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Expression of the Neutrophil Chemokine KC in the Colon of Mice with Enterocolitis and by Intestinal Epithelial Cell Lines: Effects of Flora and Proinflammatory Cytokines

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IL-10 plays an important role in preventing excessive inflammation to the normal flora in the intestinal lumen. The purpose of this study was to compare the effect of normal flora on inflammation in mice in which the IL-10 gene was disrupted. IL-10 knockout mice housed in germfree conditions remained healthy while those housed in conventional conditions developed colitis after weaning, suggesting that IL-10 inhibits the adverse responses to luminal Ag. Crypt abscesses were present in virtually all of the diseased animals as evidenced by flattening of the epithelial cells and a large number of neutrophils in the lumen of the crypt. Since KC is a chemokine that is capable of recruiting neutrophils in mice, mRNA and protein for KC was measured. Increased levels of both KC mRNA and protein were detected in the colon of diseased mice. To determine whether the epithelial cells were capable of synthesizing KC and contributing to neutrophil accumulation in the crypts, a murine intestinal epithelial cell line (Mode-K) was shown to express mRNA and protein for KC. Two cytokines induced in association with colitis in these mice, TNF-α and IFN-γ, increased the expression of KC mRNA and protein in murine epithelial cells. However, IL-10 was incapable of decreasing the induction of KC, even though the cells expressed the IL-10 receptor. These results suggest that the neutrophil chemokine KC is produced by gastrointestinal epithelial cells in response to inflammatory mediators that are expressed following exposure to normal flora in animals lacking IL-10. The Journal of Immunology, 1999, 162: 2275–2280.

The host response to the massive antigenic load in the intestinal lumen is regulated by cytokines that select for protective immunity while inhibiting excessive amounts of inflammation. The appearance of chronic intestinal inflammation in IL-10 knock-out (KO) mice suggests that this cytokine inhibits adverse responses to luminal Ag (6). In fact, the colitis in these animals is associated with an increase in T cells producing TNF-α and IFN-γ (7, 8). Moreover, the administration of exogenous IL-10 (7) or helper T cell subsets that produce high levels of IL-10 can prevent or attenuate inflammation in various models of colitis in mice (9, 10).

In addition to an increase in lymphocytes, mice with colitis, including IL-10-deficient mice (7), often have increased levels of neutrophils in their intestinal mucosa. In humans with inflammatory bowel disease, one of the characteristic lesions is the presence of crypt abscesses, accumulations of neutrophils within the luminal space of the intestinal epithelial crypt. The chemokines produced by intestinal epithelial cells are believed to be critical participants in the development of these inflammatory infiltrates (11–13). Chemokines are synthesized in response to bacterial products and cytokines secreted by a wide variety of immune, inflammatory cells, as well as epithelial and endothelial cells (11, 12, 14–16). The α chemokines, such as IL-8, are potent chemoattractants for polymorphonuclear neutrophils, whereas β chemokines, such as monocyte chemoattractant protein-1, are chemoattractant for mononuclear cells. The mouse KC gene is a member of the α chemokine family originally isolated from platelet-derived growth factor-stimulated BALB/c 3T3 fibroblasts (17). KC is a homologue of the human GRO/melanoma growth-stimulatory activity family (17, 18). Although no structural homologue of IL-8 has been identified in mice, KC shares 65% sequence identity with human gro-α and shares many functional properties with IL-8 (17). For example, KC

Abbreviations used in this paper: KO, knockout; wt, wild type.
transgenic mice show a marked infiltration of neutrophils at the sites of transgene expression (19). KC also binds the IL-8R, and mice deficient in IL-8R lack neutrophil migration to sites of inflammation (20, 21). The KC gene is also induced by LPS, thrombin, double-stranded RNA (poly IC), IFN-γ, TNF-α, and IL-1α in a number of cell types, including macrophage, fibroblasts, and endothelial cells (22).

