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Cell Differentiation Dependent Expressed CCR6 Mediates ERK-1/2, SAPK/JNK, and Akt Signaling Resulting in Proliferation and Migration of Colorectal Cancer Cells

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Abstract The expression of CCL20 (MIP-3α), which chemoattracts leukocytes to sites of inflammation, has been shown in intestinal epithelial cells (IEC). Aim of this study was to analyze the role of the CCL20 receptor CCR6 in IEC and colorectal cancer (CRC) cells. Expression of CCR6 and CCL20 was analyzed by RT-PCR and immunohistochemistry. Signaling was investigated by Western blotting, proliferation by MTS assays and chemotactic cell migration by wounding assays. The effect of CCL20 on Fas-induced apoptosis was determined by flow cytometry. CCR6 and its ligand CCL20 are expressed in IEC. Moreover, CRC and CRC metastases express CCR6, which is upregulated during IEC differentiation. Stimulation of IEC with CCL20 and proinflammatory stimuli (TNF-α, IL-1β, LPS) significantly upregulates CCL20 mRNA expression. CCL20 expression was significantly increased in inflamed colonic lesions in Crohn's disease and correlated significantly with the IL-8 mRNA expression in these lesions ($r = 0.71$) but was downregulated in CRC metastases. CCL20 activated Akt, ERK-1/2, and SAPK/JNK MAP kinases and increased IL-8 protein expression. The CCL20 mediated activation of these pathways resulted in a 2.6-fold increase of cell migration ($P = 0.001$) and in a significant increase of cell proliferation ($P < 0.05$) but did not influence Fas-induced apoptosis. In conclusion, IEC and CRC express CCL20 and its receptor CCR6. CCL20 expression is increased in intestinal inflammation, while CCR6 is upregulated during cell differentiation. CCR6 mediated signals result in increased IEC migration and proliferation suggesting an important role in intestinal homeostasis and intestinal inflammation by mediating chemotaxis of IEC but also in mediating migration of CRC cells. J. Cell. Biochem. 97: 709–723, 2006.

Key words: chemokine; chemokine receptor; CCL20; CCR6; cell migration; colorectal cancer; Crohn's disease

The interaction between intestinal epithelial cells (IECs) and immune cells is an important component of the intestinal immune response coordinating the recruitment and activation of leukocytes at sites of intestinal injury, inflammation, and wound repair through the expression of chemokines and adhesion molecules. Moreover, in addition to their function as chemoattractants for leukocytes, chemokines were recently identified as potential regulators

Abbreviations used: CRC, colorectal cancer; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IEC, intestinal epithelial cell; IL, interleukin; LPS, lipopolysaccharide; mAb, monoclonal antibody; MAP kinase, mitogen-activated protein-kinase; MEK, mitogen-activated protein kinase kinase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PI, phosphatidylinositol; RT-PCR, reverse transcriptase polymerase chain reaction; SAPK/JNK, stress-activated protein kinase/c-Jun-N-terminal kinase; TNF-α, tumor necrosis factor alpha.
Stephan Brand and Torsten Olszak contributed equally to this work.

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of IECs [Yang et al., 1997; Dwinell et al., 1999; Brand et al., 2002, 2005a; Niess et al., 2005].

Chemokines are chemotactic cytokines that induce mammalian cell movement through binding to seven transmembrane receptors [Luster, 1998]. These chemokine receptors are coupled to heterotrimeric G proteins and induce cell movement towards a concentration gradient of the cognate ligand [Luster, 1998]. Depending on their chemokine motif, chemokines can be classified into four different subfamilies: CC, CXC, XC, and CX3C chemokines.

The CC chemokine ligand (CCL)20 (MIP-3α, LARC, Exodus) is primarily produced by the gut epithelium, particularly in the dome epithelium of Peyer's patches where it induces local migration of dendritic cell (DC) subsets expressing its receptor CCR6 [Tanaka et al., 1999; Iwasaki and Kelsall, 2000] but also in liver, lung, appendix, and tonsillar crypts [Hieshima et al., 1997; Hromas et al., 1997; Dieu et al., 1998; Dieu-Nosjean et al., 1999; Tanaka et al., 1999]. The chemokine receptor CCR6 is expressed on immature DCs, B-cells, and memory T-cells expressing gut-homing (α4β7) and skin-homing (CLA) integrins [Liao et al., 1999; Nakayama et al., 2001]. In contrast, we recently demonstrated that the chemokine receptor CX3CR1 is expressed on mature DCs and has essential functions in the luminal sampling of intestinal bacteria [Niess et al., 2005]. Therefore, these two chemokine receptors are important mediators of trafficking of intestinal DCs. CCL20 is the only known chemokine ligand for CCR6, but recent studies have shown that this protein can also act as a functional receptor for the antimicrobial peptides β-defensin 1 and 2 [Yang et al., 1999]. CCR6 knockout mice show deficiencies in Peyer's patch organogenesis and impairment of mucosal responses to both oral antigens and enteric pathogens, and disturbed cutaneous hypersensitivity reactions but maintain normal systemic responses [Cook et al., 2000; Varona et al., 2001]. Interestingly, experiments in CCR6 knockout mice demonstrated that absence of CCR6 resulted in less severe intestinal pathology in animals treated with dextran sodium sulfate [Varona et al., 2003] suggesting that the CCR6/CCL20 axis has a critical, non-redundant role in the in vivo control of immune responses in the intestine.

In addition to their function as chemoattractant for inflammatory cells, chemokines mediate migration of epithelial cells. This function is likely to be important in cancer cell migration and metastasis. For example, signaling through the chemokine receptor CXCR4 mediates actin polymerization and pseudopodia formation in breast cancer cells and induces chemotactic and invasive responses [Muller et al., 2001]. In addition, organs that represent important sites of breast cancer metastasis are the most abundant sources of chemokine ligands for these tumor-associated receptors [Muller et al., 2001]. Recently, we demonstrated similar functions for CXCR4 in colorectal cancer (CRC) cells [Brand et al., 2005a,b].

The expression of CCR6 in IECs has been shown previously [Yang et al., 2005] but the detailed signaling mechanisms and specific biological functions of CCR6 in IEC are unsolved questions in determining its role in IEC biology including its role in intestinal inflammation and colorectal cancerogenesis which was the purpose of this study.

MATERIALS AND METHODS

Reagents

Polyclonal antibodies to phosphorylated extracellular signal-regulated kinase (ERK)-1/2 (Thr183/Tyr185), phosphorylated stress-activated protein kinase (c-Jun N-terminal kinase) SAPK/JNK (Thr183/Tyr185), phospho-p38 (Thr180/Tyr182), and phospho-Akt (Ser473) were purchased from Cell Signaling (Beverly, MA). Anti-ERK-1/2, anti-SAPK/JNK, anti-p38, and anti-Akt antibodies were also from Cell Signaling. Horseradish peroxidase linked anti-rabbit secondary antibody was purchased from Amersham (Arlington Heights, IL). Previously characterized [Dellacasagrande et al., 2003] monoclonal antibodies against human CCR6 (Clone 53103) and CCL20 (Clone 67310) were from R&D Systems (Minneapolis, MN). Recombinant human CCL20, TNF-α, and IL-1β were obtained from R&D Systems (Minneapolis, MN). MEK-1 inhibitor PD98059, SAPK/JNK inhibitor SP600125, p38 inhibitor SB203580, and phosphatidylinositol3-(PI3) kinase inhibitor wortmannin were from Tocris Cookson (Bristol, UK). Lipopolysaccharide (LPS) from *Escherichia coli* (O26:B6) prepared by phenol extraction was purchased from Sigma (St. Louis, MO) and prepared as dispersed sonicate in endotoxin-free water (Life Technologies, Rockville, MD) before diluting to final concentration in supplemented media.

