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Iron chelator induces MIP-3α/CCL20 in human intestinal epithelial cells: implication for triggering mucosal adaptive immunity

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Abbreviations: 5-ASA, 5-aminosalicylic acid; CCL20, CC chemokine-ligand 20; dbcAMP, dibutyryl-cAMP; DC, dendritic cells; DFO, deferoxamine; FC, ferric citrate; HerA, herbimycin A; IECs, human intestinal epithelial cells; MIP-3α, macrophage inflammatory protein 3α; OA, okadaic acid; PKC, protein kinase C; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)phrazolo[3,4-d]pyrimidine; PTK, protein tyrosine kinase; RFI, relative fluorescence intensity; rh, recombinant human; RT, reverse transcription; VA, sodium *ortho*-vanadate (Na_3VO_4)

Abstract

A previous report by this laboratory demonstrated that bacterial iron chelator (siderophore) triggers inflammatory signals, including the production of CXC chemokine IL-8, in human intestinal epithelial cells (IECs). Microarray-based gene expression profiling revealed that iron chelator also induces macrophage inflammatory protein 3 alpha (MIP-3α)/CC chemokine-ligand 20 (CCL20). As CCL20 is chemotactic for the cells involved in host adaptive immunity, this suggests that iron chelator may stimulate IECs to have the capacity to link mucosal innate and adaptive immunity. The basal medium from iron chelator deferoxamine (DFO)-treated HT-29 monolayers was as chemotactic as recombinant human CCL20 at equivalent concentrations to attract $CCR6^+$ cells. The increase of CCL20 protein secretion appeared to correspond to that of CCL20 mRNA levels, as determined by real-time quantitative RT-PCR. The efficacy of DFO at inducing CCL20 mRNA was also observed in human PBMCs and in THP-1 cells, but not in human umbilical vein endothelial cells. Interestingly, unlike other proinflammatory cytokines, such as TNF-α and IL-1β, a time-dependent experiment revealed that DFO slowly induces CCL20, suggesting a novel mechanism of action. A pharmacologic study also revealed that multiple signaling pathways are differentially involved in CCL20 production by DFO, while some of those pathways are not involved in TNF-α-induced CCL20 production. Collectively, these results demonstrate that, in addition to some bacterial products known to induce host adaptive immune responses, direct chelation of host iron by infected bacteria may also contribute to the initiation of host adaptive immunity in the intestinal mucosa.

Keywords: CCL20 protein, human; deferoxamine; immunity, active; iron; iron chelator; tumor necrosis factor-α

Introduction

Iron is a critical nutritional element, essential for a great variety of important biological processes, including cell growth and differentiation, electron transfer reactions, and oxygen transport, activation, and detoxification (Crichton *et al.,* 1992). Maintaining iron availability for cellular metabolic and growth requirements is critical to the survival of both prokaryotes and eukaryotes. Iron is also a central element in the regulation of immune function. A sufficient supply with iron is of pivotal importance for immuno-surveillence because of iron's growth-promoting role for immune cells and its interference with cell-mediated immune effector pathways and cytokine activities (Brieva *et al.,*1984; Bierer *et al.,* 1990; Golding *et al.,* 1995; Weiss *et al.,* 1995; Gray *et al.,* 2002; Lee *et al.,* 2004). Moreover, iron is directly involved in cytotoxic immune defense mechanisms in which it is needed to catalyze the formation of the hydroxyl radical (OH·) via the

Fenton reaction (Marx, 2002).

The intestinal epithelial cells (IECs) which line the mucosal surfaces of the intestinal tracts are the first to face the challenge of either normal flora or exogenous putative pathogens. Such IECs have a pivotal role in the host defense as they sense microbial infection at a very early stage, thereby producing a variety of proinflammatory cytokines affecting leukocyte activity (Finlay, 1997; Shin *et al,* 2004). This innate immune response is essential for the rapid clearance of bacteria. The CXC-chemokine IL-8 is expressed in IECs in response to pro-inflammatory cytokines (Roebuck, 1999) and cellular stress (Shapiro *et al,* 1997; Desbaillets *et al,* 1999; Narayanan *et al.,* 1999) and is recognized as a major activator of acute inflammation (Witko-Sarsat *et al.,* 2000). We recently identified that bacterial iron chelator (siderophore) can also trigger inflammatory signals, including the production of IL-8, in human IECs, demonstrating that direct chelation of host iron may be an another important mechanism to initiate the innate immune response by infected bacteria (Choi *et al.,* 2004).

During infection of the gut, microbial pathogens must acquire host iron to survive and replicate on the surface of the mucosal layer. Competition for iron between the host and microorganisms is therefore inevitable. In fact, highly virulent strains possess exceptionally powerful mechanisms for obtaining host iron from healthy hosts (Weinberg, 1989). An excellent example is bacterial siderophores. These small molecules can withdraw iron from transferrins synthesized by a variety of host species. Along with this line, iron chelators have been implicated in modulating certain inflammatory mediators and regulating inflammatory processes (Weiss *et al,* 1994; Saleppico *et al,* 1996; Dlaska *et al,* 1999; Tanji *et al,* 2001). Given the potential importance of iron in mucosal immunity, it is also of great interest to elucidate whether iron chelator can modulate the ability of IECs to have the capacity to link mucosal innate and adaptive immunities.

Recent studies have shown that intestinal epithelium have the capacity to play a role in signaling host adaptive immunity by producing the CC chemokine MIP-3α/CCL20 (Hieshima *et al.,* 1997; Schutyser *et al.,* 2003). The CCL20 is chemotatic for immature dendritic cells (DCs), effector/memory T cells, and B cells via its unique receptor CCR6 (Schutyser *et al.,* 2003) and the expression CCL20 gene in human IECs is up-regulated by pathogenic bacteria and proinflammatory cytokines, such as IL-1β and TNF-α (Fujiie *et al.,* 2001; Izadpanah *et al.,* 2001; Sierro *et al.,* 2001). However, it has not been determined whether the expression of CCL20 mRNA and its protein levels are affected or regulated by cellular iron state.

In this study, we therefore investigated whether deferoxamine (DFO), which is an actual component of bacteria to chelate iron for their growth, can trigger epithelial cells to produce a biologically active form of CCL20 as a single stimulus, so as to recruit the cells that are important components of the host adaptive immune system. In fact, microarray-based preliminary work by this laboratory demonstrated that DFO, an iron chelator, up-regulates CCL20 in human HT-29 cells (Choi *et al.,* 2004). We provide evidence in this study that multiple signaling pathways are differentially involved in the induction of CCL20 by iron chelator. Furthermore, some of these pathways are not overlapped with TNF-α-mediated CCL20 production. Moreover, culture supernatants from DFO-stimulated cells triggered CCL20-mediated migration of CCR6 expressing cells compared with that of unstimulated control or CCL20-preincubated cells, thereby suggesting that iron metabolism in IECs may have a direct role in the evocation of host innate and adaptive immune responses, presumably by modulating CCL20 as well as other inflammatory mediators.

