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Differential Pattern of Inflammatory Molecule Regulation in Intestinal Epithelial Cells Stimulated with IL-1

Sen Rong Yan,2* Robbie R. Joseph, † Jun Wang,*† and Andrew W. Stadnyk3*†

To better predict the consequences of blocking signal transduction pathways as a means of controlling intestinal inflammation, we are characterizing the pathways up-regulated by IL-1 in intestinal epithelial cells (IEC). IL-1β induced increased mRNA levels of MIP-2, MCP-1, RANTES, inducible NO synthase (iNOS), and cyclooxygenase-2 (COX-2) in the IEC-18 cell line. IL-1β activated NF-κB but not ERK or p38. Infecting cells with adenovirus expressing a mutated gene for IκBα (IκBAA) blocked IL-1-induced mRNA increases in MIP-2, MCP-1, and iNOS but not COX-2 or RANTES. Expression of IκBAA attenuated the IL-1-induced increase in COX-2 protein. Unexpectedly, RANTES mRNA increased, and protein was secreted by cells expressing IκBAA in the absence of IL-1. Adenovirus-expressing IκBα, blocking protein synthesis, and IL-1β all resulted in activation of JNK. The JNK inhibitor SP600125 prevented the RANTES increases by all three stimuli. A human enterocyte line was similarly examined, and both NF-κB and JNK regulate IL-1-induced RANTES secretion. We conclude that in IEC-18, IL-1β-induced increases in mRNA for MIP-2, MCP-1, and iNOS are NF-κB-dependent, whereas regulation of RANTES mRNA is independent of NF-κB but is positively regulated by JNK. IL-1β-induced mRNA increases in COX-2 mRNA are both NF-κB- and MAPK-independent but the translation of COX-2 protein is NF-κB-dependent. This pattern of signaling due to a single stimulus exposed the complexities of regulating inflammatory genes in IEC. The Journal of Immunology, 2006, 177: 5604–5611.

In addition to functioning as a barrier and absorptive organ, intestinal epithelial cells (IEC) play an active role in inflammatory diseases by releasing multiple cytokines and inflammatory mediators (1, 2). The constellation of IEC-derived inflammatory molecules includes interleukins, chemokines, growth factors, prostaglandins, and nitrogen radicals. Chemokines in particular contribute to the pathology by recruiting various leukocyte types into the mucosa. IEC in turn respond to the cytokines that the leukocytes make in the milieu, such as IL-1, TNF-α, IFN-γ, and IL-4 during inflammatory bowel disease (IBD) (reviewed in Ref. 3). In establishing the IEC cytokine/chemokine network using the nontransformed rat IEC-18 cell line we previously reported that IL-1-stimulated CXC2L (MIP-2) and CCL2 (MCP-1) and IL-4-stimulated MCP-1 and CCL11 (eotaxin), whereas IFN-γ only stimulated MCP-1 (4). Considering the potential for different mediators to influence pathology, our aim now is to better understand the regulation of expression of multiple molecules elicited by each single cytokine stimulus. This effect is important for predicting the response of IEC to pharmacological therapies intended to block signaling pathways in efforts to reduce inflammation. In this study, we build on our earlier data and report on the signaling dependencies of IL-1β stimulation of multiple mediators in the IEC-18 cell line.

IL-1 is a pleiotropic cytokine produced by many cell types in response to variety of stimuli. Although IL-1 consists of two agonists, IL-1α and IL-1β, IL-1β has been implicated in multiple aspects of inflammation, most notably the ability to induce the synthesis and release of other inflammatory mediators in vivo, including lipid mediators, reactive oxygen and nitrogen intermediates, cytokines, and chemokines (5). In addition to other evidence and our studies for stimulation of chemokines in IEC, IL-1 reportedly increases or induces expression of IL-6, COX-2, and the inducible NO synthase (iNOS) (6–9). The regulatory mechanisms by which IL-1 induces these changes in IEC are still not fully understood although MAPK, NF-κB, and PI3K signaling pathways have been implicated (10–13).

