Intestinal barrier function: regulation of epithelial permeability and mucin expression
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KGF selectively stimulates PGE$_1$ secretion by intestinal myofibroblasts

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Abstract
Intestinal subepithelial myofibroblasts regulate epithelial cell function, barrier integrity and are involved in mucosal healing following injury. These functions are in part mediated by secretion of paracrine factors such as prostaglandins (PG) and growth factors. Both PG (PGE$_1$ more than PGE$_2$) and keratinocyte growth factor (KGF) have been found to induce mucin synthesis and to contribute to mucosal protection and healing. We investigated the ability of KGF to contribute to mucoprotective mechanisms through the regulation of PG secretion by subepithelial myofibroblasts. KGF-induced PGE$_1$ and PGE$_2$ synthesis by myofibroblasts was measured. COX enzyme expression (protein) was determined and specific COX inhibitors were used to determine the pathway of prostaglandin production. KGF stimulated PGE$_1$ production whereas PGE$_2$ was unaffected. The KGF effects were associated with increased COX-1 protein expression. Specific inhibitors of prostaglandin synthesis supported the role for COX-1 in KGF mediated PGE$_1$ secretion by myofibroblasts, and COX-2 inhibition was found to be partially effective. In conclusion, this study revealed that the mucoprotective effects of KGF may involve stimulation of myofibroblast derived PGE$_1$ production.
KGF stimulates myofibroblast derived PGE₁ secretion

Introduction
Fibroblast growth factors (FGF) have been reported to be protective in several animal models of inflammatory bowel disease, and this effect has been associated with increased mucin synthesis. FGF-7, better known as KGF, is a trophic factor stimulating mucosal healing in various models of intestinal damage and inflammation. Proinflammatory cytokines stimulate KGF release by stromal fibroblasts, intraepithelial T-cells and subepithelial myofibroblasts and concentrations of KGF were found to be increased in mucosal biopsies of IBD patients. Myofibroblasts form a thin layer of cells underlying the intestinal epithelium and regulate epithelial cell functions e.g. proliferation, differentiation, wound healing, secretion and motility. We recently found enhanced KGF expression to be associated with amelioration of immune mediated barrier disruption in a coculture model of epithelial cells and subepithelial myofibroblasts. While using the same model, our group has reported prostaglandin(PG)E₁, to be more potent than PGE₂ in stimulating epithelial mucin expression. Myofibroblasts are an important source of PG production in the intestinal mucosa and the role of KGF in the regulation of PG synthesis by myofibroblasts has not been studied yet. These cells particularly can be of relevance since they may regulate mucin production by goblet cells which lose KGF receptor expression during differentiation. In the present study, we investigated the ability of KGF to affect PG synthesis by intestinal myofibroblasts and evaluated the role of COX-1 and COX-2 enzyme activity in the regulation of PGE₁ and PGE₂ secretion.

Materials and methods
Cell culture
Intestinal myofibroblasts CCD-18Co (α-smooth muscle actin and vimentin positive) were cultured in DMEM/F12 glutamax I (Invitrogen Life Technologies, Carlsbad, CA) with penicillin (100 IU/ml), streptomycin (100 μg/ml) (pen/strep) (Invitrogen Life Technologies) and 5% heat inactivated fetal bovine serum (Invitrogen Life Technologies). Medium was refreshed every two days.

Stimulation of CCD-18Co cells with KGF, IL-1β and COX-inhibitors
CCD-18Co cells were grown confluent in 96 or 12 wells tissue culture plates (Corning BV, Acton, MA) and incubated for 24 h with 10-100 ng/ml rec. human KGF (FGF-7) (Sigma-Aldrich BV, St. Louis, MO), 50-100 ng/ml rec. human KGF-2 (FGF-10) (PeproTech, London, UK) or 10 ng/ml rec. murine IL-1β (bioactive 17 kDa form, R&D, Minneapolis, MSA). Incubations were performed in the presence or absence of 10⁻⁸-10⁻⁹ M COX-1 inhibitor SC-560 (COX-1 IC₅₀: 9x10⁻⁹M; COX-2 IC₅₀: 6.3x10⁻⁷M) (Calbiochem, San Diego, CA) or COX-2 inhibitor 10⁻⁶ M NS-398 (COX-1 IC₅₀: 75x10⁻⁶M; COX-2 IC₅₀: 61
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1.8x10^6 M (Alexis, Lausen, Switzerland). Supernatants were collected and cells were lysed in laemml buffer to determine prostaglandin secretion and COX protein expression respectively.