Materials and Methods

Reagents and antibodies

DFO, ferric citrate (FC), mimosineosine, PHA, BAPTA-AM, thapsigargin, herbimycin A (HerA), sodium *ortho*vanadate (Na_3VO_4; VA), okadaic acid (OA), A23187, dibutyryl-cAMP (dbcAMP), 5-aminosalicylic acid (5-ASA), sulfasalazine, alkaline phosphatase-conjugated rabbit anti-goat IgG, and *p*-nitrophenyl phosphate tablets were purchased from Sigma (St. Louis, MO). Recombinant human (rh) IL-1β, TNF-α, IL-2, mouse anti-human CCL20 monoclonal antibody (clone 67310.111), mouse anti-human CCR6-PE, mouse anti-human CD3-FITC, and goat anti-human CCL20 polyclonal antibody were obtained from R&D Systems (Minneapolis, MN). 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)phrazolo [3,4-d]pyrimidine (PP2), KT5720, protein kinase C (PKC) inhibitors (rottlerin, Gö6983 and Gö6976), and MAPK inhibitors (SB202190, U0126 and PD 98059) were purchased from Calbiochem-Behring (La Jolla, CA).

Cell culture

HT-29 human colon epithelial cells [ATCC HTB 38; American Type Culture Collection (ATCC), Manassas, VA] and THP-1 human acute monocytic leukemia cells (ATCC TIB-202) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, penicillin G (100 IU/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM). T-84 human

colon epithelial cells (ATCC CCL-284) and Caco-2 human ileocecal epithelial cells (ATCC HTB 37) were grown in DMEM containing the supplements as mentioned above. Human PBMCs were isolated from normal healthy donors by dextran sedimentation followed by centrifugation through a discontinuous Ficoll gradient (Amersham Biosciences). All the cell lines or primary cell fractions mentioned above were cultured at 37°C in a humidified atmosphere containing 5% CO_2-95% air. Log-phase cells were seeded at 0.1-2 $\times$ 10^6 per 12-well, 6-well plate or 60-mm dish and were used for various experimental purposes.

Measurement of CCL20

The concentration of CCL20 in culture supernatants from HT-29 cells was measured by a previously described method (Sierro *et al,* 2001). In brief, 96-well microtiter plates (MaxiSorpTM, Nunc, Denmark) were coated with 2 μg/ml of human CCL20-specific mAb (clone 67310.111; R&D Systems) and were used to capture CCL20 in culture medium. Goat anti-human CCL20 (R&D Systems) diluted at 0.5 μg/ml was used as the detection antibody, and development was performed with alkaline phosphatase-conjugated rabbit anti-goat antibody (Sigma) diluted 1:5,000. After incubation, plates were read at 405 nm on a microplate reader (Molecular Devices Corp, Sunnyvale, CA). CCL20 concentration was calculated from a standard curve using rhCCL20 (R&D Systems). The detection threshold was 0.25 ng/ml.

RNA Isolation and RT-PCR

Human epithelial cells (5×10^6), PBMCs (5×10^6) or THP-1 cells (5×10^6) were grown in a 60-mm culture dish and were incubated for 4-24 h in a fresh medium containing stimuli as indicated. After discarding the growth medium, total RNA was isolated from the cells using easy-Blue (Intron Biotechnology, Korea) following the manufacturer's instructions. Reverse transcription (RT) of the RNA was performed using AccuPower RT Premix (Bioneer, Daejeon, Korea). The resulting first-strand DNA was amplified in a final volume of 20 μl containing RT-generated DNA (2-5 μl, 20 pmole of each primer and AccuPower PCR PreMix (Bioneer). The primers used for cDNA amplification were: 5'-ATGTGCTGTACCAAGAGTTTG-3' (sense) and 5'-TTACATGTTCTTGACTTTTTTACTGAGGAG-3' (antisense) for CCL20 (Scapini, *et al,* 2001); 5'-CGGAGTCAACGGATTTGGTCGTAT-3' (sense), 5'-AGCTTCTCCATGGTGGTGAAGAC-3' (antisense) for GAPDH (Choi *et al,* 2004). Amplification conditions were denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s for 30 cycles. The expected PCR products were 291 bp (for CCL20) and 306 bp (for GAPDH). PCR products were subjected to electrophoresis on 1.2 % agarose gel and were stained with ethidium bromide.

Real-time quantitative PCR

Real-time quantitative PCR analyses were performed using the DyNAMO SYBR Green qPCR Kit (MJ Research, Waltham, MA) in DNA Engine Opticon for continuous fluorescence detection system (MJ Research) according to the manufacturer's instructions. Briefly, 1 μl of RT product (cDNA) served as a template in a 10 μl reaction containing 10 pmol of each primer and 1$\times$ qPCR master mix. Samples were loaded into 0.2 ml thermofast low-profile white tubes and incubated in the fluorescence thermocycler (Opticon) for an initial denaturation step at 95°C for 10 min, followed by 50 cycles of amplification (95°C for 30 s, 56°C for 30 s, 72°C for 30 s). Melting curve analysis was done to characterize the dsDNA product by slowly raising the temperature (0.2°C/s) from 65°C to 95°C with fluorescence data collected at 0.2°C intervals. The levels of CCL20 mRNA normalized for GAPDH were expressed as fold change relative to the untreated controls. The fold change in gene expression was calculated using the following equation: fold change = $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = (C_{T,Target}-C_{T,GAPDH})_{time\ x}-(C_{T,Target}-C_{T,GAPDH})_{time\ 0}$, where time x is any time point and time 0 represents the 1 $\times$ expression of the target gene of untreated cells which was normalized to GAPDH (Livak *et al,* 2001).

Measurement of $[Ca^{2+}]_i$

An inverted confocal microscope was used with a $\times$63 objective (LSM 410, C-APO 63/1.2 water, Zeiss) equipped with an argon laser (457, 488, and 514 nm). For the Ca^{2+} experiments, the scanning speed was set to 1.08 s for a 512$\times$512-pixel image and the pinhole was set to achieve a full-width, half-maximum *z* resolution of approximately 1 μm. Cells were loaded for 30 min with fluo-3-AM (2 μM) at 37°C; fluorescence staining of the cells was then directly followed by visualization at 488 nm excitation with the LSM. Images were then recorded at a scanning speed of 2 or 4 s per full-size image with a two-frame average. The fluorescence signal at 488 nm excitation consisted of more than 95% from the fluo-3 emission signal. The results were expressed as the relative fluorescence intensity (RFI).

Chemotaxis assay

An *in vitro* chemotaxis assay was performed using

the 96-well chemoTx #106-5 microplate (Neuro Probe, Gaithersburg, MD) by a method described previously (Liao *et al,* 1999). Briefly, PBMCs or EBV-transformed B cells were resuspended in prewarmed RPMI 1640 containing 1% FBS and 10 mM HEPES at 2×10^6 cells/ml. rhCCL20 (1 μg/ml), control medium or basal medium of untreated, DFO-, DFO plus FC- or TNF- α-treated HT-29 cells in complete RPMI 1640 were added to the bottom wells. The plate was incubated at 37°C in 5% CO_2-95% air for 1-3 h. Following incubation, cells on top of the filter were removed and 0.5 mM cold-EDTA in Dulbecco's phosphate buffered salt solution was added for 20 min at 4°C before centrifugation to dislodge any cells on the filter's underside. The migrated cells in the bottom wells were collected, counted, and stained for flow cytometric analysis.

Flow cytometric analysis

PBMCs obtained from the above chemotaxis assay were stained with monoclonal antibodies against CD3 (FITC-conjugated) and CCR6 (PE-conjugated); the percentages of $CD3^+$ and $CCR6^+$ cells were then determined by flow cytometry on a FACSCalibur (Becton Dickson, San Jose, CA).