NF-κB, an inducible transcription factor composed of Rel A (p65) and NF-κB1 (p50) in IEC, is widely regarded as the master control over IEC inflammatory gene expression (14). The activation of NF-κB is regulated by an endogenous cytoplasmic inhibitor, IκB; in responding to certain stimuli IκB becomes phosphorylated at serine residues 32 and 36 and then selectively ubiquitinated and degraded (15). In the course of our investigations into the IκB/NF-κB pathway in IL-1-stimulated IEC, we detected differential patterns of mRNA changes in cells infected with an adenovirus expressing a mutated gene for IκBα (IκBAA) that suppresses the activation of NF-κB. We report that although the IL-1β-induced increases of some inflammatory molecules are positively regulated by NF-κB, others are not and in fact increase despite inhibition of NF-κB. The results will need to be taken into consideration in strategies intended to dampen NF-κB activation as a means of controlling intestinal inflammation.

Materials and Methods

Materials

The IEC-18 cell line and a PCR-based mycoplasma detection kit used to determine our cultures were mycoplasma free were both purchased from...
American Type Culture Collection. The human fetal enterocyte line HIEC, established with funding from the Medical Research Council of Canada, was generously provided by Dr. J.-F. Beaulieu, Université de Sherbrooke (Sherbrooke, Québec, Canada) (16). Cell culture polystyrene flasks and Multiwell plates were from BD Biosciences. DMEM, HEPES, t-glutamine, FBS, newborn cow serum, penicillin, streptomycin, and bovine insulin were purchased from Invitrogen Life Technologies. Rat IL-1β and human IL-1β were purchased from PeproTech. Nitrocellulose membrane, ECL Western blotting detection reagents, and γ-32P]ATP were purchased from Amersham Biosciences. SB203580, PD98059, and SP600125 were obtained from Calbiochem. Rabbit anti-IκBα protein and anti-phosphorylated p38 antisera and mouse anti-phosphorylated IκBα mAb were from Cell Signaling Technology. Rabbit anti-NF-κB (p65), ERK2 and p38 antisera, and a mouse anti-phosphorylated ERK1/2 mAb were obtained from Santa Cruz Biotechnology. A protein assay kit (Bradford method) and other reagents for electrophoresis were from Bio-Rad. HRP-conjugated goat anti-mouse IgG and anti-rabbit IgG Abs, cycloheximide, Wortmannin, and all the other reagents were obtained from Sigma-Aldrich.

**Cell culture**

The IEC-18 cell line was maintained in DMEM supplemented with 10 mM HEPES, 2 mM t-glutamine, 5% heat-inactivated newborn cow serum, 50 U/ml penicillin and 50 μg/ml streptomycin, hereafter referred to as complete DMEM, at 37°C and 5% CO2. The passage and periodical mycophenolic acid treatments were performed as described elsewhere (17).

Experiments were performed 1 wk postpassage on confluent cells (using 18–20h passage cells) in 6- or 12-well polystyrene plates. For experimental treatments the cells were replenished with fresh complete DMEM, and in some experiments, with PD98059, SB203580, SP600125, or cycloheximide, each dissolved in DMSO then incubated for 20 min at 37°C before IL-1β addition (diluted with complete DMEM/5% FCS to a final concentration of 0.5 μg/ml unless otherwise indicated) and further incubation. The IEC line was cultured in DMEM (high glucose) supplemented with 4 mM L-glutamine, 20 mM HEPES, 5% FBS, and 0.2 IU/ml insulin.

IL-1 treatments were conducted on confluent cultures in serum-free medium.