The studies described above suggest that the disruption in the regulation of the host response to microbial products can contribute to the induction of chemokines and inflammatory infiltrates in colitis. The present study examined the effect of normal flora on the development of colitis and KC expression in the IL-10-deficient mouse model by comparing these parameters in animals housed under germfree or conventional conditions. Our results suggest that expression of the neutrophil chemokine KC in the intestinal mucosa of mice with enterocolitis was regulated by the balance between proinflammatory cytokines (such as TNF-α and IFN-γ) and anti-inflammatory cytokine IL-10.

Materials and Methods

Animals

IL-10-deficient mice were generated by Kuhn et al. (6) by the insertion of neomycin resistance gene (neo) into the IL-10 gene as previously described. Heterozygous animals were provided by DNAX (Palo Alto, CA) and bred in conventional housing facilities at University of Texas Medical Branch (UTMB, Galveston, TX). Offspring were screened for the defect in the IL-10 gene by PCR of DNA isolated from blood. Briefly, 10 μl of blood was washed in 200 μl of TED buffer, and proteins were digested by heating the pellet in 40 μl of 0.1% Triton X-100 and 10 μl of 0.4 N NaOH at 95°C for 5 min. After neutralization with 1 M Tris (pH 7.5), the material is stored until use. DNA solution (10 μl) are added to a PCR mixture containing 1 sense primer (position 1523, CGG TTC AGT ATA AAA GGG GGA CC) and 2 antisense primers, the first to native IL-10 (position 1723, GTG GTC GCA GGT ATT GTC TCC CCG) and the second, to the neo insert (position 338 of neo, inserted 111 bases 3’ to the IL-10 sense primer, CCT GCG TGC AAT CCA TCT TG). The wild-type (wt, +/+ ) gene yields a product of 200 bases, while the neo primer yields a product of ~449 bases in the disrupted gene. The effect of the genetic mutation was confirmed by the absence of detectable levels of IL-10 protein by ELISA of supernatants of stimulated splenocytes (our unpublished observation).

The IL-10 homozygous (−/−), heterozygous (+/−), and wt (+/+ ) mice were housed at UTMB in conventional conditions free of specific pathogen-free system SAS (SAS Institute, Cary, NC). All procedures were approved by the Animal Care and Utilization Committees at the respective institutions.

Evaluation of Disease

Clinical Evaluation. Animals (~160) from the breeding colony were examined on a daily basis over a period of 18 mo. Illness was described as mild, moderate, and severe according to the following criteria: mild, no obvious change in behavior or temperament, occasional diarrhea or blood on stool or around anus; moderate, animal obviously sick, blood in stool and around anus, may have a rectal prolapse; severe, animals appeared depressed and cachectic, evidence of bloody diarrhea, and a rectal prolapse was present. If animals did not die suddenly, they were euthanized when their body weight was ~20% less than the controls.

Evaluation at necropsy. The gross pathology was scored as mild, moderate or severe at the time of euthanasia: mild, a single area of grossly obvious involvement in the large intestine, no obvious cachexia, formed stool; moderate, ~1 cm of involvement in one or more areas of the large intestine, some formed stool; severe, blood in the stool, evidence of cachexia. Histopathological analyses were performed on approximately half of the affected mice and controls. Control or IL-10-deficient mice were sacrificed at weaning and when moderately ill. Tissues were fixed in 10% buffered formalin and embedded in paraffin blocks. Subsequently, 4–6-μm thick sections were cut and stained with hematoxylin and eosin before being examined by light microscopy. Each mouse was scored using a modification of a technique that was previously described (23). Briefly, one point was given for each of the following criteria: number of areas of involvement (lower colon, upper colon, cecum), epithelial disruption, branching of crypts, epithelial dysplasia, and crypt abscesses.