Results

Iron chelators induce CCL20 protein secretion and stimulate CCL20 mRNA accumulation in human IECs

In a previous report, we documented for the first time that iron chelator triggers inflammatory signals, including the production of CXC chemokine IL-8, in human IECs even in the absence of conventional immuno-stimulatory/inflammatory stimuli (Choi *et al.,*

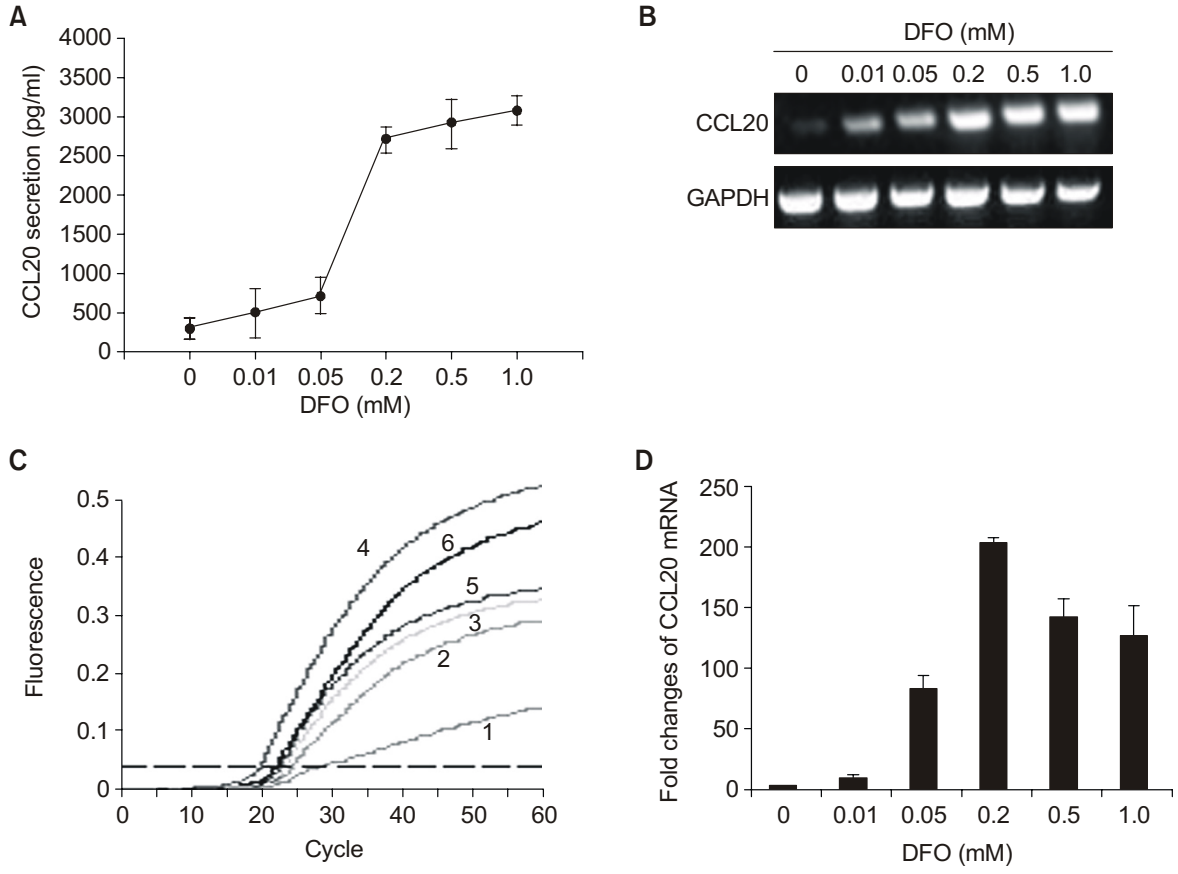


Figure 1. Iron chelator induces CCL20 production in human intestinal epithelial HT-29 cells. Cells were treated for 20 h with the indicated concentrations of DFO (0-1 mM). Levels of CCL20 secretion and mRNA were determined by ELISA (A) and semi-quantitative RT-PCR (B), respectively. (C and D) Real-time quantitative RT-PCR was performed to confirm the results of the RT-PCR where the numbers 1, 2, 3, 4, 5, and 6, respectively, are the DFO concentrations, 0.01, 0.05, 0.2, 0.5, and 1.0 mM (C). The data represented as the relative fold change of the initial CCL20 mRNA as normalized to GAPDH (D). Results are expressed as means ± SD of three independent experiments.

2004). In addition, by utilizing the cDNA microarray technique, we found that a number of both novel and known genes responded to the cellular iron availability (Choi *et al.,* 2004). Interestingly, we identified that iron chelator also induces CC chemokine MIP-3α/CCL20 in human IECs. As MIP-3α/CCL20 is known to link innate and acquired immunity by attracting immature dendritic cells and effector memory T and B cells via its receptor CCR6, this implies that iron metabolism in IECs may have a direct role in the evocation of host innate and adaptive immune responses, presumably by modulating CCL20 as well as other inflammatory mediators.

In this study, we therefore wished to examine the mechanism of CCL20 expression by iron chelator in the intestinal epithelial-type cells. The human colon epithelial HT-29 cells were cultured under the standard conditions as described in the Materials and Methods. Treatment of HT-29 cells with DFO, an iron chelator, markedly induced CCL20 secretion (Figure 1A) as well as mRNA accumulation (Figure 1B-D). The effect of DFO was concentration-dependent in the range of 0–1 mM. Maximal increase of CCL20 mRNA expression was achieved at the concentration of 0.2–1 mM as assessed by real-time quantitative PCR (Figure 1C and D). The CCL20 concentrations induced by DFO were comparable with those elicited by IL-1β (5 ng/ml) and TNF-α (10 ng/ml) and the combinations of DFO and either IL-1β or TNF-α showed additive effects on CCL20 secretion (Figure 2A). Mimosine, an iron