**Adenovirus constructs and infection**

Replication defective adenovirus lacking segments of the E1 and E3 regions, described in detail elsewhere (18) and kindly provided by Dr. C. Jobin, University of North Carolina (Chapel Hill, NC), encode a CMV-driven mutated IκBα resistant to degradation (Ad5IκBα). Adenovirus 5expressing GFP, which was confirmed by fluorescent microscopy of infected cells, was used as an infection control. Virus was replicated by infection of the E1-transformed HEK293 cells and purified from cell lysate. Titers were estimated by OD260 absorbance, and viral stocks were stored at −20°C in storage buffer (5 mM Tris-HCl (pH 8.0), 50 mM NaCl, 500 μM MgCl2, 25% glycerol). IEC-18 of near confluence were infected overnight with recombinant adenovirus expressing GFP, which was confirmed by fluorescent microscopy of infected regions, described in detail elsewhere (18) and kindly provided by Dr. C. Jobin, University of North Carolina (Chapel Hill, NC). Replication defective adenovirus lacking segments of the E1 and E3 regions, described in detail elsewhere (18) and kindly provided by Dr. C. Jobin, University of North Carolina (Chapel Hill, NC), encode a CMV-driven mutated IκBα resistant to degradation (Ad5IκBα). Adenovirus 5expressing GFP, which was confirmed by fluorescent microscopy of infected cells, was used as an infection control. Virus was replicates by infection of the E1-transformed HEK293 cells and purified from cell lysate. Titers were estimated by OD260 absorbance, and viral stocks were stored at −20°C in storage buffer (5 mM Tris-HCl (pH 8.0), 50 mM NaCl, 500 μM MgCl2, 25% glycerol). IEC-18 of near confluence were infected overnight with recombinant adenovirus expressing GFP, which was confirmed by fluorescent microscopy of infected regions, described in detail elsewhere (18) and kindly provided by Dr. C. Jobin, University of North Carolina (Chapel Hill, NC).

**Preparation of cell lysates**

After incubation cells were washed once with ice-cold PBS containing 1 mM DFP (diisopropyl fluorophosphate) then lysed with M2 buffer (20 mM Tris (pH 7.0), 0.5% Nonidet P-40, 200 mM NaCl, 3 mM EDTA, 0.5 mM DTT, 2 mM DTT, 5 mM PMSF, 0.5 μg/ml leupeptin, 5 μg/ml pepstatin, 10 μg/ml aprotinin, 0.2 mM Na2VO3, 10 mM NaF, 10 μM phenylarsine oxide) (19). The lysates were clarified by centrifugation at 12,000 × g for 10 min at 4°C and assessed for protein concentration using the Bradford method (Bio-Rad). Samples were kept at −80°C before use.

**Preparation of nuclear extracts**

The experimental procedure of Ward et al. (20) was used with some modifications. Cells were washed once with ice-cold PBS/DFP then collected into buffer A (10 mM Tris (pH 7.8), 1 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 0.1 mM Na2VO3, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 10 μg/ml aprotinin, 10 mM NaF, 10 μM phenylarsine oxide). Then 0.1 volume of 10% Nonidet P-40 was added and the samples vortex-mixed for 4 s followed by centrifugation at 12,000 × g for 2 min. The supernatants were aspirated, and the pellets washed once with buffer A. The nuclei were collected by centrifugation as described and the nuclear proteins were extracted with buffer B (20 mM Tris (pH 7.8), 150 mM NaCl, 50 mM KCl, 1.5 mM EDTA, 5 mM DTT and protease, and phosphatase inhibitors as in buffer A) for 1 h at 4°C. The residues of the nuclei were removed by centrifugation at 12,000 × g for 10 min at 4°C. Protein concentrations in the Nuclear extracts were determined using the Bradford method.

**Western blot analysis**

Equal amounts of protein from the preparations were mixed with one-third volume of 4% SDS-PAGE sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 100 mM 2-ME, and 0.01% bromophenol blue) and boiled for 10 min. Proteins were then separated by SDS-PAGE on 10% acrylamide gels and transferred to a nitrocellulose membrane through electrophoresis. The membranes were blocked with 5% skim milk and analyzed by Western blotting using the indicated Abs as described elsewhere (21). The same membranes were also used for blotting for the second or third proteins after the bound Abs were removed with a stripping buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM 2-ME) at 55°C for 30 min followed by blocking with milk.

