- α exposure. Since they are functionally active, the data support a role of MMPs in the pathogenesis of mucosal damage in IBD that involve epithelial cells. Modulation of MMP expression and activity has been proposed as a possible new therapeutic approach to reduce intestinal mucosal injury in IBD. Viewed in the light of the involvement of the epithelial cells in this process, further studies are warranted to show if a topical approach may be a useful adjunct to other treatments in this context.

The novel demonstration that colonic epithelial cells express TLR9, a pattern recognition receptor, extends the emerging view that the epithelium represents an important frontline cellular component of the innate immune system in the gut. Interestingly, the differentiated epithelial cells, which have been exposed to the luminal bacterial flora in vivo, were unresponsive to TLR9 ligand stimulation, contrasting findings in the epithelial cell line HT-29 that is cultured continuously in bacterial free environment. This may suggest, theoretically, that the normal human colonic epithelium can avoid inappropriate immune responses to microbial antigens including commensal bacterial DNA through modulation of the TLR9 pathway. It has been suggested that the increasing incidence of IBD at least partly may be linked to environmental factors, including the luminal content. Further studies are therefore relevant to describe the interactions between the intestinal epithelium, as the first line defence to luminal antigens, and components of the innate and acquired immune system. The presently devised cell model was shown to be a valuable technique in a number of expression and functional studies in vitro. Additionally, it was also found to have the potential to explore new targets for therapeutic intervention in a clinical setting. Hence, our results from the functional PPAR γ experiments in vitro, prompted us to design a small controlled, clinical study exploring the possible stimulatory effects of rosiglitazone (a PPAR ligand) in vivo. Interestingly, it was found that topical application of rosiglitazone enemas in patients with active distal ulcerative colitis (UC) reduced clinical activity and mucosal inflammation similar to the effect measured in the UC patients treated with mesalazine enemas. Moreover, rectal application of rosiglitazone induced PPAR γ signalling in the colonic epithelium in vivo, supporting the view that activation of PPAR γ may be a new potential therapeutic target in the treatment of UC in the future. Overall, the in vitro model of representative human colonic epithelial cells presented here, has shown to be a widely useful technique for detailed studies of a range of metabolic and immunological functions that are important for homeostasis of the healthy as well as diseased epithelium. Currently, the findings support the view that intestinal epithelial cells actively participate in immunological processes in the colonic mucosa. Additionally, the model seems to be applicable for generating and evaluating new therapeutic approaches from laboratory bench to bed line as illustrated by the PPAR γ study where findings in an in vitro setting, initiated the hypothesis and experimental design of an in vivo clinical trial. Models based on representative colonic epithelium

lial cells, as the one described here, have increasingly been used in combination with other models in immunological (and other) studies of the human colonic mucosa. Thus, it is probable, that studies in these cell models could contribute with important knowledge about the pathogenesis of human inflammatory and neoplastic colonic diseases also in the future.

ENGLISH SUMMERY

Ulcerative colitis (UC) and Crohn's disease (CD), collectively referred to as inflammatory bowel disease (IBD), are chronic immune disorders affecting the gastrointestinal tract. The aetiology of IBD remains an enigma, but increasing evidence suggests that the development of IBD may be triggered by a disturbance in the balance between gut commensal bacteria and host response in the intestinal mucosa. It is now known that epithelial cells have the capacity to secrete and respond to a range of immunological mediators and this suggests that these cells play a prominent role in the pathogenesis of IBD. Current knowledge about the intestinal epithelium has mainly been obtained using models based on animal cells, transformed human intestinal cell lines and isolated cells from resected colonic bowel segments. Species difference, malignant origin and confounders related to surgery, obviously make these cell models however less applicable for pathophysiological studies. Consequently, there was a clear need for models of representative intestinal epithelial cells that would allow functional and dynamic studies of the differentiated human colonic epithelium *in vitro*.

The primary purpose of this thesis was to explore and validate the optimal conditions for establishing a model based on short-term cultures of human colonic epithelial cells obtained from endoscopic biopsies. The cell cultures were accordingly used to describe the interplay between proinflammatory cytokines and colonic epithelium, with focus on alterations in viability, butyrate metabolism and secretion of a chemokine and metalloproteinases (MMP). Finally, the model was used to characterize expression and activation of receptors like toll like receptor (TLR)9 and peroxisome activated proliferators (PPAR) —known to be important players in regulation of innate and adaptive immune responses in human colonic epithelium.