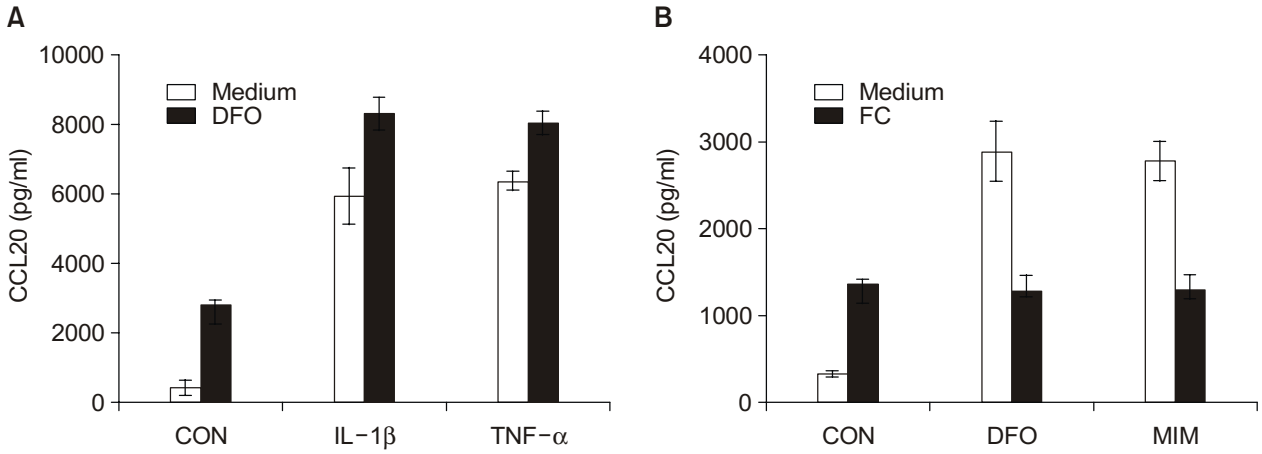


Figure 2. Iron chelator-induced CCL20 production is up-regulated by proinflammatory cytokines but decreased by the addition of iron supplement. Cells were treated for 20 h with DFO (0.2 mM), IL-1β (1 ng/ml), TNF-α (10 ng/ml) (A), FC (Fe^{3+}, 0.5 mM), mimosine (MIM, 0.2 mM) (B) or a combination thereof. The levels of CCL20 secretion were determined by ELISA. Results are expressed as means ± SD of three independent experiments.

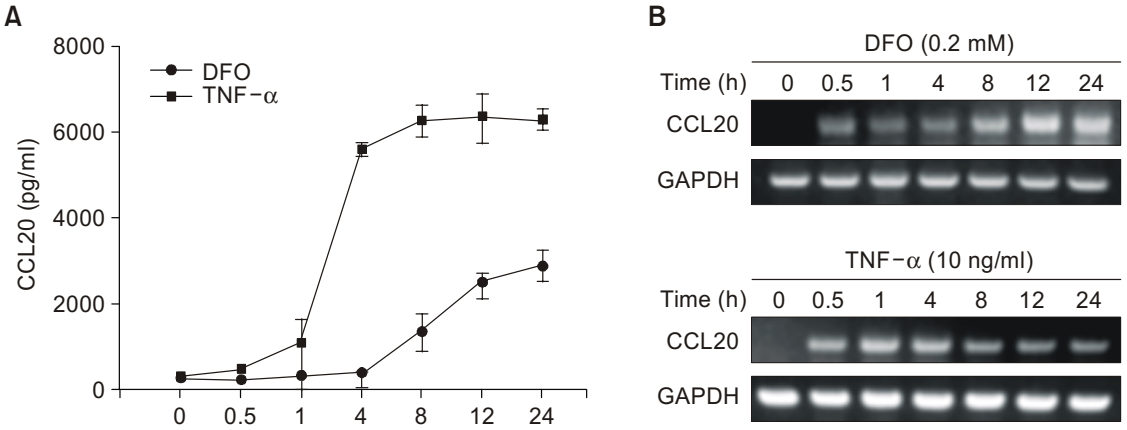


Figure 3. Iron chelator induces CCL20 protein secretion and CCL20 mRNA accumulation in HT-29 cells in a time-dependent manner. Cells were incubated with DFO (0.2 mM) or TNF-α (10 ng/ml) for the indicated time periods. Levels of CCL20 protein (A) and mRNA (B) were determined by ELISA and RT-PCR, respectively. These data are representatives of two independent experiments.

chelator structurally distinct from DFO, also induced CCL20 secretion in HT-29 cells. Conversely, the addition of FC (Fe^{3+}, 0.5 mM) prevented the production of CCL20 induced by DFO or mimosine (Figure 2B), indicating that the target of iron chelators is specific for the intracellular iron in HT-29 cells. Surprisingly, FC alone could also induce a minimal amount of CCL20 secretion in HT-29 cells (Figure 2B), suggesting that both iron deficiency and overload are involved in modulating host immunity.

Time-dependent experiments revealed that DFO slowly induces CCL20 secretion and that maximal induction is seen after 8-12 h of incubation (Figure 3A). This is different from what is observed in TNF-α-elicited CCL20 secretion where a plateau of CCL20 concentration is reached within 4 h after TNF-α treatment. We also found that the increase of CCL20 protein secretion appears to correspond to the increase of CCL20 mRNA accumulation levels (Figure 3B).

To investigate whether the induction of CCL20 by iron chelator is of broader significance for other epithelial cell lines, we used two transformed epithelial cell lines, T84 and Caco-2, and treatment was performed under identical conditions. Induction of CCL20 mRNA was also observed in T84 exposed to 0.2 mM DFO (Figure 4A). However, DFO could not induce CCL20 mRNA accumulation in Caco-2 cells, thus suggesting that the extent of responsiveness may vary depending on cell types or state of differentiation. Because CCL20 is one of the major chemokines produced by human monocyte or neutrophils in response to inflammatory stimuli (Schutyser *et al,* 2000; Scapini *et al,* 2001; Scapini *et al,* 2002; Akahoshi *et al,* 2003), we also tested whether DFO could activate monocyte-like THP-1 cells or human PBMCs to produce CCL20. As shown in Figure 4B, treatment with DFO significantly up-regulated CCL20 mRNA as well as the release of CCL20 protein in these cells (*data not shown*). Taken together, these results suggest that induction of inflammatory signals by iron chelator may have a broader relevance for the cells that are the first to face the challenge of exogenous putative pathogens, thereby initiating and maintaining inflammatory responses.

Roles of p38 kinase and extracellular signal-regulated kinase-1/2 (ERK1/2) in the stimulation of CCL20 by iron chelator

We previously found that both p38 and ERK1/2 activation crucially contribute to the iron chelator-induced IL-8 production in HT-29 cells, presumably by modulating post-transcriptional regulation of the IL-8 gene transcript (Choi *et al,* 2004). In this study, we further investigated the involvement of these two MAPK sub-family members as well as other potential signal transducers on the production of CCL20 in DFO-treated HT-29 cells; we then compared with that of TNF-α-mediated signals. DFO-induced CCL20 production was almost completely inhibited by SB202190 (20 μM), a specific p38 kinase inhibitor, and by PD98059 (20 μM) or U0126 (10 μM),

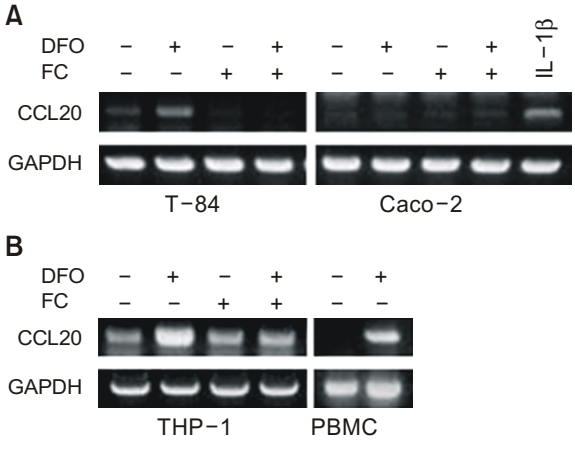


Figure 4. Iron chelator up-regulates CCL20 mRNA in human IECs as well as in THP-1 and PBMCs. Human epithelial cells, T-84 and Caco-2 (A) and THP-1 and PBMCs (B) were treated for 20 h with DFO (0.2 mM) or DFO in combination with FC (0.5 mM). For some experiments, IL-1β was treated for 20 h to reveal the positive control of CCL20 mRNA. Levels of mRNA were determined by semi-quantitative RT-PCR. These data are representatives of three independent experiments.