**Immunoprecipitation and in vitro kinase assays**

The MAPKs (ERK1/p38) were immunoprecipitated from cell lysate proteins using Abs bound to protein A-agarose beads by rotating at 4°C for 2 h. The precipitates were intensely washed with lysis buffer and the precipitates were assessed by in vitro kinase assay using myelin basic protein or activating transcription factor-2 as substrates for ERK1/2 and p38, respectively, in the presence of (γ-32P]ATP as described earlier (22).

**RNA extraction and relative RT-PCR**

RNA was extracted using TRIzol reagent (Invitrogen Life Technologies) following the manufacturer’s instructions. Reverse transcription and PCR were performed as previously described (17). Briefly, 1 μg of total cellular RNA from each sample was reverse transcribed using Maloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies) with 0.01 mM dNTPs and 1 μg of random hexamers (both from Pharmacia). The reverse transcriptase product was diluted 1/10 and 4 μl used for measurement of β-actin, otherwise an equal undiluted volume was used as template for each cytokine determination. PCR mixes contained (in final concentrations) 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM MgCl2, 0.1 μg/ml BSA, 0.2 mM dNTPs, and 2.5 pmol of each primer. Most primer sequences have been published previously, the exceptions being RANTES (all reading 5′ to 3′) sense ATATGGGCCTCCGACACACTC, antisense CTCTTCTCTGTTGGTGCA; COX-2 sense GACTTTCGATCTCCTTGTTG, antisense CAATCAGCAAGTCTCAGACG; and iNOS sense TGCTCTCTCTGTTGAGCATC, antisense CACGAACTGTG GGTCATGC. PCR was conducted under the following cycling conditions: 93°C, 60°C, and 72°C for 30 s or 35 cycles. Products were visualized on 1.5% agarose gels containing 5 μg/ml ethidium bromide and photographed using Polaroid 667 film.

**ELISA measurements of RANTES protein concentration**

Culture supernatants were recovered after overnight incubation of cells treated with IL-1 or following infection with adenovirus, some in combination with SP600125. Rat and human RANTES protein concentrations were measured using a commercial ELISA prepared to measure human RANTES (PeproTech). Recombinant human RANTES was used as the standard for quantifying HIEC culture supernatants. Considering the ELISA cross-reacts with rodent RANTES, recombinant rat RANTES (PeproTech) was used to prepare the standard curve for reading rat supernatant samples.

**Results**

**IL-1β up-regulates multiple inflammatory factors in adherent IEC-18**

IL-1β plays an important role and is commonly reported elevated in the mucosa of a number of intestinal inflammatory diseases. Not only does this cytokine prime endothelial cells with adhesion molecules and activate leukocytes but it also stimulates IEC into cytokine expression that in turn further contribute to the inflammatory pathology. The IEC-18 rat crypt-like cell line is a good model to represent normal cell function because it is not transformed and typically expresses inflammatory mediators only following stimulation. When added to adherent IEC-18, IL-1β induced the expression of multiple genes for factors that have been strongly associated with inflammation. Shown in Fig. 1 are mRNA increases for...
COX-2, MCP-1, MIP-2, RANTES, and INOS. Detection of β-actin mRNA, which in our experience does not change in concentration in cells treated with IL-1β, was used to demonstrate similar first strand cDNA loading between the treatment groups.

**IL-1β time- and dose-dependently activates NF-κB**

Added to confluent IEC-18, IL-1β induced a rapid and intense phosphorylation of IκBα that first appeared within a few minutes as a transient phase followed in 30 min by a second, protracted episode (Fig. 2A, left). The first phase of IκBα phosphorylation was closely followed by the degradation of the protein that resulted in a low IκBα level between 10 and 20 min. Levels of IκBα protein then increased and remained constant after exposure to IL-1β for 1 h, despite the second phase of IκBα phosphorylation, probably due to de novo protein synthesis. Shown in Fig. 2A, right, IκBα phosphorylation was increased in response to increasing concentrations of IL-1β as was IκBα degradation. IEC-18 responded to a concentration of IL-1β as low as 0.02 ng/ml with detectable phosphorylation of IκBα.