Epithelial cell lines

The murine intestinal epithelial cell line Mode-K (24) was maintained as adherent cells, at 37°C in a humidified atmosphere of 5% CO2 in air in DMEM media containing 1% nonessential amino acids, 0.055% (w/v) sodium pyruvate, 10% FCS, 4 mM glutamine (complete DMEM medium). The cells were detached three times a week, using a solution of 0.25% trypsin in 0.5 M EDTA (Sigma, St. Louis, MO). For analysis of the regulation of KC mRNA and protein, the Mode-K were treated with varying doses of recombinant murine TNF-α (PharmMingen, San Diego, CA), IFN-γ (Boehringer Mannheim, Mannheim, Germany), or IL-10 (kindly provided by Schering-Plough Research Institute, Kenilworth, NJ). For data presented in this report, a single, optimal concentration of these cytokines was used as noted in Results. The supernatants or the cells were collected after the culture for 4 h in vitro and assayed for KC mRNA or protein as described below.

RNA preparation and analysis

RNA was extracted from the epithelial cell line (Mode-K) or the intestinal tissue from control or IL-10 KO mice using RNAzol (Biotech Laboratories, Houston, TX), following specifications from the manufacturer. Total RNA (20 μg) per sample was separated by electrophoresis on 1.4% agarose-formaldehyde gel and transferred to a Hybond N membrane (Amersham, Buckinghamshire, England) and hybridized with random primed 32P-labeled cDNA probes (106 cpm/ml hybridization solution) for KC and G3PDH (Clontech Laboratories, Palo Alto, CA) for 16 h at 65°C. The filter was then submitted to high stringency washes (at room temperature by 2× SSC, 0.1% SDS at three times and at 55°C by 0.1× SSC, 0.1% SDS at three times), dried, and exposed to Kodak XAR-5 x-ray films at −70°C.

KC protein preparation and analysis

For KC protein determination, colonic tissue from IL-10−/−, +/−, and +/+ mice in the germfree or conventional conditions were homogenized in 2 ml of PBS (pH 7.4) (for 1 min in ultraturrax homogenizer) and supernatants obtained by centrifugation (1800 rpm for 10 min.), and frozen at −80°C in polypropylene tubes until assayed. KC protein was detected by a commercially available ELISA kit (R&D Systems, Minneapolis, MN) and used according to manufacturer’s instructions. The reaction was terminated by adding 100 μl of stop solution to each well with 30 min, and absorbance was measured at a wave length of 450 nm. Tissue KC levels were expressed relative to total protein, which was measured using a commercial protein quantification kit (Pierce, Rockford, IL).

Statistical analysis

Levels of cytokine expression are presented as the mean ± SEM. These data were analyzed by the Student’s t test and considered significant if p values were <0.05. Comparison of mortality rates was evaluated using Kaplan-Meier techniques followed by log-rank tests. All calculations were performed using a microcomputer implementation of the statistical software system SAS (SAS Institute, Cary, NC).

Results

IL-10 KO mice show increased morbidity and mortality when exposed to normal flora

Age-matched homozygous, IL-10-deficient (−/−) mice were combined into groups, housed under conventional or germfree conditions, and their mortality was compared with littermates with a normal phenotype (+/+ or +/−) under identical housing. As shown in Fig. 1, 100% of the male IL-10-deficient mice housed in conventional conditions died by 4 mo. During this time, the female mice displayed mild to moderate disease (data not shown), but 50% of them were still alive after 4 mo. All of the male or female control mice (+/+ or +/−) housed in conventional conditions were alive and healthy for up to a year while both IL-10 KO male and female mice were also clinically normal when they were housed in germfree conditions (up to 8 mo).

Tissues collected from IL-10-deficient mice housed in germfree conditions and conventionally housed IL-10-deficient mice were examined histologically and compared with those from normal.
controls. As shown in Fig. 2, marked inflammation was observed in the IL-10-deficient animals that were housed in conventional conditions. Abnormal changes included: 1) thickening of the mucosa, 2) disorganization and hyperplasia of crypts, 3) epithelial erosion/ulceration, 4) accumulation of bacteria, 5) marked infiltration of leukocytes, and 6) crypt abscess formation. None of these inflammatory changes were detected in the IL-10-deficient animals housed in the germfree conditions or in the wt control mice housed in conventional conditions.