**PGE\(_1\) and PGE\(_2\) production and Cell proliferation and viability**

Supernatants from CCD-18Co cells were analyzed for prostaglandin production using ELISAs for PGE\(_1\) (R&D, 21% cross reactivity with PGE\(_2\)) and PGE\(_2\) (Biotrak, 4% cross reactivity with PGE\(_1\))\(^{20}\) according to manufacturers instructions. BrdU (Roche Diagnostics, Indianapolis, IN) incorporation was measured to determine the effect of KGF on cellular proliferation. Therefore CCD-18Co cells were incubated for 24 h with 0-100 ng/ml KGF and BrdU, the assay was performed according to manufacturers instructions. Additionally, after 24 h incubations the viability of the cells was determined using the WST-1 assay (Roche diagnostics) according to manufacturers instructions.

**Western Blotting COX-1 and COX-2**

Cell samples were homogenized in laemml buffer and protein concentrations determined using the DC-protein assay (Biorad, Hercules, CA) according to the manufactures protocol with minor modifications. After protein determination 2,5% β-mercaptoethanol was added, samples were boiled and centrifuged at 10,000 rpm. Cell lysates (30 μg) were separated in 7.5% SDS gels and transferred to polyvinylidene difluoride (PVDF) membranes (Roche Diagnostics). Membranes were subsequently blocked in TBST/ 5% Protivar (Nutricia, Zoetermeer, The Netherlands) and incubated with mouse anti-COX-1 monoclonal or goat anti-COX-2 polyclonal antibodies (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), washed and subsequently incubated with conjugated antibodies anti-goat/mouse-HRP (1:2000; Santa Cruz Biotechnology). ECL (Roche Diagnostics) was used as substrate, signal detection was performed with the Lumi-Imager (Boehringer Mannheim BV) and expressed in Boehringer Light Units (BLUs). The level of expression was presented relative to controls (%BLU).

**Data analysis**

All data are presented as mean +/- SEM. Data were analyzed with the univariate ANOVA using SPSS software version 10.

**Results**

**Modulation of prostaglandin production by KGF**

Unstimulated CCD-18Co secreted comparable amounts of PGE\(_1\) and PGE\(_2\) (248±75 vs 291±154 pg/ml) (fig 1). Incubations with KGF revealed dose-dependent stimulation of PG 62
production. KGF tripled the PGE\textsubscript{i} concentrations (758 ± 186 pg/ml, 100 ng/ml KGF) as compared with controls in the highest dose groups (p<0.01) whereas it did not affect PGE\textsubscript{2}. In all dosage groups KGF increased PGE\textsubscript{i} concentrations compared with PGE\textsubscript{2} (# p<0.05; ## p<0.01). KGF-2 (50-100 ng/ml) also selectively enhanced PGE\textsubscript{i} secretion (n=5, p<0.05, data not shown). That the KGF effects observed could not simply be attributed to increased proliferation was also investigated. KGF did not affect proliferation or cell viability as determined by BrdU incorporation and WST-1 assay analyses (data not shown).

**COX protein expression after KGF incubations**

A representative immunoblot of COX-1 and COX-2 expression in subepithelial myofibroblasts after 24 h exposure to KGF is shown in figure 2A (IL-1\textbeta was used as positive control for COX-2 induction). In addition, densitometric analysis of multiple experiments revealed that COX-1 expression was significantly increased compared with control incubations in the highest dose group (fig 2B; **p<0.005) whereas COX-2 expression was not changed (n=3, data not shown).