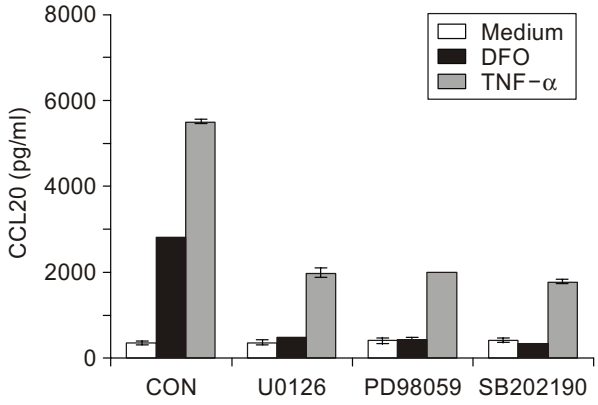
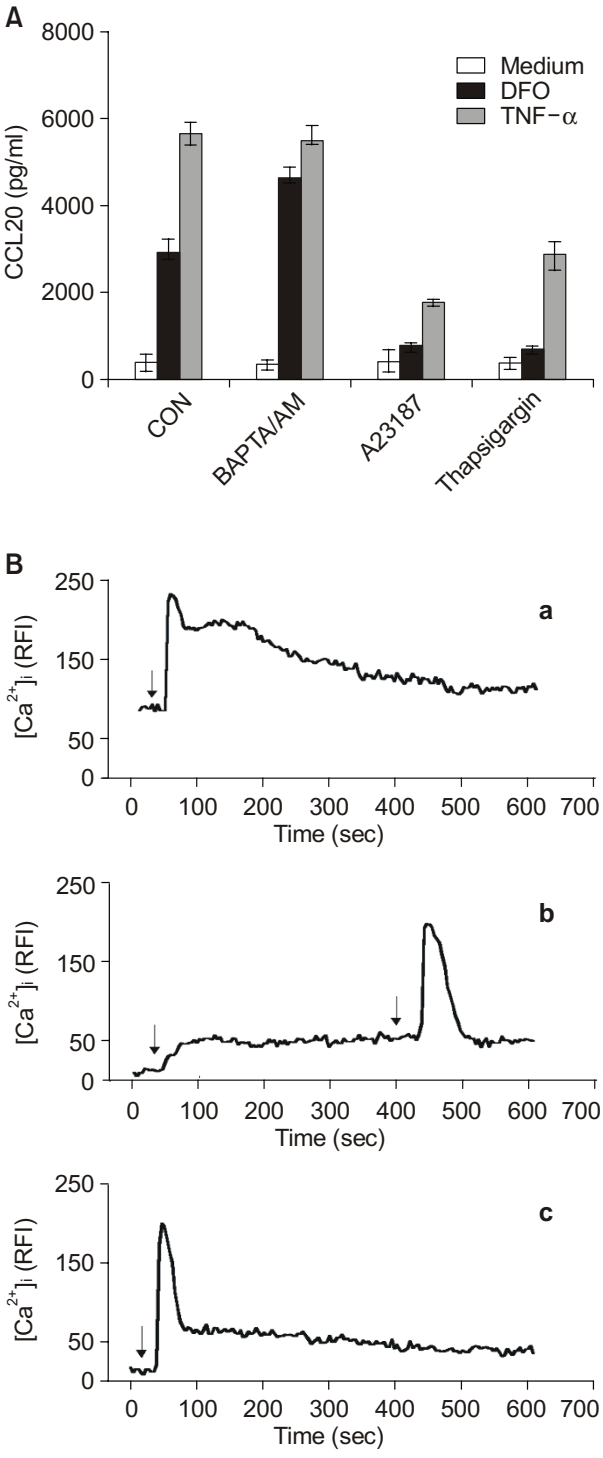


Figure 5. Effects of MAPK manipulation on CCL20 release by iron chelator in HT-29 cells. Cells were pre-treated for 1 h with or without p38 kinase inhibitor, SB202190 (20 μM), or MEK1 inhibitors, U0126 (10 μM) and PD98059 (20 μM). The cells were then further incubated for 20 h with DFO (0.2 mM) or TNF-α (10 ng/ml). Levels of CCL20 protein were determined by ELISA. These data are representative of three independent experiments.

selective ERK1/2 pathway inhibitors, thus suggesting that both p38 and ERK1/2 have pivotal roles in the induction of CCL20 by DFO (Figure 4). However, these MAPK inhibitors showed only modest effects on TNF-α-induced CCL20 production in HT-29 cells (Figure 5), thus suggesting that the signaling pathways induced by DFO are somehow distinct from that of TNF-α.



Effects of intracellular calcium modulators in the stimulation of CCL20 by iron chelator

As calcium is required for the activation of many calcium-dependent kinases, we therefore tested the effects of intracellular calcium modulators on DFO-induced CCL20 production. Interestingly, exposure to the BAPTA-AM, an intracellular calcium chelator, increased the level of CCL20 production by DFO in HT-29 cells. In contrast, exposure of HT-29 cells to A23187, a calcium ionophore, or thapsigargin, an IP_3-independent intracellular calcium releaser, significantly reduced the level of CCL20 production induced by DFO (Figure 6A). In consistent with this result, treatment of HT-29 cells with DFO drastically reduced A23187-induced intracellular Ca^{2+} mobilization (Figure 6B). Treatment with A23187 or thapsigargin also inhibited TNF-α-induced CCL20 production (Figure 5A), thus suggesting that calcium signals may negatively regulate iron chelator- or TNF-α-induced signals in terms of CCL20 production in HT-29 cells.

Roles of PKC, protein tyrosine kinase (PTK), and protein phosphatases in the stimulation of CCL20 by iron chelator

Interestingly, among three different PKC isotype inhibitors, only rottlerin, a selective PKCδ inhibitor, fully inhibited DFO-induced CCL20 production (Figure 7A). Rottlerin also inhibited TNF-α-induced CCL20 production although the degree of inhibition was smaller than that to DFO (Figure 7A), suggesting the important role of PKCδ in the induction of inflammatory signals, including CCL20 expression, in human IECs.

We next examined whether tyrosine and serine/threonine kinase activities are required for CCL20 production by DFO in HT-29 cells. Pretreatment of HT-29 cells with inhibitors of PTK, HerA (1 μg/ml) or PP2 (10 μM), almost completely blocked DFO-induc-

Figure 6. (A) Effects of calcium manipulation on CCL20 release by iron chelator in HT-29 cells. Cells were pre-treated for 1 h with or without BAPTA/AM (10 μM), A23187 (10 μM) or thapsigargin (1 μM). Then the cells were further incubated for 20 h with DFO (0.2 mM) or TNF-α (10 ng/ml). Levels of CCL20 protein were determined by ELISA. These data are representative of three independent experiments. (B) Effect of iron chelator on the change of $[Ca^{2+}]_i$ induced by calcium ionophore in HT-29 cells. Cells were treated with A23187 (10 μM) (*a*) or DFO (0.2 mM) for 400 s followed by A23187 (*b*), or pretreated with DFO for 30 min, and then treated with A23187 (*c*). Each trace is a single cell representative of three independent experiments. RFI is an abbreviation of relative fluorescence intensity.