**KC mRNA expression by IL-10 KO mice varies with age and gender**

Other studies suggest that crypt abscesses form during chronic intestinal inflammation due to the production of epithelial chemokines that are stimulated by bacteria and/or cytokines (11–13). In view of the presence of these abscesses in virtually all of the IL-10-deficient mice with colitis, samples of colonic tissue were screened for the presence of KC, a murine neutrophil chemoattractant. The levels of KC mRNA and protein in the colonic tissue of IL-10 KO mice differed based on the presence of colitis, which was affected by age, gender, and housing conditions. For example, by 12 wk, the male IL-10 KO mice housed in conventional conditions demonstrated high levels of KC expression compared with the similarly aged female mice or weaning mice (Fig. 3). The frequency of mice expressing KC and the magnitude of expression were affected by age. KC mRNA expression was also compared in samples of colonic tissue from animals housed in conventional or germfree conditions. As shown in Fig. 4, KC mRNA was virtually undetectable in IL-10 KO mice housed in germfree conditions. The presence or absence of KC generally correlated with the presence or absence of colitis and the neutrophil infiltrate detected in the colonic tissues.

**Association of KC mRNA and protein levels in IL-10 KO mice exposed to normal flora**

To confirm that KC mRNA expression correlated with the presence of KC protein, samples of colonic tissue were homogenized and assayed by ELISA. As shown in Fig. 5, KC protein was not detected in healthy control mice. In contrast, a significant induction of KC was detected in colonic tissue from the IL-10 KO mice housed in conventional conditions. Moreover, the level of KC was lower in the cohorts (i.e., the females) that showed a more mild form of the colitis as scored based on clinical appearance and degree of inflammation detected by histology (mean score and SEM: 4.2 ± 0.5 for males, 2.1 ± 0.3 for females at 4 mo of age; *p* < 0.05).

**The regulation of KC expression in epithelial cells by proinflammatory cytokines**

Several studies have suggested that crypt abscesses in human disease likely reflect the expression of chemokines in epithelial cells (11–13). Thus, using the murine intestinal epithelial cell line Mode-K, we examined the expression of KC mRNA and protein. As shown in Fig. 6, Mode-K cells constitutively expressed low levels of KC mRNA and protein. As intestinal T cells from IL-10-deficient mice produce IFN-γ and TNF-α during inflammation (7, 8), KC expression was measured in Mode-K cells after exposure to these cytokines. TNF-α mediated a dose-dependent induction of KC mRNA and protein (data not shown). Subsequently a
suboptimal dose of TNF-α (50 ng/ml) was combined with other cytokines to evaluate cytokine interactions. Although IFN-γ (100 U/ml) had little ability to increase KC mRNA and protein, it markedly enhanced KC expression when combined with TNF-α (Fig. 6).

Previous studies from our laboratory have shown that freshly isolated or cultured intestinal epithelial cells express the IL-10R (Campbell et al., manuscript in preparation), so the effects of IL-10 on KC expression were examined. In contrast to other cells in which IL-10 can inhibit inflammatory responses, IL-10 did not affect the constitutive or cytokine-induced expression of KC mRNA or protein in the epithelial cell line tested (Fig. 6). The lack of an inhibitory effect by the IL-10 was not due to a lack of bioactivity as this preparation did induce proliferation of an IL-10 dependent cell line (data not shown). These findings suggest that the effect of IL-10 in vivo may be targeted toward cells other than the epithelium.

Discussion

The development of spontaneous colitis in humans and animal models has provided compelling evidence for the importance of immune regulation in controlling local inflammatory responses. For example, in the original description of colitis in IL-10-deficient...
mice, housing the animals in specific pathogen-free conditions partially decreased the inflammation (6), presumably due to a decrease in bacterial load in the lumen. This report provides definitive evidence that conventional flora are sufficient to trigger the development of colitis in IL-10-deficient mice, thus reaffirming the importance of IL-10 in local immune regulation. Moreover, this report demonstrates that the neutrophil infiltrate and crypt abscess formation that is observed in the inflamed colon are associated with the increased expression of the murine neutrophil chemokine, KC.