Shown in Fig. 2B, increased levels of nuclear NF-κB, identified in the Western blot as p65, were detected as early as 10 min after the addition of IL-1β, and levels increased up to 2 h during IL-1β stimulation, but returned to a basal level by 4 h. As seen in the phosphorylation and degradation of IκBα, increasing levels of nuclear p65 paralleled the increases in concentration of IL-1β (Fig. 2B). We also detected NF-κB activation by IL-1β using adenovirus expression of a κB-driven luciferase gene (data not shown in this study, but published elsewhere (23)).

**IL-1β-induced increases in mRNA for iNOS, MCP-1, and MIP-2 are NF-κB-dependent**

To confirm the NF-κB dependency of the various mediator mRNA changes seen in Fig. 1, we infected cells with an adenovirus expressing a mutated and tagged gene for IκBα, which is resistant to phosphorylation by IκB kinase and therefore is inactive (18). The mutant protein is larger than native IκBα when detected by Western blot analysis (IκBα-Tag in Fig. 3A). Prior infection of IEC-18 with this virus but not the GFP-expressing virus almost abolished the phosphorylation/degradation of IκBα (Fig. 3A) and the nuclear translocation of p65 induced by IL-1β (Fig. 3B). When these cells were assessed for mRNA we found that the IκBAA-expressing virus but not the GFP-expressing control virus essentially blocked the IL-1β-induced increase in mRNA for iNOS, MCP-1, and MIP-2 (Fig. 4A). IL-1β up-regulated mRNA levels for both RANTES and COX-2 were not suppressed by IκBAA infection. In a pattern unlike any of the other mediators, infection by IκBAA-expressing adenovirus caused a marked increase in the expression of RANTES, even greater than that induced by IL-1 alone. We noticed that the control virus infection resulted in a low but detectable RANTES mRNA increase (Fig. 4A) so we explored whether there was a dose relationship between virus infection and any RANTES mRNA increase. Indeed, higher infectious doses of
GFP-expressing virus did result in increased RANTES mRNA in otherwise untreated IEC-18 (data not shown). This result was interpreted to mean that virus infection alone could contribute to increased RANTES (GFP-expressing viral infection) in an NF-κB-independent manner (IxBAA-expressing virus). The heightened RANTES mRNA levels in IxBAA-expressing virus may therefore be an effect of virus directly combined with inhibition of NF-κB in the cells.

Considering the COX-2 mRNA levels were increased in IL-1β-treated cells expressing IxBAA, we thought to examine intracellular protein levels (after 3 h) and observed that the infection did prevent the expected increase in protein otherwise seen in IL-1-treated cells (Fig. 4B). This effect was not from viral infection as the GFP-expressing virus did not have the same effect on COX-2 protein levels.