Cell Culture

The human CRC cell lines T84, SW480, Caco-2, HT-29, HCT116, and DLD-1 were obtained from American Type Culture Collection (Rockville, MD). While T84 cells were grown in Dulbecco's modified Eagle medium/F-12 (GIBCO BRL/Life Technologies, Gaithersburg, MD), the other cell lines were grown in Dulbecco's modified Eagle medium (GIBCO) with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum (PAA, Pasching, Austria) in a humidified 5% CO_2 atmosphere at 37°C. For signal transduction experiments with CCL20, cells were starved overnight in serum-free medium.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated using Trizol reagent (GIBCO BRL/Life Technologies, Gaithersburg, MD). For RT-PCR, RNA was treated with ribonuclease (RNase)-free deoxyribonuclease (DNA-free™-Kit, Ambion) to remove potential genomic DNA contaminants. Three micrograms of total RNA were reverse transcribed using Roche first strand cDNA synthesis kit. To control for genomic contamination, an identical parallel PCR reaction was performed containing starting material that had not been reverse transcribed. The following conditions were used for semi-quantitative PCRs: 25 or 35 cycles (depending on the specific PCR) of denaturing at 95°C for 45 s, annealing temperature at 61°C for 45 s, extension at 72°C for 45 s. All PCR reactions included GAPDH primers to quantify PCR products. The primers for the PCR reactions were as follows: CCR6 forward and reverse 5′-atttcagcgatgttttcgactc-3′ and 5′-ggagaagcctgaggacttgta-3′, CCL20 forward and reverse 5′-ttgctcctggctgctttgatg-3′ and 5′-tctttctgttcttggcgtatg-3′, GAPDH forward and reverse 5′-catgtgggccatgaggtccac-3′ and 5′-tgaaggtcggaagtcaactgat-3′. The PCR products were subcloned into pCR 2.1 vector (Invitrogen, Carlsbad, CA) and sequenced.

Quantitative PCR

Real-time PCR was performed with a Rotorgene RG-3000 cycler (Corbett Research, Sydney, Australia) using the Quantitect SYBR Green PCR Kit from Qiagen (Hilden, Germany) following the manufacturer's guidelines. Oligonucleotide primers were designed according to the published sequence, and the following primer pairs were used: CCL20: forward 5′-ctactccacctctgcggcgaa-3′, reverse 5′-ttttactgaggagacgcacaa-3′; beta-actin: forward 5′-gccaaccgcgagaagatga-3′, reverse 5′-catcacgatgccagtggta-3′; interleukin-8 (IL-8): forward 5′-ccaggaagaaaccaccgga-3′, reverse 5′-gaaatcaggaaggctgccaag-3′ (MWG-Biotech, Ebersberg, Germany). CCL20 mRNA expression was normalized to beta-actin expression in the respective cDNA preparation. To compare CCL20 expression levels between inflamed and non-inflamed colonic lesions, CCL20 expression in non-inflammatory tissue was arbitrarily set to 1.0 (i.e., 100%).

Gel Electrophoresis and Immunoblotting

Cells were solubilized in lysis buffer containing 1% Nonidet P-40, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 10 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and phosphatase inhibitors (400 mM sodium orthovanadate and 4 mM NaF) and were passed six times through a 21G needle. After 30 min on ice, lysates were cleared by centrifugation at 10,000*g* for 20 min. The protein concentration of each sample was quantified by the Bradford method. Immunoblotting was performed as previously described [Muehlhoefer et al., 2000].

Enzyme-Linked Immunosorbent Assay (ELISA)

For the quantification of IL-8 release, BD OptEIA Human IL-8 Elisa Kit II (BD Biosciences, Bedford, MA) was used according to the manufacturers instructions.

Immunohistochemistry

Surgical specimens from patients undergoing colectomy or lymph node resection were taken after obtaining patient's consent. Immunohistochemistry was performed as previously described [Muehlhoefer et al., 2000] using a standard streptavidin-peroxidase technique. Previously characterized [Dellacasagrande et al., 2003] monoclonal antibodies against human CCR6 and CCL20 were from R&D Systems (Minneapolis, MN) and were used according to the manufacturer's guidelines.

Cell Proliferation Assay

HT-29 cells were seeded onto 96-well plates at a density of 10,000 cells/well and were allowed to attach overnight. The cells were stimulated

with 10, 100, or 1,000 ng/ml CCL20, or with CCL20-free medium (negative control). The cell proliferation rate was determined by MTS assay on day 2 using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's instructions.

Apoptosis Assays

Apoptosis assays were performed as we described previously [Eichhorst et al., 2004]. SW480 cells, which are less resistant to Fas induced apoptosis than HT-29 cells [Abreu et al., 2000], were used in these assays. For induction of CD95-mediated cell death ligand specific anti-APO-1 mAb at concentrations of 100 and 500 ng/ml was used.

Wounding Assay

Wounding assays were performed as previously described [Dignass and Podolsky, 1993]. Briefly, SW480 cells, which were the most suitable CRC cell line in pilots experiments, were grown in 6-well plates to complete confluence. Using a sterile razor blade, six standardized wounds were created in each plate. Detached cells were removed by three washes with PBS, and the cell medium was changed from 10% fetal calf serum (FCS) containing medium to 1% FCS containing medium. The cells were stimulated with CCL20 (10 and 100 ng/ml) or 1% FCS. The cells were washed with PBS after 24 h and fixed with ethanol. The number of migrated cells (over the wounding edge) was counted under a microscope (Olympus IX50, 10× magnification). For each group (CCL20 stimulated and medium stimulated) three dishes were analyzed, whereas for each dish six separate fields were counted.

Statistical Analysis

Statistical analysis was performed using two-tailed Student's t-test. P-levels <0.05 were considered as significant.

RESULTS

Expression of CCR6 and its Ligand CCL20 in IEC and CRC Cells

To determine if CCR6 is expressed in IEC and CRC cells and to utilize an IEC model to study the CCL20-CCR6 ligand-receptor system, we analyzed CCR6 and CCL20 mRNA expression in several human CRC derived IEC cell lines (HCT116, Caco-2, DLD-1, HT-29, SW480, T84). RT-PCR analysis demonstrated CCR6 and CCL20 mRNA expression in all cell lines tested (Fig. 1A). Using colonic biopsy samples, we demonstrated by immunohistochemical analysis that CCR6 is expressed in primary IEC (Fig. 1B).