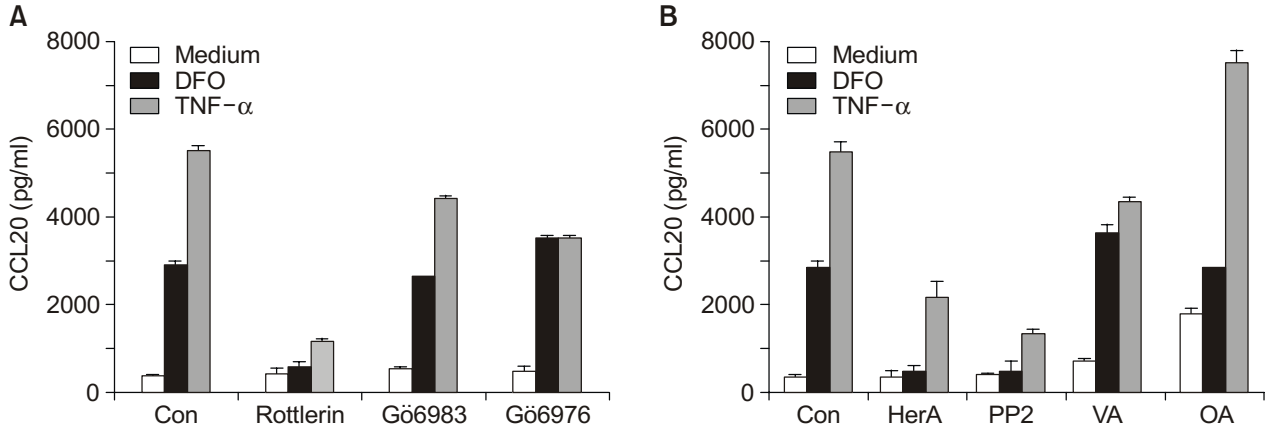


Figure 7. Effects of PKC, PTK, and protein phosphatase modulators on CCL20 release by iron chelator in HT-29 cells. Cells were pre-treated for 1 h with or without selective PKC inhibitors (12 μM of rotterlin, 100 nM of Gö6983 and Gö6976) (A), inhibitors of tyrosine kinases (1 μg/ml of HerA and 10 μM of PP2), tyrosine phosphatases (20 μM of VA) or serine/threonine phosphatases (10 nM of OA) (B). The cells were then further incubated for 20 h with DFO (0.2 mM) or TNF-α (10 ng/ml). Levels of CCL20 protein were determined by ELISA. These data are representative of three independent experiments.

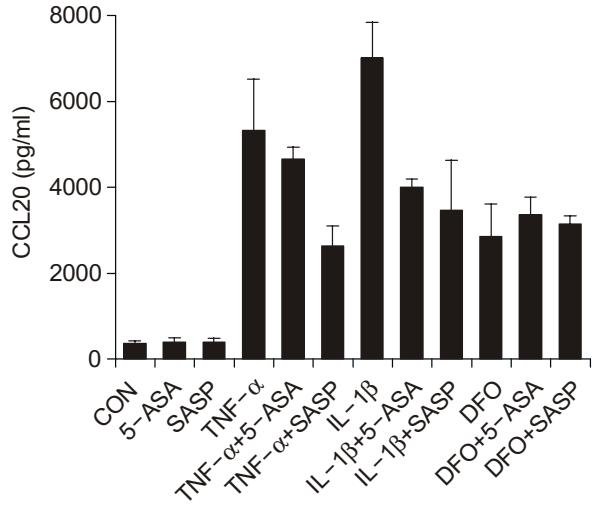


Figure 8. Effects of NF-κB inhibitors on CCL20 release by iron chelator in HT-29 cells. Cells were pre-treated for 1 h with or without 5-ASA (10 mM) or sulfasalazine (5 mM). Then the cells were further incubated for 20 h with DFO (0.2 mM), TNF-α (10 ng/ml) or IL-1β (1 ng/ml). Levels of CCL20 protein were determined by ELISA. These data are representative of three independent experiments.

ed CCL20 production (Figure 7B). On the other hand, specific inhibitors of both tyrosine phosphatase, VA (Na_3VO_4; 20 μM), and serine/threonine phosphatase, OA (10 nM), showed additive effects on DFO-induced CCL20 production (Figure 7B). Interestingly, these two phosphatase inhibitors by themselves could induce a minimal amount of CCL20 production (Figure 7B), thus suggesting that both tyrosine and serine/threonine kinase systems are required for iron chelator-induced CCL20 production in HT-29 cells.

Iron chelator induces CCL20 production *via* NF-κB-independent mechanism

In a previous report we also demonstrated that iron chelator induces IL-8 secretion via a NF-κB independent mechanism, while the responsible transcription factor is not currently determined (Choi *et al.,* 2004). Thus, we further questioned whether NF-κB inhibitors such as 5-ASA and sulfasalazine could inhibit DFO- or TNF-α-induced CCL20 production in HT-29 cells. As shown in Figure 8, both 5-ASA and sulfasalazine only significantly inhibited TNF-α-induced CCL20 production, thereby confirming that NF-κB is not involved in iron chelator-induced CCL20 production in human IECs.

Medium from DFO-treated HT-29 cells induces migration of CCR6-expressing cells

We finally determined that the basal medium obtained from iron chelator-treated human epithelial HT-29 cells can induce migration of CCR6-expressing cells. PBMCs from normal healthy donors were resuspended in prewarmed chemotaxis medium and added to the upper wells of the chemotaxis chamber. Control medium, rhCCL20 (1 μg/ml), or basal medium from DFO (0.2 mM)-, DFO plus FC (0.5 mM)-, or TNF-α (1 ng/ml)-treated or untreated for 24 h, were added to the bottom wells. As shown in Figure 9A, the PBMCs of normal healthy donors were able to migrate in response to rhCCL20. The migration was inhibited by pre-incubation of PBMCs on the upper