The results showed that it is possible to establish short-term cultures of representative, viable human colonic epithelial cells from endoscopic mucosal biopsies of patients with IBD. Short-time isolation by EGTA /EDTA from colonic biopsies allowed establishment of small scale cultures of epithelial cells which were viable and metabolic active for up to 48 hours *in vitro*. The cell model preserved important cellular metabolic and immunological functions of the human colonic epithelium, including the ability to oxidate butyrate, detoxificate phenolic compounds and secrete the chemokine interleukin (IL)-8 *in vitro*.

Tumour necrosis factor (TNF)- α and interferon (IFN)- γ are pro-inflammatory cytokines, which are present in increased amounts in inflamed colonic mucosa. The precise mechanisms of cytokine-mediated mucosal injury are unknown, but one might be that TNF- α and IFN- γ , directly impair epithelial cell function similar to effects seen on distinct target cells in other autoimmune diseases. Using the model, both cytokines were found directly to impair the viability of colonic epithelial cells and to induce secretion of IL-8 *in vitro*. Interestingly, the cells from inflamed IBD mucosa were less sensitive to cytokine-induced damage, which suggests that an intrinsic defense mechanism is triggered in these cells, perhaps as a result of exposure to toxic luminal factors or high local cytokine levels *in vivo*.

TNF- α and IFN- γ may also be involved in regulation of intestinal inflammation through stimulation of MMP expression and proteolytic activity. We found that colonic epithelial cells express a range of MMPs and moreover that expression of distinct MMPs is increased in cells from inflamed IBD mucosa. Using a functional peptide cleavage assay it was shown that epithelial cells secreted proteolytic active enzymes and that the functional MMP activity was increased in inflamed IBD mucosa. This suggests that colonic epithelial cells, like myofibroblasts and immune cells, may contribute to local intestinal mucosal damage, through secretion of active MMPs.

Disturbance of recognition and discrimination of potentially harmful pathogens from commensals in the intestinal mucosa have increasingly been implicated in the pathogenesis of IBD. Our results revealed that colonic epithelial cells express TLR9, a key pattern recognition receptor. Interestingly, the differentiated epithelial cells, which have been exposed to the luminal bacterial flora *in vivo*, were unresponsive to TLR9 ligand stimulation, contrasting findings in the epithelial cell line HT-29 that is cultured continuously in bacterial free environment. These findings suggest, theoretically, that colonic epithelium may regulate immune responses to microbial antigens including commensal bacterial DNA through modulation of the TLR9 pathway. Currently, the results are in line with the emerging view, that the epithelium represents an important frontline cellular component of the innate immune system in the gut.

PPAR γ is a nuclear receptor involved in the regulation of lipid and carbohydrate metabolism. Recent studies in rodent colitis models suggest that PPAR γ also is involved in modulation of inflammatory processes in the colon. Using the model, we characterise expression and activity of PPARs in human colonic epithelium and, additionally, evaluated the functional significance of a possible imbalanced PPAR γ regulation in relation to inflammation. Our experiments showed that colonic epithelial cells express PPAR γ and furthermore that PPAR γ signalling was impaired in inflamed UC epithelium. It was possible to restore PPAR γ signalling in the cell cultures by stimulation with rosiglitazone (a synthetic PPAR γ ligand) *in vitro*. Hence, these experiments prompted us to design a small controlled, clinical study exploring the possible stimulatory effects of rosiglitazone (a PPAR ligand) *in vivo*. Interestingly, it was found that topical application of rosiglitazone in patients with active distal UC reduced clinical activity and mucosal inflammation similar to the effects measured in patients treated with mesalazine enemas. Moreover, rectal application of rosiglitazone induced PPAR γ signalling in the epithelium *in vivo*, supporting the view that activation of PPAR γ may be a new potential therapeutic target in the treatment of UC.

Overall, the *in vitro* model of representative human colonic epithelial cells has shown to be a useful technique for detailed studies of metabolic and immunological functions that are important for homeostasis of the colonic epithelium. Currently, the findings support the view that intestinal epithelial cells actively participate in immunological processes in the colonic mucosa. Additionally, the model seems to be applicable for generating and evaluating new therapeutic approaches from laboratory bench to bed line as illustrated by the PPAR γ study. It is therefore probable, that studies in models of representative colonic epithelial cells, as the one described here, could contribute with important knowledge about the pathogenesis of human inflammatory colonic diseases also in the future.

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