Next, we analyzed the influence of cell differentiation on the CCR6 expression levels

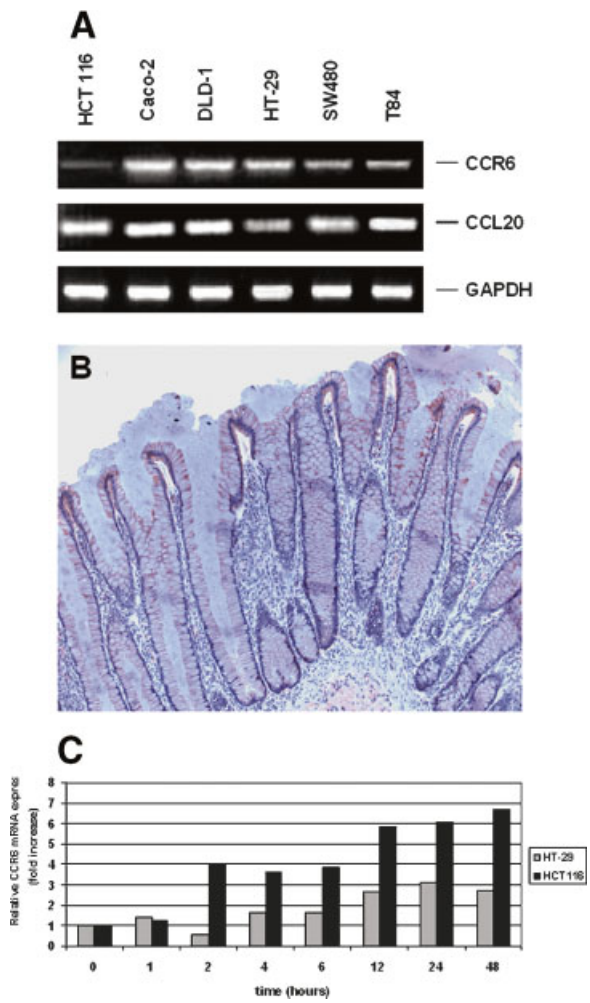

Fig. 1. CCR6 is expressed in IECs and its expression increases with cell differentiation. **A**: Expression of CCR6 and CCL20 in various IEC lines using RT-PCR analysis of mRNA derived from IECs as indicated. **B**: Immunohistochemistry of normal colonic tissue using a previously characterized [Dellacasagrande et al., 2003] human monoclonal anti-CCR6 antibody. CCR6 is predominantly expressed in differentiated, apical intestinal epithelial cells. **C**: Quantitative PCR analysis of HT-29 cells and HCT116 cells stimulated with 5 mM sodium butyrate for time intervals as indicated. The mRNA expression level of CCR6 was normalized to actin levels and is presented as -fold increase compared to CCR6 mRNA levels at time point 0 h. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

in IEC using sodium butyrate stimulation as an established model of IEC differentiation [Chung et al., 1985; Jordan et al., 1999]. Increasing cell differentiation enhanced CCR6 mRNA expression in HT-29 cells up to 3.1-fold after 24 h and in HCT116 cells up to 6.7-fold after 48 h (Fig. 1C). Similarly, there was a clear distribution pattern of CCR6 staining in normal IECs beginning in the middle and increasing to the upper third of the crypt with highest expression in the most differentiated apical IECs (Figs. 1B and 2A).

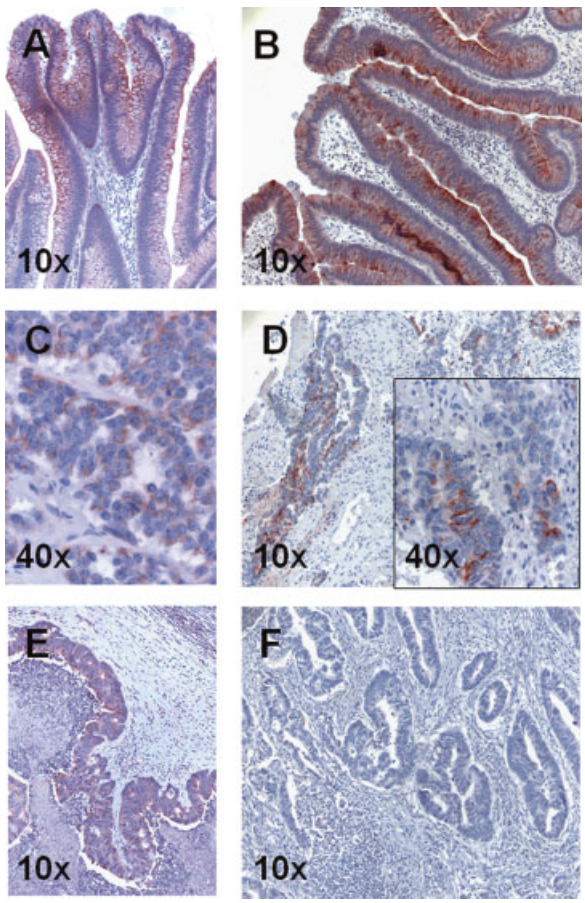

Fig. 2. Intestinal epithelial cells in colorectal adenomas, CRC, and CRC metastases express CCR6. Examples of the immunohistochemical analysis of 4 colorectal adenomas, 15 CRC, 4 CRC lymph node metastases, and 6 CRC liver metastases are shown. **A**: Immunostaining with a human monoclonal anti-CCR6 antibody (R&D Systems, Minneapolis, MN) of a histological section of a colorectal adenoma demonstrating that CCR6 expression is increased in the more differentiated, apical IEC. **B**: Colorectal adenoma stained with a human monoclonal anti-CCL20 antibody (R&D Systems, Minneapolis, MN) demonstrating high CCL20 expression. **C**: CRC stained with anti-CCR6. **D**: CRC stained with anti-CCL20. **E**: Lymph node metastasis stained with anti-CCR6. **F**: CRC section stained with anti-CCL20 demonstrating loss of CCL20 expression. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Having demonstrated that CCR6 and its ligand CCL20 are expressed in CRC derived cell lines (Fig. 1A), we next performed a comprehensive immunohistochemical analysis to study CCR6 and CCL20 expression in primary human CRC tissue and CRC metastases. This included tissue of a variety of CRCs of different stages of invasion and differentiation (histological grading G1-G4) taken from 15 different patients. In addition, we analyzed tissue from CRC lymph node metastases ($n = 4$), liver metastases ($n = 6$), and colorectal adenomas ($n = 4$). While normal colonic mucosa and all four colorectal adenomas demonstrated staining positive for CCL20 protein expression (Fig. 2B), none of the liver metastases and only half of the lymph node metastases expressed CCL20 according to the immunohistochemical analysis. Similarly, in 9 of 15 CRCs CCL20 expression was lost or decreased in comparison to normal colonic tissue suggesting that CCL20 is downregulated in cancerous and metastatic tissue (Table I, Fig. 2D,F). Moreover, the more invasive the specific CRC or CRC metastases were according to the TNM classification, the less likely was CCL20 expression (Table I). For example, none of the three T4 tumors/metastases expressed CCL20 (Table I). All CRC and CRC metastases stained, at least focally, positive for CCR6 (Table I, Fig. 2C,E). However, the number of high or moderate CCR6 expressing tissue samples decrease from 100% in colorectal adenomas to 66.6% CRC to 50% in lymph node metastases and only 33.3% in liver metastases suggesting, similar to the results of Figure 1B,C, that CCR6 expression is higher in differentiated tissues.