**Selectivity of COX-1 and COX-2 inhibitors revealed by IL-1\textbeta induced PG production**

COX-inhibitors were used to determine whether the KGF stimulation of PGE\textsubscript{i} production involved COX-1 or COX-2 enzyme activity. IL-1\textbeta is known to induce COX-2 expression thus the PG production induced by IL-1\textbeta is largely COX-2 dependent. IL-1\textbeta induced both PGE\textsubscript{i} and PGE\textsubscript{2} production (fig 3, ## p<0.002). The IL-1\textbeta induced PGE\textsubscript{2} secretion was completely inhibited by the COX-2 inhibitor NS-398 (10\textsuperscript{-9}-10\textsuperscript{-6} M gave comparable results, 10\textsuperscript{-6} M is shown) (**p<0.005). Several concentrations of the COX-1 inhibitor SC-560 (10\textsuperscript{-9}-10\textsuperscript{-6} M) were tested during IL-1\textbeta stimulation (10\textsuperscript{-8}-10\textsuperscript{-9} M are shown). The concentration of 10\textsuperscript{-9} M SC-560 did not inhibit IL-1\textbeta enhanced PG production while higher concentrations were found to be effective (**p<0.005). Hence, in CCD-18Co cells COX-1 inhibitor SC-560 appears to inhibit also COX-2 at concentrations of 10\textsuperscript{-8} M or higher.

*Figure 1* KGF increases PGE\textsubscript{i} production by subepithelial myofibroblasts. KGF incubation (24 h; 10-100 ng/ml) dose dependently stimulated PGE\textsubscript{i} secretion by CCD-18Co cells (50-100 ng/ml, p<0.01), whereas PGE\textsubscript{2} production was not affected. All KGF dose groups enhanced PGE\textsubscript{i} production compared with PGE\textsubscript{2} production (# p<0.05, ## p<0.005, n=5). Moreover, basal PGE\textsubscript{i} and PGE\textsubscript{2} production by CCD-18Co was not found to be different.
Figure 2 KGF enhances COX-1 expression in CCD-18Co. A) Representative western blot showing the differential effects of KGF on COX-1 and COX-2 expression. Dose dependent stimulation of COX-1 expression was observed after incubation with KGF. IL-1β (10 ng/ml) was used as positive control since it is known to induce COX-2 expression. B) Densitometric analysis revealed KGF (100 ng/ml) to enhance COX-1 protein expression (**p<0.005; n=3).

Selectivie inhibition of COX prevents KGF stimulated PGE₁ production
Stimulation of PGE₁ production by KGF was inhibited by 10⁻⁸ M SC-560 but more relevant also by 10⁻⁹ M SC-560 (fig 4). 10⁻⁹ M SC-560 selectively inhibits COX-1 since IL-1β induced PG secretion was not inhibited at this concentration. COX-2 inhibitor NS-398 was less effective in inhibiting KGF stimulated PGE₁ secretion. Hence, the increase in PGE₁ production (fig 4, ## p<0.002) following KGF incubation was found to be predominantly abrogated via selective inhibition of COX-1 (**p<0.005) although some effect of COX-2 inhibition was observed (*p<0.02).

Discussion
KGF (keratinocyte growth factor) regulates gastrointestinal morphogenesis and enhances proliferation and differentiation of normal intestinal epithelial cells, goblet cells in particular. KGF secretion is enhanced during mucosal injury or inflammation and like other FGFs induces epithelial restitution, reduces ulceration, and increases the number of goblet cells. By enhancing the number of goblet cells, KGF contributes to the increased mucin production which may result in enhanced mucosal protection. Interestingly, KGF cannot directly increase mucin production in differentiated goblet cells since, during differentiation in these cells KGF receptor expression is lost. We hypothesized that KGF may further regulate
epithelial mucin production by intermediate regulation of myofibroblasts derived prostaglandin production.