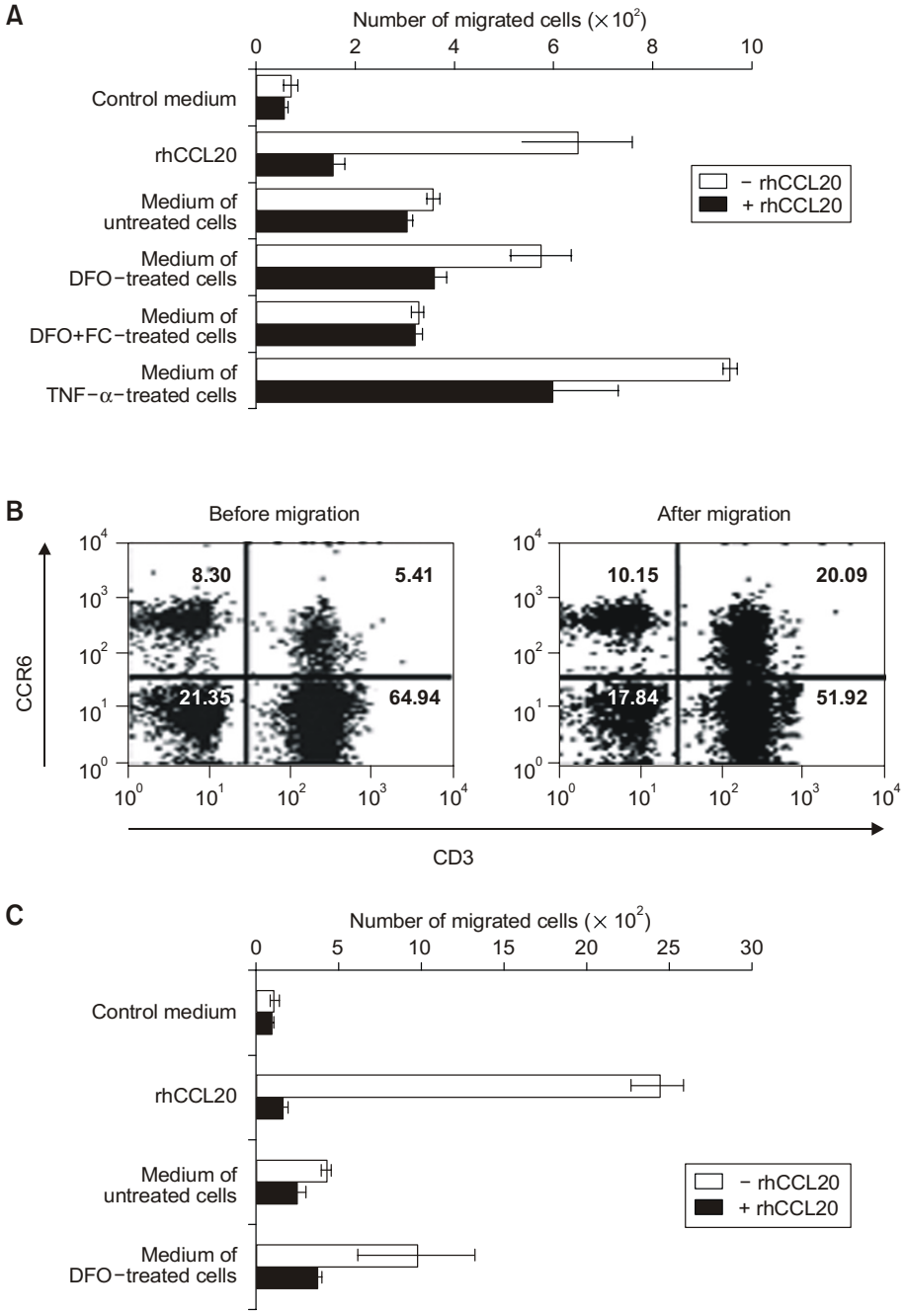


Figure 9. Medium from DFO-treated HT-29 cells triggers migration of CCR6 expressing cells. (A) Human PBMCs (2×10^6 cells/ml) were resuspended in chemotaxis medium as described in the Materials and Methods. Cells were applied to the upper wells of the chemotaxis chamber. Control medium, rhCCL20 (100 ng/ml), or basal medium of untreated, DFO-, DFO plus FC- or TNF-α-treated HT-29 cells in complete RPMI 1640 were applied to the bottom wells. When specified, rhCCL20 (100 ng/ml) (■) was mixed with cells during assay to eliminate CCL20 gradients. The results are representative of two independent experiments. (B) The migrated cells in the bottom wells of above (A) were collected, counted, and stained with CD3-FITC and CCR6-PE for flow cytometric analysis. Results are representative of two independent experiments. (C) The migration of EBV-immortalized B cells was determined as described in (A) above. Results are representative of three independent experiments.

well with the same amounts of rhCCL20. Similarly, the basal medium from DFO- or TNF-α-treated HT-29 cells was as chemotactic as rhCCL20, while the migration was slightly but significantly reduced by treatment with rhCCL20 to the upper well. A small amount of migration of PBMCs was also observed with basal medium from untreated cells, probably reflecting the constitutive secretion of CCL20 or other chemokines including IL-8, by HT-29 cells. As shown in Figure 9B, although approximately 20% of gated lymphocyte populations were $CCR6^+$ before chemotaxis, migrated cells toward the basal medium treated with DFO, revealed a higher percentage of $CCR6^+$ cells, thus suggesting that DFO-treated HT-29 cells secrete a physiologically active form of CCL20.

Since a previous report demonstrated that human B cells immortalized with EBV upregulate CCR6, after which these cells vigorously respond to the ligand of CCR6 (Nakayama *et al,* 2002), we further

examined whether the basal medium from DFO-treated HT-29 cells could efficiently support the migration of this cell line. As expected, the addition of DFO-treated basal medium significantly increased the migration of EBV-transformed B cells as compared to untreated control medium (Figure 9C). Collectively, the migration of PBMCs or EBV-immortalized B cells in response to the basal medium from DFO-treated HT-29 cells, specifically depends on CCL20 activity.

Discussion

Iron is vital for microorganisms (Weinberg, 1978; Neilands, 1981). To obtain iron from the iron-restricted environment, micro-organisms have developed many strategies. Molecules involved in iron uptake and transport may be receptors or channels (localized in the outer and inner membrane of the microbe) and siderophores (Greek for 'iron bearers'), which are high-affinity specific iron-binding molecules that are shuttled to the environment to catch iron. These compounds are secreted into the environment where they extract iron from other ligands. The siderophore-iron complex returns to the microorganism where iron is incorporated and used for replication and growth (Marx, 2002).

Iron is also important for host immunosurveillance because of its growth-promoting role for immune cells and its interference with cell-mediated immune effector pathways and cytokine activities (Laskey *et al.,* 1988; Seligman *et al.,* 1992). It has been demonstrated that iron deficiency as well as iron overload can exert subtle effects on immune status by altering the proliferation and activation of T-, B-, or NK-cells (Mainou-Fowler *et al.,* 1985; Brekelmans *et al.,*1994). In this study, we provide the evidence that bacterial siderophore DFO specifically stimulate CCL20 chemokine expression and secretion by human IECs, resulting in chemotaxis of $CCR6^+$ PBMCs and EBV-transformed B cells. Such CCL20 expression and the resulting migration of $CCR6^+$ cells may be essential for the induction of an adaptive immune response in the gut. Although several investigators have reported that both iron and iron chelators may modulate certain inflammatory mediators and regulate inflammatory processes (Weiss *et al.,* 1992; O'Brien-Ladner *et al.,*1998), our study is the first to demonstrate that iron chelator triggers mucosal adaptive immune response even in the absence of conventional immunostimulatory/ inflammatory stimuli.

The time course of the iron chelator-mediated CCL20 production in human IECs is much slower than that of TNF-α. This agrees to our previous work in which we found that DFO slowly induces IL-8 secretion in HT-29 cells, while IL-1β induces it rapidly (Choi *et al.,* 2004), suggesting that iron chelator may induce CCL20 production via different signaling systems from that of such proinflammatory mediators as IL-1β and TNF-α. Evidence of this is that iron chelator does not require NF-κB-dependent transcriptional activity to induce CCL20 production in human IECs, as proven by NF-κB inhibitor treatment (Figure 8), I-κBα degradation, ELISA-based NF-κB binding activity, and NF-κB-luciferase report gene assay (Choi *et al.,* 2004). These, coupled with the fact that the signaling systems evoked by cellular iron imbalance are largely unknown, urged us to determine which signaling pathways are predominantly involved iron chelator-induced CCL20 production.