Proinflammatory Cytokines Increase CCL20 mRNA Expression

Several chemokines have been shown to be upregulated by proinflammatory cytokines. Therefore, we analyzed if LPS and the proinflammatory cytokines TNF-α and IL-1β regulate CCL20 mRNA expression. In these experiments, the human IEC cell line HT-29 was stimulated with LPS (1 μg/ml), TNF-α (50 ng/ml), or IL-1β (10 ng/ml). LPS increased CCL20 mRNA expression nearly twofold with a peak after 4 h (Fig. 3A). Similar to LPS, the proinflammatory cytokines TNF-α and IL-1β upregulated CCR6 mRNA expression up to 2.4- and 2.5-fold, respectively (Fig. 3B,C). In addition to proinflammatory stimuli (Fig. 3A–C), CCL20 increased its own mRNA expression in

TABLE I. Results of Semiquantitative Immunohistochemical Analysis of CCL20 and CCR6 Protein Expression in Colorectal Adenomas, CRC, CRC Lymph Node Metastases, and Liver Metastases

	TNM	Grading	CCL20 expression	CCR6 expression
Colorectal adenomas (CRA)				
CRA1	N/A	N/A	++	+
CRA2	N/A	N/A	++	+
CRA3	N/A	N/A	+	+
CRA4	N/A	N/A	+	+
Colorectal cancers (CRC)				
CRC1	T1N0M0	G1	−	(+)
CRC2	T2N0M1	G2	+	++
CRC3	T3N0M0	G2	(+)	+
CRC4	T3N0M0	G2	(+)	+
CRC5	T3N0M0	G2	+	(+)
CRC6	T3N0M0	G3	−	+
CRC7	T3N0M0	G3	+	+
CRC8	T3N0M1	G2	+	(+)
CRC9	T3N1M0	G2	(+)	+
CRC10	T3N1M1	G2	(+)	+
CRC11	T3N2M0	G2	+	+
CRC12	T3N2M0	G4	−	++
CRC13	T3N2M1	G3	+	(+)
CRC14	T4N2M0	G3	−	(+)
CRC15	T4N1M1	G2	−	++
Lymph node metastases (LNM)				
LNM1	T3N2M0	G2	+	++
LNM2	T3N1M1	G2	+	+
LNM3	T3N2M1	G3	−	(+)
LNM4	T4N2M0	G3	−	(+)
Liver metastases (LM)				
LM1	T2N0M1	G2	−	(+)
LM2	T3N0M1	G2	−	+
LM3	T3N1M1	G2	−	(+)
LM4	T3N1M1	G2	−	(+)
LM5	T3N1M1	G3	−	(+)
LM6	T3N1M1	G3	−	+

CRCs and CRC metastases are ranked according to the results of their TNM classification. All samples were scaled regarding their CCL20 and CCR6 immunostaining intensity by two senior investigators blinded to clinical, TNM, and treatment information. Scale: −, no expression; (+), partial/low expression; +, moderate expression; ++, strong expression.

IEC (Fig. 3D). This induction of CCL20 mRNA expression was biphasic with peak expression levels at 2 and 24 h, an expression pattern previously described for other proinflammatory cytokines including the chemokine ENA-78/CXCL5 after cell stimulation with proinflammatory cytokines [Schnyder-Candrian and Walz, 1997] suggesting that CCL20 increases the expression of proinflammatory cytokines resulting in the second CCL20 mRNA peak. This hypothesis was analyzed in the following experiments (Fig. 4).

CCL20 mRNA is Increased in Inflammatory Colonic Lesions in Crohn's Disease and Correlates Highly With IL-8 mRNA Expression Levels

Next, we analyzed if CCL20 mRNA expression correlates with the level of inflammation in vivo using real-time PCR. In these experiments, we compared CCL20 mRNA expression levels in biopsy samples taken from 14 different sites (of 7 patients with Crohn's disease) with endoscopically (macroscopic) inflamed colonic mucosa with those of endoscopically non-inflamed colonic mucosa taken from 14 different sites of the same 7 patients. The IL-8 expression, which was used as a control marker for inflammation, was significantly increased ($P < 0.05$) in the inflamed biopsy samples (Table II). The increase in IL-8 mRNA expression ranged from 1.4 up to 31.1-fold compared to the non-inflamed tissues. Similarly, CCL20 mRNA expression levels were significantly higher ($P < 0.05$) in biopsy samples with inflamed mucosa when compared with non-inflamed lesions (increase between 1.9- and 16.7-fold; Table II). Moreover, the CCL20 mRNA levels correlated highly with the IL-8 mRNA expression levels ($r = 0.71 \pm 0.09$), demonstrating its association with intestinal inflammation.

Having demonstrated that CCL20 mRNA expression correlates highly with IL-8 levels in Crohn's disease and that CCL20 upregulates its own mRNA expression in IEC, we analyzed next

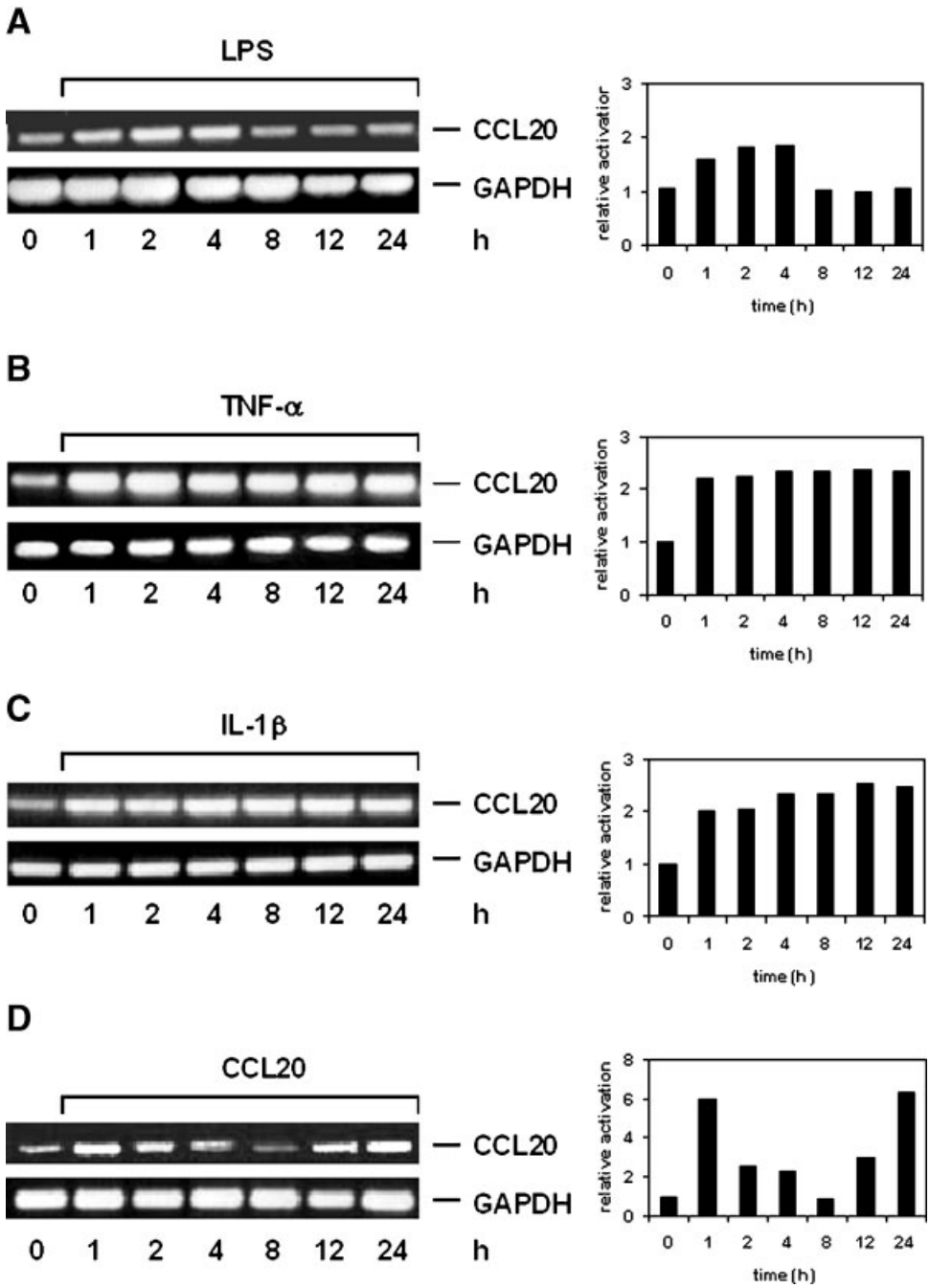

Fig. 3. CCL20 mRNA expression is upregulated after stimulation with various proinflammatory stimuli. Regulation of CCL20 mRNA expression after stimulation with LPS (1 μg/ml; **Panel A**) with TNF-α (50 ng/ml; **Panel B**), IL-1β (10 ng/ml; **Panel C**) and CCL20 (100 ng/ml; **Panel D**). The expression levels of CCL20 mRNA were normalized to GAPDH mRNA levels using TINA software.

if CCL20 increases IL-8 levels in IEC. In these experiments, HT-29 cells were stimulated with 100 ng/ml CCL20 and IL-8 protein expression was measured by ELISA. CCL20 stimulation increased IL-8 protein levels 4.6-fold after 24 h (Fig. 4).