**IL-1β does not activate MAPKs (ERK1/2 and p38) in IEC-18**

In many cell types, including IEC, the MAPKs are involved in a broad range of cellular responses so we explored whether they had a role in IL-1β-induced increases of these mediators in IEC-18. Adherent IEC-18 cultured in 5% FCS demonstrated high levels of ERK1/2 phosphorylation (Fig. 5A) and activity (Fig. 5B) both of which were inhibited by the MAPK kinase inhibitor PD98059. Surprisingly, the addition of IL-1β to the cells did not cause any detectable change in the phosphorylation or activity of ERK1/2 (Fig. 5). The high constitutive activity of ERK1/2 was unaffected by infection with either IxBAA or the control GFP-expressing virus (data not shown). In contrast to ERK, only a low level of p38 phosphorylation and activity was detected in cells, but like ERK, levels remained unchanged by IL-1β (Fig. 5). This basal level of p38 activity could be reduced by the inhibitor SB203580 (Fig. 5B), but this had no effect on IL-1β-stimulated mediator mRNA levels (data not shown). These data suggest that the MAPks are unlikely to be involved directly in the IL-1β responses of IEC-18. Considering the high level of phosphorylated ERK we proceeded to examine the effects of preincubation with PD98059 on constitutive and IL-1β-induced activation of the NF-κB pathway and COX-2 production (Fig. 4B). Shown in Fig. 6, the inhibitor had no apparent effect on IxBB phosphorylation or degradation or p65 nuclear translocation triggered by IL-1β yet consistently resulted in lower COX-2 protein levels. In fact, the 25-min incubation in PD98059 resulted in a reduction in COX-2 protein levels below vehicle-treated control cells implying that activated ERK has a role in the regulation of constitutive COX-2 protein levels, presumably at a posttranscriptional level. Nevertheless, COX-2 protein levels were clearly increased when cells were stimulated by IL-1β even in the presence of the ERK inhibitor (Fig. 6).

Further to determining the intracellular signals leading to RANTES up-regulation, activation of the IL-1R type 1 complex of proteins can trigger the recruitment of PI3K, which in turn acts through Akt to induce NF-κB (24). We sought to test whether there may be an NF-κB-independent role for PI3K in the up-regulation of RANTES by IL-1 by using wortmannin to inhibit PI3K activity. However applying wortmannin in a range of doses as high as 100 μM did not prevent the IL-1-induced increase in any of the mediators mRNA, suggesting this role for PI3K is not the case (data not shown).

**JNK is a positive regulator of RANTES expression**

The high levels of RANTES mRNA achieved using the IxBAA-expressing virus, shown in Fig. 4, led us to consider whether NF-κB is a negative regulator of RANTES expression in these cells, possibly by promoting the expression of a repressor of
RANTES transcription. To address this hypothesis, we treated cells with cycloheximide to inhibit protein translation on the premise that constitutive NF-κB-mediated proteins would be interrupted and the repression of RANTES transcription released. Indeed, as shown in Fig. 7, incubation of IEC-18 in 10 μg/ml cycloheximide without any other stimulation results in increased RANTES mRNA levels. However another effect of protein translation blockade is the activation of the stress-activated protein kinase JNK (25). Activation of JNK even shows a reciprocal relationship with NF-κB (25). Activation of JNK even shows a reciprocal relationship blockade is the activation of the stress-activated protein kinase JNK (25). Activation of JNK even shows a reciprocal relationship with NF-κB (25), thus we determined whether JNK became activated in the IEC-18. Fig. 8 shows that the IκBα, cycloheximide, and infection of cells with IxBAA-expressing virus all result in increased RANTES mRNA levels. The phosphorylation and activation of JNK is not inhibited by preincubation of cells with the activity inhibitor, SP600125 (Fig. 8B). In contrast, SP600125 dose-dependently blocked the mRNA increases in RANTES induced by each of these stimuli (Fig. 9). We confirmed that the SP600125 was not lethal during the incubation period as the cells remained adherent in a monolayer (data not shown). This pattern of JNK inhibition resulting in a failure to increase RANTES mRNA levels led us to conclude that the IL-1-stimulated increase in RANTES mRNA is mediated by JNK in the IEC-18 line.

In some experiments we replaced the IxBAA-expressing adenovirus with curcumin as a pharmacological means of inhibiting NF-κB (27). The 25 μM curcumin blocked RANTES mRNA increases following IL-1 treatment but curcumin also reportedly blocks JNK activation (28), so its use was not evaluated further.