Our data reveal differential effects of KGF on PGE₁ versus PGE₂ production by myofibroblasts. KGF dose dependently stimulated PGE₁ production whereas PGE₂ remained unchanged. Selective stimulation of PGE₁ secretion may favor mucosal protection since we have previously found PGE₁ rather than PGE₂ to stimulate mucin expression in intestinal epithelial cells. KGF has been found to enhance goblet cell differentiation, cellular mucin expression and the number of goblet cells in the normal rat ileum and colon. KGF receptor expression is typically localized in the crypts and less prominently in the more differentiated villi. Goblet cell differentiation was associated with loss of KGF receptor (KGFR) expression. This implies that despite KGF being responsible for goblet cell differentiation it will not contribute directly to the mucin synthesis. According to our observations in the present study, we suggest that KGF may play an important role in mucoprotection through both regulation of both goblet cell differentiation during epithelial maturation and PGE₁ production by subepithelial myofibroblasts. The latter cell type underlies the epithelial cell layer and could support mucin production in differentiated goblet cells through secretion of the soluble mediator PGE₁.

Prostaglandins are converted from specific substrates by cyclooxygenase (COX) enzymes. In the gastrointestinal tract COX-1 is constitutively expressed in the crypts and considered to be involved in the regulation of epithelial proliferation. COX-2 is induced during processes of inflammation and COX-2 derived prostaglandins cause oedema and hyperalgesia. On the other hand, recent data have indicated that both COX-1 and COX-2 derived prostaglandins may play an essential role in maintenance of intestinal mucosal integrity during mucosal injury. Colon derived myofibroblasts (CCD-18Co) constitutively express...
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Figure 4 Effect of COX inhibitors on KGF stimulated PGE$_1$ production. KGF (100 ng/ml) enhanced PGE$_1$ production as compared with basal production (### p<0.002, n=5). Although both COX inhibitors could abrogate this effect, the COX-1 inhibitor was particularly effective (*p<0.02, ** p<0.005).

COX-1 mRNA while COX-2 can be induced. Our data show dramatic induction of COX-2 expression by IL-1β stimulating both PGE$_1$ and PGE$_2$ production by these cells. It was previously reported that IL-1 induces COX-2 expression in CCD-18Co cells with no or only limited effects on COX-1 mRNA and protein expression. In contrast, KGF enhanced COX-1 expression without affecting COX-2 protein expression and our data showed selective COX-1 inhibition could abrogate the KGF stimulated production of PGE$_1$. COX-2 seems to be only partially involved since COX-2 inhibition reduced PGE$_1$ production by KGF less effectively than selective COX-1 inhibition. Several growth factors have been reported to stimulate COX-1 protein expression. In contrast, in endothelial and intestinal epithelial cells FGF-2, FGF-10 or FGF-20 were found to slightly upregulate PGE$_2$ production which correlated with enhanced COX-2 mRNA expression. Our data may reflect differential responses of FGF depending on the cell type, as in myofibroblasts KGF particularly enhanced PGE$_1$ production without affecting PGE$_2$. Similar, TGF-β$_1$ increased COX-1 protein expression in human lung fibroblasts without changing the PGE$_2$ production.

KGF is considered to be a paracrine mediator of epithelial and mesenchymal cell interaction. It is produced by mesenchymal cells and binds to the KGF receptor (KGFR or FGFR-2IIIb) present on for instance epithelial cells but not on several other cell types including fibroblasts. However, KGFR expression indeed has been observed in tissues of non-epithelial origin such as the periostium, perichondrium, testicular mesenchyme, skeletal muscle and intestinal smooth muscle which all simultaneously express KGF mRNA. Our data show specific effects of both KGF and KGF-2, which are known to use the same receptor, on myofibroblasts.

In conclusion, KGF increased the PGE$_1$ production by myofibroblasts and was found to enhance COX-1 expression in these cells. Abrogation of prostaglandin production with selective COX inhibitors revealed COX-1 activity and to a lesser extent COX-2 to be responsible for this selective increase in PGE$_1$ production. Thus, apart from its effect on
goblet cell differentiation, the mucoprotective effect of KGF may also involve selective stimulation of PGE$_1$ production by intestinal subepithelial myofibroblasts.

References