CCL20 Induces ERK-1 and ERK-2 Activation by MEK-1 Dependent Mechanism

Having demonstrated that CCR6 is functional in IEC resulting in increased expression of the proinflammatory chemokines CCL20 and

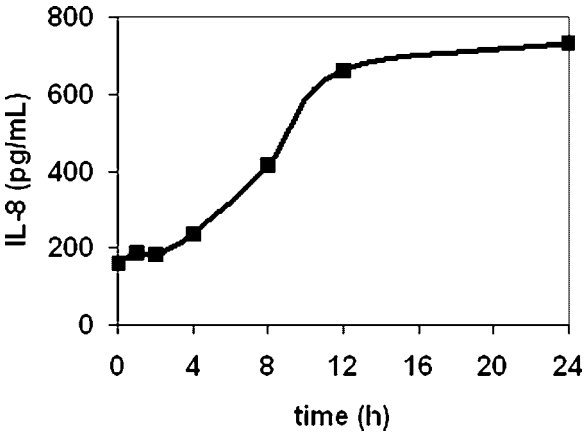

Fig. 4. CCL20 increases IL-8 protein expression in HT-29 cells. IL-8 protein expression measured in ELISA assays increased 4.6-fold after stimulation with recombinant human CCL20 (100 ng/ml).

IL-8, we next analyzed major signaling pathways of CCR6 in IEC. In these experiments, CCR6 expressing SW480 and HT-29 cells were stimulated with the CCR6 ligand CCL20. Stimulation of chemokine receptors can result in transient activation of mitogen-activated protein (MAP) kinases [Ganju et al., 1998a; Tilton et al., 2000]. Similarly, 100 ng/ml CCL20 induced transient activation of ERK-1/2 (Fig. 5A). To identify the upstream signaling events, we investigated the effect of the MEK-1 inhibitor PD98059 on the CCL20 mediated ERK regulation. PD98059 downregulated ERK-1/2 phosphorylation after CCL20 stimulation (Fig. 5B), suggesting MEK-1 as an upstream signal transducer of the CCL20 induced ERK activation. Crosstalk between the PI3-kinase and the MEK-ERK pathway has been proposed [Rommel et al., 1999]. However, ERK activation after CCL20 stimulation was not significantly affected by pre-treatment with wortmannin (Fig. 5C), suggesting a PI3-kinase-independent activation of ERK-MAP kinases by CCL20. Similarly, the JNK kinase inhibitor SP600125 did not influence the CCL20 induced ERK activation (Fig. 5D).

CCR6 Activation Results in SAPK/JNK- and Akt Phosphorylation

Activation of chemokine receptors may also result in activation of p38 and SAPK/JNK kinases and Akt [Ganju et al., 1998b; Sotsios et al., 1999; Robledo et al., 2001]. Stimulation of SW480 and HT-29 cells with CCL20 resulted in phosphorylation of SAPK/JNK kinases

TABLE II. Comparison of CCL20 and IL-8 mRNA Expression in Inflamed and Non-Inflamed Colonic Lesions of Patients With Crohn's Disease

		Anatomic site of biopsy sampling				
Patient no.	Current medication	Non-inflamed	Inflamed	CCL20 expression level inflamed versus non-inflamed	IL-8 expression level inflamed versus non-inflamed	Correlation CCL20/IL-8
1*	AZA, IFX	Cecum*	Terminal ileum*	1.93	4.50	0.985
2	Mesalazine, AZA	Descending colon	Descending colon	3.69	11.67	0.636
3	AZA	Cecum	Cecum	16.73	11.08	0.372
4*	Mesalazine, cortico-steroids	Cecum*	Terminal ileum*	3.41	1.52	0.497
5	No medication	Descending colon	Descending colon	2.15	1.41	0.999
6	MTX	Transverse colon	Transverse colon	12.27	31.12	0.955
7	AZA	Transverse colon	Transverse colon	2.00	2.24	0.542
Average ± SEM				6.03 ± 2.11	9.08 ± 3.76	0.71 ± 0.09

CCL20 expression was measured by quantitative PCR and normalized with respect to beta-actin expression levels in the respective cDNA preparation. To compare expression levels between inflamed and non-inflamed colonic lesions, mRNA expression in non-inflamed tissue was arbitrarily set to 1.0 (100%). The current medical therapy, under which biopsy samples were taken, and the anatomic site from which the samples were taken, are given for all patients. Biopsy sampling for inflamed and non-inflamed lesions was intended to be performed in the same colonic or ileal segment. This was not possible in patients marked with an asterisk (*) due to severe inflammation in the whole anatomic segment. Therefore, biopsies from a bordering (non-inflamed) segment were included for comparison. Abbreviations: AZA, azathioprine; IFX, infliximab; MTX, methotrexate.