Considering the difficulty in relating changes in JNK phosphorylation and mRNA levels to a biological outcome, we measured the levels of secreted RANTES protein after overnight culture in the presence of the various stimuli and the JNK inhibitor. Fig. 10A shows the 24 h RANTES protein concentrations achieved by IEC-18 cells left untreated, treated with IL-1, or these same conditions combined with 25 μM SP600125. Fig. 10A also shows that DMSO, used as diluent for SP600125, had no direct effect or any detectable effect on IL-1-stimulated RANTES secretion. Fig. 10B shows that the IxBAA expressing adenovirus directly resulted in secretion of RANTES and that this result was prevented if cells were also incubated with SP600125 at the time of virus infection (first 24 h). IL-1 added 24 h after the virus infection was initiated had some added effect but nevertheless was largely susceptible to the JNK inhibitor. We conclude from this pattern of results that the IxBAA-expressing adenovirus infection induces JNK and that RANTES up-regulation is largely if not entirely JNK-dependent because NF-κB is inhibited in these cells.

We next explored whether the effect of IL-1 that we detect on the rat IEC could be directly translated to HIEC. In our experience, freshly isolated adult human colonocytes rapidly succumb to apoptosis and are unresponsive to IL-1 or TNF-α even in short term cultures (see (www.broadmedical.org/printable_pages/Final%20Progress%20Reports/FPR_IBD-0056_stadnyk.htm)). Various transformed human colon carcinoma cell lines are available but these have serious shortcomings in representing normal cells. For example, abundant expression of the IL-1R type II, which IEC-18 only expresses following detachment, dampens the T84 cell line response to IL-1 (29). We therefore chose to use a nontransformed human fetal ileal cell line, HIEC (16). These cells secreted RANTES in the presence of IL-1 under serum-free conditions (Fig. 11). Fig. 11 further shows that 25 μM SP600125 reduced the total IL-1-stimulated and secreted RANTES by roughly half, which confirms the observations using IEC-18. Furthermore, infection with adenovirus-expressing IxBAA did not directly stimulate RANTES secretion (data not shown) but did
damper the levels achieved using IL-1 (Fig. 11). The combination of superrepressor expression and JNK inhibitor essentially ablated RANTES secretion (Fig. 11). We interpret this pattern of results in IEC-18 to be the combined effect of IL-1 to activate NF-κB (reduced by the superrepressor) and JNK (further reduced in presence of superrepressor).

Discussion

IL-1β is a potent and pleiotropic proinflammatory cytokine produced by a variety of cell types in response to inflammatory stimulation. IL-1β has been reported increased in the mucosa of patients with IBD and in intestinal tissues of animal models of colitis and therefore is believed to have a role in the development of IBD (30). Importantly, apart from its broad effects on inflammatory cells and endothelium, IL-1β is a powerful stimulus for epithelial cells. In this study, we observed that IL-1β up-regulates the expression of genes for a panel of inflammatory factors including COX-2, MCP-1, MIP-2, iNOS, and RANTES in IEC-18. With such broad cell targets and pleiotropic effects, inhibiting IL-1β becomes an attractive approach to controlling inflammation.

It has been reported by multiple studies that IL-1 induces expression of various inflammatory mediators in IEC through an NF-κB-dependent manner. Our results using the 1xBAA superrepressor to selectively inhibit NF-κB activation and p65 nuclear localization confirm this response is the case in the IEC-18 for iNOS, MCP-1, and MIP-2. In addition, we build on this understanding by showing that IL-1-stimulated IEC-18 up-regulates RANTES and COX-2 mRNA levels by an NF-κB-independent mechanism but that COX-2 translation is NF-κB-dependent. These regulatory pathways are summarized in Fig. 12. Unstimulated adherent cell COX-2 mRNA levels appear to depend on ERK, which is phosphorylated and highly active in confluent cells cultured in complete serum. Otherwise in our experiments these mediators in IL-1-treated IEC-18 are not influenced by ERK or p38. One difference between our study and other reports using human cells is that the IEC-18 cells are nontransformed and nontumorigenic. In fact most human carcinoma lines constitutively produce a number of proinflammatory molecules including IL-1β, IL-8, MCP-1, and the IL-1R type II (29, 31, 32), which confluence IEC-18 only make following cytokine stimulation (4) or detachment (17). Thus access to the promoter regions may be different in transformed cells vs nontransformed cells or in dividing cells vs nondividing cells or in the presence of serum.