The presence of crypt abscesses is a very reliable marker of some forms of inflammatory bowel disease in humans. These accumulations of neutrophils have been implicated in the perpetuation of chronic inflammation as they can disrupt the epithelial barrier function. For example, migration of neutrophils across the epithelium has been shown to increase permeability (25). Moreover, luminal neutrophils may also migrate back into the lamina propria (26, 27). Thus, antigenic material may come in contact more easily with immune/inflammatory cells if the epithelial barrier is compromised, or if the material is engulfed by neutrophils that then return to the lamina propria. The ubiquitous presence of crypt abscesses in this model will permit experimental manipulations directed toward disrupting neutrophil migration to evaluate the role of the neutrophil in the pathogenesis of this disease.

One of the explanations for the accumulation of neutrophils in human inflammatory bowel disease is the presence of IL-8 (28, 29) that contributes to the recruitment and activation of neutrophils. KC is a chemokine in mice that is similarly capable of recruiting and activating murine neutrophils (19). Whereas KC mRNA and protein were absent from normal mouse intestine, the expression of KC was induced significantly as colitis developed. Furthermore, KC was expressed at higher levels in the groups with the worst disease, namely the older, male mice.

In view of the presence of neutrophils adjacent to the crypt epithelium, the expression of KC by an epithelial cell line was tested. The data show that both KC mRNA and protein are produced by epithelial cells. In addition, cytokines that have previously been reported to be increased in IL-10-deficient mice, including TNF-α and IFN-γ, could boost the expression of both KC mRNA and protein by the epithelial cell line. TNF-α was able to increase KC expression directly, whereas IFN-γ increased KC only when given in combination with TNF-α. Interestingly, KC expression by the epithelial cells was not regulated by IL-10, even though these cells express IL-10R (Campbell et al., manuscript in preparation). Thus, it is possible that the production of KC by epithelial cells in mice is regulated indirectly by IL-10, perhaps through its effects on the production of TNF-α and IFN-γ by adjacent immune or inflammatory cells.

Although the precise mechanisms for the pathogenesis of spontaneous colitis have yet to be elucidated, several factors may play a role. The complexity of the immunological interactions is supported by the fact that modification of various immunological molecules through genetic engineering can predispose an animal to develop colitis. In addition to IL-10, mice deficient in IL-2, TGF-β, class II MHC molecules, and the α/β TCRs, all develop colitis (30). Other reports have suggested that genetic diversity also greatly affects the development of disease (31). While specific species of bacteria may be sufficient to cause colitis in susceptible mice, what has otherwise been considered as “normal flora” is sufficient to trigger colitis in some of these animal models (23, 30).

The data in this report show that the intensity of the inflammation, as evidenced by the level of KC and the clinical presentation, are also affected by the age and gender of the mice. Several other factors may account for the effect of age on the manifestation of colitis. For example, inflammation may be delayed initially since nursing animals receive substantial levels of IL-10 from the colostrum (32). In these experiments, mice were reared by dams that were heterozygous for the IL-10 gene mutation. In addition, the intestinal flora changes dramatically in young mice, and it may take some time to colonize the animals with the appropriate species to trigger colitis. In addition, older mice may have more severe forms of colitis since they have been exposed to the bacterial Ags for longer periods of time. The differences in the development of colitis among reports may be accounted for by the fact that their respective animal facilities vary in their flora and housing practices. Thus, several factors affecting colonization may contribute to the intensity of the disease or the rate at which it develops.

The observation that male mice succumbed more quickly to the complications associated with the colitis was unexpected. The male and female animals in these studies were all age-matched and housed in adjacent cages in the same room, suggesting that real gender effects might exist. Given that males were housed next to females, it is possible that sexual behavior triggered aggression and stress in the males that exacerbated the disease. Alternatively, hormones themselves may also modify the expression of spontaneous colitis in these animals. Additional studies will be required to discriminate between these possibilities.

In summary, these studies document that the development of colitis in IL-10-deficient mice is dependent on the presence of microbial flora. In addition to flora, inflammation is regulated by both age and gender. The detection of KC production may provide a reliable marker to objectively evaluate the development of inflammation in these models of colitis and the role of the neutrophil in the pathogenesis of colitis.

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References