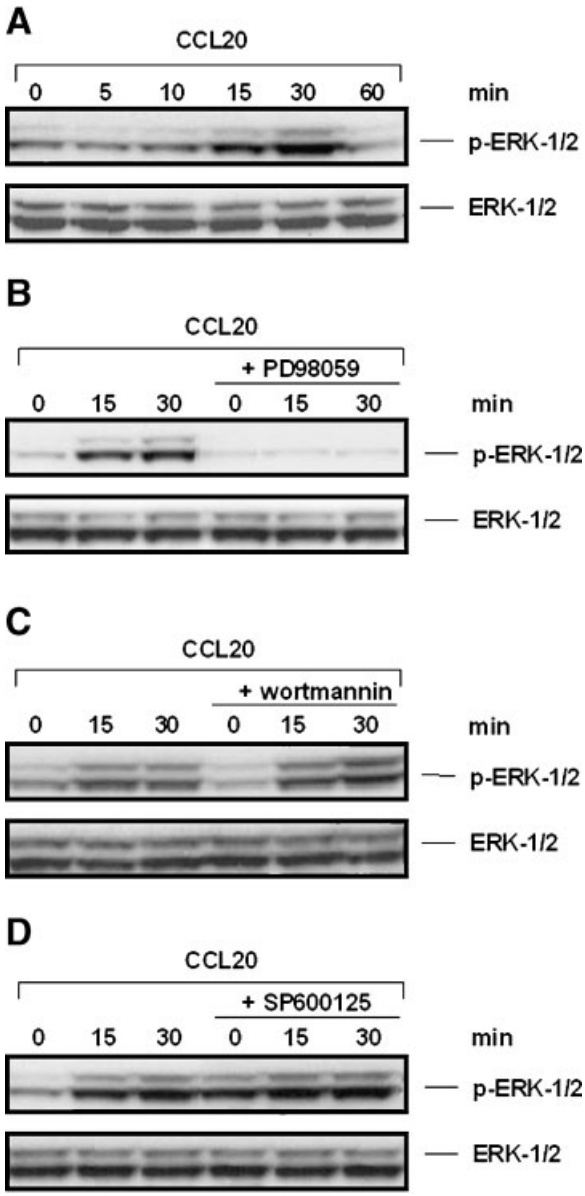

Fig. 5. CCL20 activates ERK-MAP kinases in SW480 cells. Activation and expression of phospho-ERK-1/2 was assessed by immunoblotting. **A**: Phospho-ERK-1/2 activation after CCL20 stimulation (100 ng/ml). **B**: The CCL20-induced ERK activation is MEK-1 dependent. Pretreatment with the MEK-1 inhibitor PD98059 (10 μmol/L 1 h before CCL20 stimulation) resulted in decreased ERK activation. **C**: The CCL20 induced ERK activation is PI3 kinase independent. Pretreatment with the PI3 kinase inhibitor wortmannin (25 μmol/L) did not influence ERK-1/2 activation. **D**: Similarly, ERK activation is independent from SAPK/JNK signaling. Pretreatment with the SAPK/JNK inhibitor SP600125 (20 μmol/L) did not influence ERK-1/2 activation. Similar results were found in HT-29 cells (data not shown). One representative experiment (n = 3) is shown.

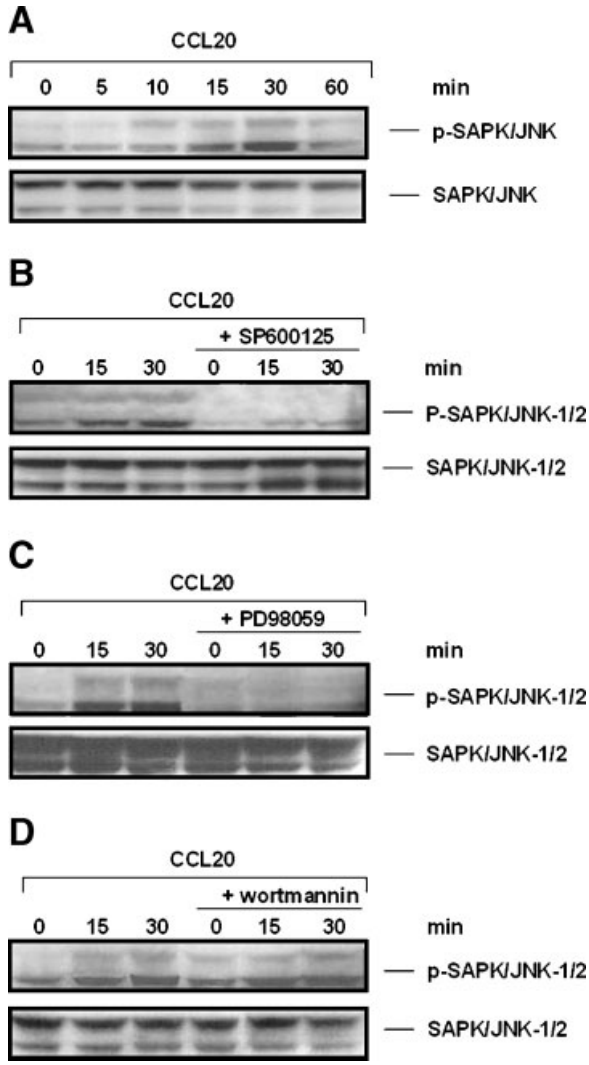

Fig. 6. CCL20 stimulation activates SAPK/JNK. **A**: Stimulation of SW480 cells with CCL20 (100 ng/ml) resulted in increased phosphorylation of SAPK/JNK kinases. **B**: Pretreatment with the SAPK/JNK inhibitor SP600125 (20μmol/L) decreased SAPK/JNK activation. **C**: SAPK/JNK activation is MEK-1 dependent. Pretreatment with the MEK-1 inhibitor (PD98059, 10 μmol/L) decreased JNK activation. **D**: The PI3 kinase inhibitor wortmannin (25 μmol/L) did not abrogate the CCL20 induced SAPK/JNK activation. Similar results were found in HT-29 cells (data not shown). One representative experiment (n = 3) is shown.

(Fig. 6A). Pretreatment with the JNK inhibitor SP600125 significantly suppressed phosphorylation of SAPK/JNK kinases (Fig. 6B), as did pretreatment with the MEK-1 inhibitor PD98059 suggesting an MEK-1-dependent pathway (Fig. 6C). In contrast, the PI3 kinase inhibitor wortmannin did not influence phosphorylation levels (Fig. 6D). In addition, CCL20 stimulation resulted only in a weak increase of phosphorylation levels of p38 (data not shown)

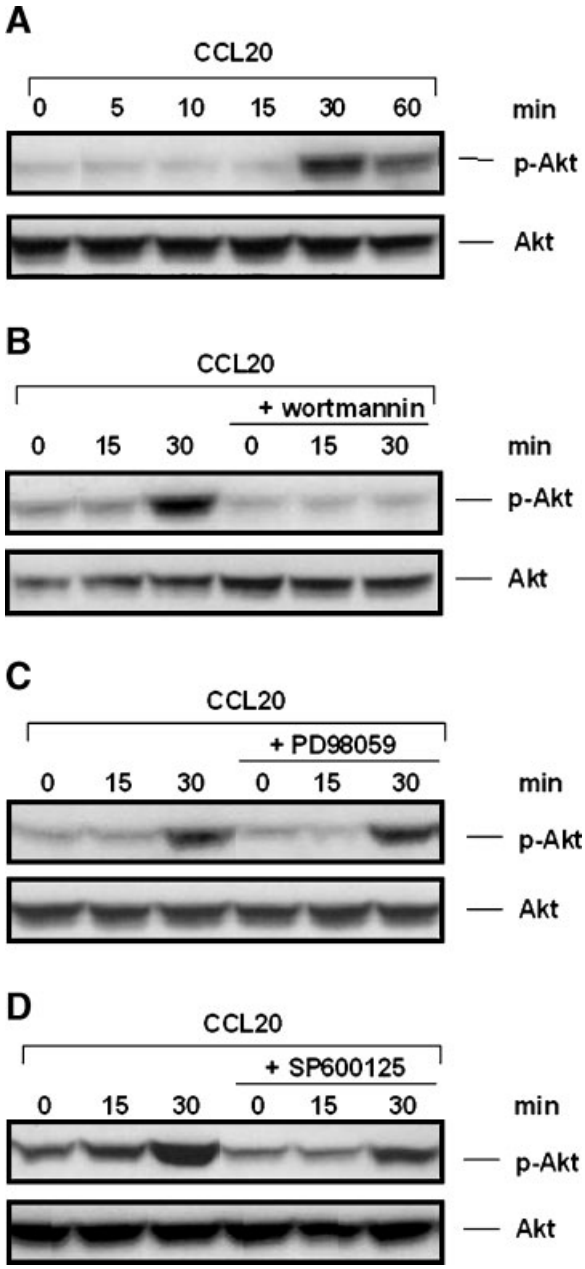

Fig. 7. CCL20 induces Akt activation in SW480 cells. **A**: CCL20 (100 ng/ml) induces Akt phosphorylation. **B**: Pretreatment with the PI3 kinase inhibitor wortmannin (25 μmol/L) inhibited Akt phosphorylation. **C**: Pretreatment with the MEK-1 inhibitor PD98059 had no effect on Akt phosphorylation levels. **D**: Similarly, the JNK inhibitor SP600125 (20 μmol/L) did not influence Akt phosphorylation levels. Similar results were found in HT-29 cells (data not shown). One representative experiment (n = 3) is shown.

suggesting that CCL20 activate primarily ERK and JNK MAP kinase signaling in IEC. Furthermore, CCL20 binding to its receptor CCR6 also resulted in increased phosphorylation of Akt (Fig. 7A). Pretreatment with the PI3 kinase inhibitor wortmannin completely inhibited CCL20-induced Akt phosphorylation (Fig. 7B), while pretreatment with the MEK-1 inhibitor PD98059 (Fig. 7C) and the SAPK/JNK inhibitor SP600125 (Fig. 7D) did not influence Akt activation suggesting a PI3 kinase and SAPK/JNK-independent pathway.