The putative role of COX-2 and prostaglandins influencing the establishment of inflammation-induced colon cancer has resulted in considerable interest in the regulation of this mediator in the colon and IEC. Infection of IEC-18 cells with virus-expressing 1xBAA did not affect the COX-2 mRNA increase induced by IL-1 (Fig. 4A), yet blocked the IL-1 up-regulation of COX-2 protein (Fig. 4B). This outcome rules out JNK as an exclusive regulator of COX-2 mRNA up-regulation by IL-1 otherwise levels would be increased in concentration in 1xBAA-expressing cells similar to RANTES. This outcome is compatible with IL-1 stabilizing COX-2 mRNA in absence of NF-κB with a consequential decline in translation, and does not rule out a role for NF-κB acting to enhance transcription of the gene. Otherwise the underlying mechanism for this pattern of changes is not yet entirely clear. Other reports indicate that it is multifactorial. For example, using the closely related IEC-6 line, investigators found that COX-2 up-regulation was p38-dependent when cells were challenged with endotoxin but that ERK was a negative regulator when cells were challenged with a stressor that induced both ERK and p38 activation (33). In the human cell line HT-29, IL-1 stimulated COX-2 was reduced following preincubations in PD98059, SB203580,
mokine response (37). In this case a high viral dose likely indirectly stimulates the cells through an IFN response, and evidence is emerging that IFN-β impacts on JNK activation (38).

Our investigations show that the RANTES up-regulation by IL-1 in the IEC-18 is entirely JNK-dependent, as was the expression in IxBAA-expressing cells. The finding of JNK-dependent RANTES expression is similar to the regulation reported in airway smooth muscle cells also stimulated with IL-1 (39). Yet our results contrast other reports using transformed human IEC cell lines. In one report RANTES mRNA increases in HT-29 required a combination of TNF-α and IFN-γ, and expression was relatively late (40). In another study using TNF-α or bacterial infection to stimulate HT-29 or Caco-2 cells, RANTES expression was again delayed by several hours (41). This delay in expression is incompatible with a direct effect of JNK or NF-κB on RANTES gene transcription in these lines and is more likely due to an indirect effect. Considering these differences between carcinoma lines and other confounders related to constitutive inflammatory cytokine expression (for example, IL-8) by carcinomas (31), we chose to use a nontransformed fetal human enterocyte line. The results obtained with HIEC differ not only from our rat cell results but also from the published data from human colon carcinomas. Our experiments indicate that the HIEC respond to IL-1 with RANTES in an NF-κB- and JNK-dependent manner. Unfortunately, limitations in our ability to culture primary human adult colonocytes remains an obstacle to improving our understanding of the biology of these cells using in vitro paradigms. We have isolated colonocytes from the normal-appearing margins of human cancer resections and observed these cells rapidly succumb to apoptosis when prepared in single cell suspension. None of the strategies in which we are aware will delay apoptosis of detached IEC-18 in culture (42) work to keep human colonocytes viable, and indeed these cells are refractory to IL-1 and TNF-α.

In summary, we observed that IL-1 induced increases in mRNA levels for COX-2, MCP-1, MIP-2, iNOS, and RANTES in adherent IEC-18. NF-κB signaling was direct and critical for MCP-1, MIP-2, and iNOS mRNA changes in these cells; however, COX-2 and RANTES were not. COX-2 protein levels were regulated by NF-κB. RANTES mRNA changes were entirely due to the activation of JNK. The multiple means by which IL-1 can lead to increased mediator expression will need to be closely considered.
when strategies intended to block NF-κB are used to control inflammation. Though these changes were not entirely mirrored in a nontransformed human enterocyte line, advances need to be made in sustaining healthy human primary IEC to confidently appreciate the contribution of these cells to inflammation.

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Disclosures

The authors have no financial conflict of interest.

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