To understand the mechanism whereby iron chelator induces CCL20 production in human IECs, we primarily utilized pharmacologic agents that could modulate intracellular signaling systems. We found that treatment with p38 or ERK inhibitor almost completely blocks DFO-induced CCL20 production, while either of these inhibitors has only minimal effects on TNF-α-induced CCL20 production in HT-29 cells, thus suggesting major roles for MAPKs in iron chelator-mediated signaling systems. However, in contrast to iron chelator, TNF-α-induced chemokine gene expression may require at least two different signaling systems, *i.e,* MAPKs and NF-κB-dependent pathways, as previous report demonstrated a complete suppression of IL-8 expression by both NF-κB and MAPK inhibitors (Bian *et al.,* 2001). In this regard, we insist that the activation of both NF-κB and p38/ERK are mediated by distinct and separate pathways, especially in human IECs, as previously demonstrated by another group (Wesselborg *et al.,* 1997).

It was surprising to note that a transient increase of intracellular calcium negatively regulates the level of CCL20 production by iron chelator in HT-29 cells. In fact, our present results are inconsistent with the previous report in which the authors demonstrated that calcium modulators A23187 and thapsigargin increase IL-8 gene expression in human T84 colonic epithelial cells (Yu *et al.,* 2001). Furthermore, it has been reported that *Helicobacter pylori*-induced IL-8 secretion is abolished by treatment with intracellular calcium chelators (BAPTA-AM and TMB-8) in human gastric epithelial cells (Nozawa *et al.,* 2002). This discrepancy is difficult to explain with the currently available knowledge, but a possible explanation may be as follows: Although calcium by itself serves as a positive signal for the induction of CCL20, it could be directly antagonistic to iron chelator. Indeed, we observed that A23187 alone slightly increases

CCL20 in HT-29 cells and significantly increases it in T84 cells (data not shown). On the other hand, we found that DFO diminishes the tentative calcium spike evoked by either A23187 or thapsigargin (Figure 6), suggesting a crosstalk between the two signals, *i.e,* calcium and iron depletion. Moreover, chelation of intracellular calcium could amplify iron chelator-induced signaling systems in terms of CCL20 production in HT-29 cells.

Although PKC is an important regulator for chemokine IL-8 expression in human IECs (Gross *et al.,* 1995; Yu *et al.,* 1998), it has been demonstrated that the synthesis of IL-8 by either TNF-α or IL-1β is independent from PKC because the PKC inhibitor staurosporin does not block the effects of these proinflammatory cytokines (Gross *et al.,* 1995). However, in the present study, rottlerin, a selective inhibitor of PKCδ, significantly inhibited iron chelator-induced CCL20 production in HT-29 cells. Rottlerin also inhibited TNF-α-induced CCL20 production although the degree of inhibition was less than that to DFO, thereby suggesting the possible role of PKCδ in the induction of inflammatory signals. However, as several reports have demonstrated that the effect of rottlerin does not always correlate with inhibition of PKCδ rmacol (Yu *et al.,* 1998; Davies *et al.,* 2000), these effects in the inhibition of DFO- or TNF-α CCL20 production require further characterizations.

HerA is a specific inhibitor of receptor *src* tyrosine kinases and has no effect on PKC or PKA (Beales *et al.,* 1997). In this study, the stimulatory effect of iron chelator was completely abolished by pretreatment with HerA, showing that PTK activity is important in signal transduction by iron chelator. As previously demonstrated (Beales *et al.,* 1997), HerA was also effective in reducing the effects of TNF-α, suggesting that both iron chelator and TNF-α share the same PTK-dependent signaling system. When PP2, an alternative *src* tyrosine kinase inhibitor, was used, a similar pattern of inhibition was seen. On the contrary, protein phosphatase inhibitors such as OA and VA not only induced CCL20 by themselves, respectively, but also slightly augmented iron chelator-mediated CCL20 production in HT-29 cells. These results are in agreement with the previous reports showing that OA is able to stimulate the accumulation of various inflammation-related proteins including TNF-α, IP-10, and IL-8 (Tebo *et al.,* 1994; Sonoda *et al.,* 1997). As both OA and VA are well documented for the induction of phosphorylation of ERK1, a member of the MAPK family (Sonoda *et al.,* 1997), it is conceivable that OA and VA may thus prolong the half-life of phosphorylated proteins (ERK1 and others) with regard to their signal transduction, thereby leading to sustained CCL20 gene activation.

It was interesting to note that the iron chelator also induces CCL20 in other cell types such as monocytes and human primary blood leukocytes. These indicate that induction of inflammatory signals by iron chleator may have a broader relevance for those cells that are the first to face the challenge of exogenous putative pathogens (Kunkel *et al.,* 1990). However, unlike our findings in the above cells, our preliminary results revealed that HUVECs show no response against iron chelator even in high concentrations (~1 mM) (data not shown). Because HUVECs are also known to induce CCL20 in response to various stimuli (Hromas *et al.,* 1997; Dieu-Nosjean *et al.,* 2000), this result implies that the effect of iron chelator is somewhat cell-type specific. Furthermore, the extent of iron chelator-responsiveness may also vary depending on the state of cellular differentiation, as evidenced by Caco-2 cells. Another interesting point found in this study is that supplementation of iron (FC) also shows a modest effect on CCL20 production in HT-29 cells, while other cell types such as THP-1 and PBMC, reveal unresponsiveness against FC. This result suggests that iron overload as well as iron deficiency may also participate in the induction of inflammatory processes, especially on the mucosal surfaces of the intestinal tracts, via distinct cellular mechanisms from iron deficiency. In strong agreement with this, a previous report demonstrated that oral iron supplements anecdotally exacerbate inflammatory bowel disease and that iron levels are elevated in the inflamed mucosa (Millar *et al.,* 2000).

Whereas intestinal epithelial cells do not produce the cytokines which are essential components of host adaptive immune responses, recent studies have shown that they do produce CCL20 in response to proinflammatory stimuli, infected enteric pathogens or bacterial products, thereby recruiting cells that are responsible for host adaptive immunity (Fujiie *et al.,* 2001; Izadpanah *et al.,* 2001; Sierro *et al.,* 2001; Nakayama *et al.,* 2002). Because the basal medium from iron chelator-treated epithelial cells is also highly chemotactic in attracting $CCR6^+$ PBMCs or EBV-immortalized B cells, our data extend the current view and suggest that rather than classical immuno-stimuli, direct chelation of host Fe by infected bacteria may also have the critical function of initiating host adaptive immunity. Based on the highly sophisticated and diverse mechanisms that micro-organisms have developed to acquire iron from their environment, it can be postulated that this element must be an extremely important microbial virulence factor. Therefore, mammals should have developed a more precise mechanism to compete with microbial invasion. This is part of the inflam-

matory response in which iron has a pivotal role in orchestrating innate and adaptive mucosal immune responses.

In summary, our experiments using IECs demonstrate that direct chelation of intracellular iron induces the expression of CCL20 mRNA as well as the release of CCL20 protein. This stimulation is independent from the NF-κB pathway but depends on other multiple signaling pathways, including MAPK (p38 and ERK), calcium, PKC, and PTK, thereby suggesting a distinct and novel mechanism of action from other known inflammatory agonists. Our results further imply, for the first time, that modulation of the iron status by infected bacteria may also contribute to the evocation of host innate and adaptive immune responses even in the absence of conventional immunostimulatory/inflammatory stimuli.

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