CCL20 Does not Influence Fas Ligand Induced Apoptosis but Stimulates IEC Proliferation

We demonstrated that CCL20 stimulation results in ERK-1/2 and Akt activation which have been shown to mediate anti-apoptotic pathways and increase cell proliferation [Fridell et al., 1996; Dudek et al., 1997]. Both, cell proliferation and apoptosis are important mechanisms by which the intestinal barrier and its restitution (e.g., in intestinal inflammation) are controlled. Moreover, increased cell proliferation and decreased apoptosis have been demonstrated in cancerous tissue resulting in enhanced tumor growth and resistance to "apoptotic" and antiproliferative therapy strategies [Eichhorst and Krammer, 2001]. Therefore, we investigated the chemokine-mediated effect on apoptosis using experimental conditions which we previously established [Eichhorst et al., 2004]. In these experiments, SW480 cells were used which are less resistant to Fas induced apoptosis than HT-29 cells [Abreu et al., 2000]. However, no significant difference between the number of apoptotic cells in the chemokine stimulated group and the unstimulated group was found (Fig. 8A). In contrast, CCL20 at concentrations of 10 ng/ml and 100 ng/ml increased significantly cell proliferation ($P < 0.05$ and $P = 0.006$, respectively), while higher concentrations did not change the cell proliferation rate significantly (Fig. 8B).

CCR6 Activation Results in Increased Cell Migration

The activation of MAP kinases such as ERK-1/2 and the activation of Akt have been linked to cell migration [Bonacchi et al., 2001] which is a major mechanism of wound healing after injury of the IEC barrier, for example, in severe inflammation but also a main characteristic of CRC cancer growth. Therefore, we analyzed in cell migration assays if CCL20 is chemotactic for IECs. In these "wounding" assays, standardized sterile wounds were created in SW480 cell monolayers, which were the most suitable CCR6-expressing IEC line for wounding assays

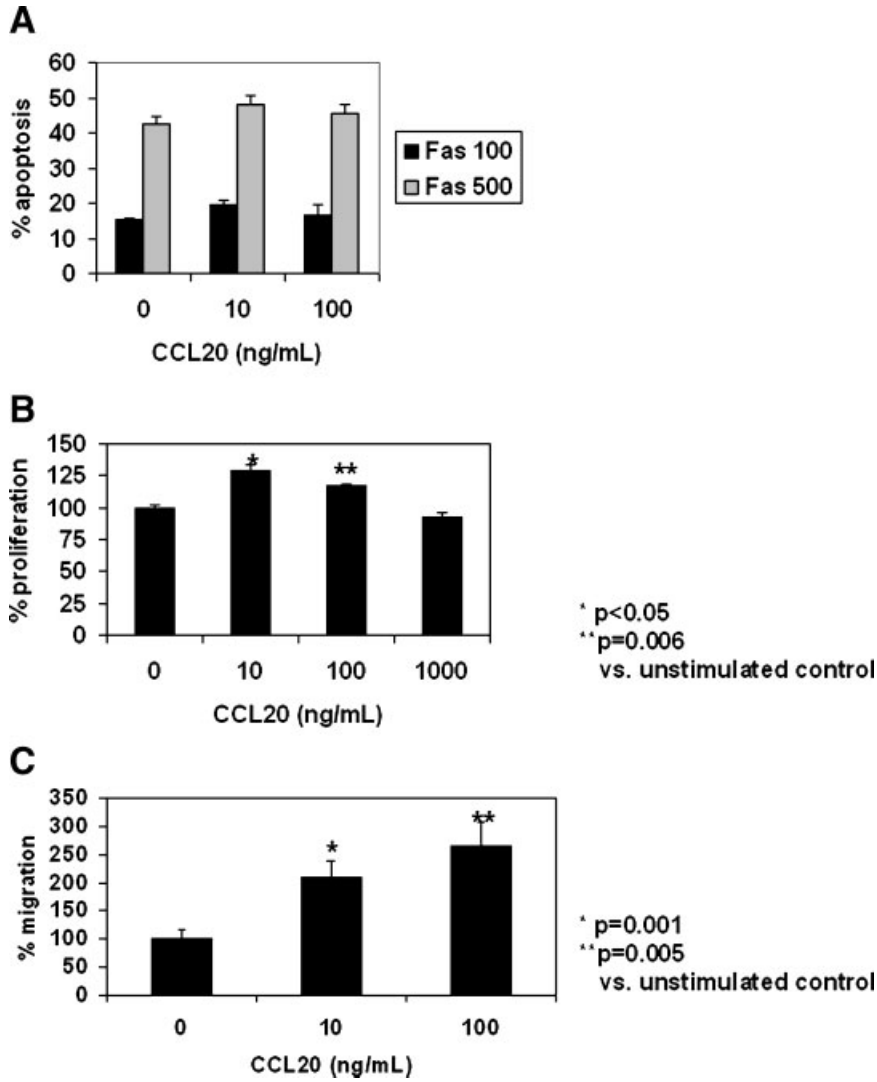

Fig. 8. CCL20 does not regulate apoptosis but increases cell proliferation and cell migration in IEC cells. **A**: CCL20 does not influence Fas ligand mediated apoptosis in SW480 cells. Cells were treated with 100 and 500 ng/ml anti-APO-1 antibody and either CCL20 stimulated or remained unstimulated for 24 h. Apoptosis was measured by FACS analysis. **B**: CCL20 increases cell proliferation in HT-29 cells. HT-29 cells were seeded onto 96 well plates at a density of 10,000 cells/well and were grown for 1 day. After starvation in serum-free medium overnight, the cells were stimulated with CCL20 as indicated, or with CCL20-free medium (negative control). The cell proliferation rate was determined by MTS assay after 48 h. There was a statistical significant ($P < 0.05$ and $P = 0.006$) increase in cell proliferation when the cells were stimulated with CCL20 (10 ng/ml and 100 ng/ml, respectively). Relative means ± SEM from three independent experiments are shown. **C**: ''Wounding'' assays were used to analyze the influence of CCL20 on IEC migration. Standardized, sterile wounds were created in SW480 cell monolayers as described in the Method section. Twenty-four hours after wounding the number of migrated cells (over the wounding edge) was counted under the microscope. CCL20 induced a significant increase of the cell migration rate (10 ng/ml CCL20: $P = 0.001$; 100 ng/ml CCL20: $P = 0.005$ vs. medium stimulated controls).

in pilot experiments. Twenty-four hours after wounding, the number of migrated cells over the wounding edge was counted under the microscope. To quantify the CCL20 mediated cell migration, we analyzed a total of 18 fields in 6 separate dishes for each group containing more than 300 migrated cells per group. This experiment demonstrated a statistically significant increase of the cell migration rate in the CCL20 stimulated cells in comparison to medium stimulated controls ($P = 0.001$ for 10 ng/ml CCL20; $P = 0.005$ for 100 ng/ml CCL20; Fig. 8C).

DISCUSSION

Chemokines are chemotactic cytokines, which recruit leukocytes to sites of infection

or inflammation [Butcher and Picker, 1996; Baggiolini, 1998; Zlotnik and Yoshie, 2000; Niess et al., 2005]. However, recent studies indicate that certain chemokines and chemokine receptors also play an important role in IEC biology [Dwinell et al., 1999; Jordan et al., 1999; Brand et al., 2002, 2005a,b]. Moreover, several studies demonstrated that chemokine receptors are critical in determining homing of metastatic tumor cells [Geminder et al., 2001; Muller et al., 2001; Robledo et al., 2001; Taichman et al., 2002; Brand et al., 2005a].

Previous studies demonstrated that IEC express the CCR6 ligand CCL20 [Tanaka et al., 1999; Iwasaki and Kelsall, 2000; Kwon et al., 2002; Puleston et al., 2005]. Here, we show that IEC also express its receptor CCR6. Furthermore, we demonstrated that CCR6 is functional in IEC. Stimulation of IEC with CCL20 resulted in activation of distinct signaling pathways. We also demonstrated that this chemokine ligand receptor pair is an important mediator of intestinal inflammation. Stimulation of IEC with proinflammatory cytokines and LPS significantly upregulated CCL20 mRNA expression. Moreover, CCL20 increased its own mRNA expression, a mechanism previously shown for other proflammatory cytokines and chemokines such as fractalkine [Brand et al., 2002]. In addition, we demonstrated that CCR6 activation in IEC resulted in increased protein expression of the proinflammatory chemokine IL-8. Quantitative PCR analysis demonstrated that CCL20 is upregulated in inflamed colonic biopsy samples taken from patients with Crohn's disease. The CCL20 mRNA levels correlated highly with the IL-8 mRNA levels in these biopsy samples suggesting an important role in intestinal inflammation. These results are consistent with previous studies demonstrating increased CCL20 expression levels in colonic biopsy samples of patients with inflammatory bowel disease [Kwon et al., 2002; Kaser et al., 2004; Puleston et al., 2005].

A multitude of studies indicates that chronic intestinal inflammation as seen in ulcerative colitis and Crohn's disease is a risk factor for developing CRC [Katzka et al., 1983; Ekbom et al., 1990; Choi and Zelig, 1994; Gillen et al., 1994]. CRC is characterized by metastasis into abdominal lymph nodes and liver. Tumor metastasis is of high clinical relevance since patients with advanced CRC rarely live beyond 5 years. In this study, we demonstrate that CRC cells express functional CCR6 chemokine receptors, which was also detected in all primary CRC cells and in all metastases analyzed. The liver is a main producer for CCL20 [Hieshima et al., 1997], therefore liver metastasis of CCR6-expressing CRC cells is likely to be facilitated by this chemokine system. This is supported by a recent study, which demonstrated that CCR6 is over-expressed in liver metastases of colon, thyroid, and ovarian carcinomas compared with normal liver [Dellacasagrande et al., 2003]. In contrast to CCR6, the expression of CCL20 was downregulated in several CRCs and CRC metastases (Table I). Stimulation of CRC cells by the CCR6 ligand CCL20 triggers responses that are similar to leukocyte responses to chemoattractants, such as CRC migration. Our results demonstrating a role for CCR6 in CRC cell migration are supported by a very recent study [Yang et al., 2005], which showed that apical stimulation of polarized CCR6 expressing CRC cells with CCL20 resulted in tyrosine phosphorylation of the p130 Crk-associated substrate (Cas), an adaptor/scaffolding protein that localizes in focal adhesions and has a role in regulating cytoskeletal elements important for cell attachment and migration.

Moreover, we demonstrate that CCL20 is expressed by normal IECs possibly facilitating migration and invasion of cancer cells into the normal intestinal epithelium. In addition to CRC, functional CCR6 receptors have been detected in several other malignancies such as pancreatic cancer [Kleeff et al., 1999; Kimsey et al., 2004; Campbell et al., 2005], hepatic cancer cells [Fujii et al., 2004], multiple myeloma [Moller et al., 2003], and certain B-cell non-Hodgkin's lymphomas [Rodig et al., 2002] suggesting similar mechanisms of tumor cell migration and metastasis in a variety of malignancies.

Interestingly, we demonstrate an increasing CCR6 expression along the intestinal crypt axis with low expression levels at the base of the crypts to high expression levels in the more differentiated apical cells. Although CCR6 expression increased in normal IEC with cell differentiation, this association was less clear in CRC tissue. However, high CCR6 expression was less common in CRC metastases than in normal colonic tissue and colorectal adenomas supporting the observation that CCR6 expression is higher in differentiated tissues. Our observation that CCR6 is a marker of normal

IEC differentiation, is also supported by a recent complex gene analysis of markers of differentiation in keratinocytes and IECs which found CCR6 primarily in IEC at later stages of differentiation [Dabelsteen et al., 2003]. Based on the IEC migration induced by CCL20 found in our experiments, this CCR6 gradient within the intestinal crypt is likely to be a factor involved in the directed IEC movement during cell differentiation from the base of the crypt to the apical part.

CCL20 predominantly activates the MEK-ERK MAP kinase signaling pathway in IEC which has also been shown to be the major signaling pathway of other chemokine receptors such as CX3CR1 [Brand et al., 2002] and CXCR4 [Tilton et al., 2000; Brand et al., 2005a,b]. However, in contrast to our analysis of CX3CR1 signaling [Brand et al., 2002], CCR6 activation also resulted in increased phosphorylation of SAPK/JNK kinases as well as increased Akt phosphorylation levels. Interestingly, two recent studies demonstrated that SAPK/JNK is activated in Crohn's disease [Hommes et al., 2002; Waetzig et al., 2002], and that inhibition of SAPK/JNK resulted in significant clinical benefit and rapid endoscopic ulcer healing [Hommes et al., 2002].

Particularly, the activation of ERK-MAP kinases and Akt has been implicated in cell migration [Sotsios et al., 1999; Bonacchi et al., 2001; Yap, 2001; Graness et al., 2002]. Our experiments demonstrated that CCR6 activation results in increased IEC migration. Increased cell migration is a major feature of malignant tumor growth. In addition, CCR6-induced IEC migration is also important during normal IEC development and under inflammatory conditions. For example, an impairment of the integrity of the mucosal epithelial barrier is observed in the course of various intestinal disorders including inflammatory bowel diseases. As demonstrated in wounding assays in this study, CCL20 stimulation facilitates the epithelial restitution.

In summary, we demonstrate that the chemokine receptor CCR6 is expressed by normal IEC and CRC cells. Upon stimulation with its ligand CCL20 several distinct signaling pathways including ERK-MAP kinases and Akt are activated. CCL20 mRNA expression is upregulated in intestinal inflammation but downregulated in CRC metastases. CCR6 expression in CRC and CRC metastases indicate a role for this chemokine system in CRC migration and metastasis. This is supported by our results of in vitro models demonstrating increased CRC proliferation and migration after CCR6 activation. Therefore, CCR6 and its ligand, which are essential in controlling immune cell trafficking in response to inflammatory stimuli, may have also an important role in determining the metastasis of CRC cells in vivo.

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