Interleukin-10 and nerve growth factor have reciprocal upregulatory effects on intestinal epithelial cells

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Ma, Donglai, Danielle Wolvers, Andrzej M. Stanisz, and John Bienenstock. Interleukin-10 and nerve growth factor have reciprocal upregulatory effects on intestinal epithelial cells. Am J Physiol Regul Integr Comp Physiol 284: R1323–R1329, 2003. First published January 23, 2003; 10.1152/ajpregu.00756.2002.—The intestinal mucosa is in a constant state of controlled inflammation, but the processes whereby this occurs are poorly understood. The aims of this study were to look at the role of IL-10 and nerve growth factor (NGF) in intestinal epithelial cell regulation. The human colon epithelial cell lines T84, HT-29, and CACO-2 were used. RT-PCR, flow cytometry analysis, and immunohistochemistry were applied to measure the cytokine changes in epithelial cells induced by recombinant cholera toxin and its B subunit, IL-10, and NGF. Cholera toxin B subunit caused selective dose-dependent increased mRNA for IL-10 in T84 cells and the protein in T84, HT-29, and CACO-2 cells. IL-10 dose dependently selectively increased NGF mRNA in T84 cells and intracellular protein synthesis in all three epithelial cell lines. The effect of NGF was reciprocal, selective, and dose dependent because it increased mRNA for IL-10 and IL-10 synthesis. Our results suggest that the epithelium may actively participate in downregulation through innate mechanisms involving IL-10 and NGF. The reciprocal interaction suggests for the first time that NGF may be involved in local downregulation by mucosal epithelium and thus may play a potent protective role in response to injury, by prevention of undue inflammation.

cholera toxin; cholera toxin B subunit

INTERESTNATIONAL MUCOSAL TISSUE is minimally inflamed and is in a constant state of downregulation under normal physiological circumstances, but the processes whereby this occurs are poorly understood. Recent work has suggested that nonpathogenic Salmonella organisms inhibit the synthesis of IL-8 by blocking a nuclear transcription degradation system (37). Little information exists as to other possible mechanisms of downregulation of local mucosal inflammation.

We have shown that a component of cholera toxin (CTX), cholera toxin B subunit (CTB), a potent factor that when conjugated to a variety of antigens promotes oral tolerance (8), upregulated and caused epithelial synthesis of IL-10, a significant immunodownregulatory molecule. This effect was selective, and no effects were seen on IL-6, IL-8, or transforming growth factor (TGF)-β1. IL-10 dose dependently selectively upregulated synthesis of a neurotrophin, nerve growth factor (NGF), which in a variety of model systems appears to promote tissue repair and protection (28, 36, 41). Furthermore, we showed that NGF itself, which is found in relatively large amounts in normal saliva and other external secretions and is constitutively made in intestinal epithelial cells (31), selectively and reciprocally upregulated intestinal IL-10 synthesis. These findings raise the possibility that the epithelium can be induced to synthesize downregulatory molecules such as IL-10 and thus play a significant physiological role in mucosal homeostasis.

MATERIALS AND METHODS

Reagents

Recombinant CTX and recombinant CTB were gifts from Prof. T. R. Hirst, Univ. of Bristol. The following reagents were obtained from the companies listed: DMEM, Eagle’s minimum essential medium with Earle’s salts, F12 nutrient mixture (Ham) medium, McCoy’s medium, L-glutamine, non-essential amino acids, penicillin-streptomycin (GIBCO BRL, Grand Island, NY); IL-10, diethyl pyrocarbonate, Brefeldin A, saponin (Sigma, Oakville, ON); Caltag Fixation Medium (Reagent A) and Permeabilization Medium (Reagent B) (Caltag Laboratories, Burlingame, CA); phycoerythrin (PE)-conjugated rat anti-human IL-10 monoclonal antibody (mAb) and PE-conjugated R3–34 (rat IgG1 isotype) (PharMingen, Mississauga, ON); affinity-purified FITC-conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA); and rabbit IgG (DAKO Diagnostic Canada, Mississauga, ON). All primers (Table 1) were synthesized by the Institute for Molecular Biology and Biotechnology, McMaster University.

NGF was isolated from male mouse submandibular glands according to the methods of Petrides and Shooter (39). Rabbit anti-NGF was a generous gift from Dr. M. Coughlin, McMaster Univ. (7), and was affinity purified by passage over a CN-Br Sepharose 4B (Amersham Pharmacia Biotech, Piscataway, NJ) column coupled to NGF. Antibody was eluted with 0.5 M acetic acid, and the eluate was neutralized, concentrated, and tested for activity (48).

Cells

The T84, HT-29, and CACO-2 cell lines (human colon epithelial cells) were obtained from the American Type Cul-
Table 1. Oligonucleotide primers and PCR product sizes used in experiments

<table>
<thead>
<tr>
<th>mRNA Species</th>
<th>Sequences (5’–3’)</th>
<th>Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5’TGA AGG TCG GAG TCA AGC GAT TGG GT</td>
<td>983</td>
</tr>
<tr>
<td>IL-6</td>
<td>5’AAA TTC GGT AGA TCC TCG AC</td>
<td>295</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>5’ACC ACA TCA GAG CTC GGA</td>
<td>500</td>
</tr>
<tr>
<td>IL-8</td>
<td>5’CTG GGC GTG GCT CTC TTG GCA GGA</td>
<td>395</td>
</tr>
<tr>
<td>IL-10</td>
<td>5’GCC TAA CAT GCT TCG AGA</td>
<td>204</td>
</tr>
<tr>
<td>NGF</td>
<td>5’TGA AAA GGG GGG ACT CGG TC</td>
<td>167</td>
</tr>
</tbody>
</table>

TGF-β1, transforming growth factor-β1; NGF, nerve growth factor.

Flow Cytometry Analysis of Intracellular Cytokines

The methods used followed those described elsewhere (2), but with some changes. Based on RT-PCR results, optimal concentrations of CTB, NGF, or IL-10 were added, respectively, to the culture and incubated with T84 cells. Flow cytometry was used to analyze the expression of target transcripts.

Immunohistochemistry Staining of Intracellular IL-10 and NGF

To look at the effect of CTB, NGF, and IL-10 on all three cell lines, immunohistochemistry staining was also used to view intracellular IL-10 and NGF. Each of the cell lines was grown on coverslips in six-well plates for 3 days at a starting concentration of 10^6/ml. Cells were incubated with CTB or NGF for 72 h or with IL-10 for 48 h, followed by incubation
IL-10 and NGF Have Reciprocal Effects

Cytokine mRNA Expressions

CTX and CTB Induce Differential Patterns of Cytokine mRNA Expressions

We determined by RT-PCR the effects of different concentrations (0.1, 1, 10, 100, and 1,000 ng/ml) for 2, 6, and 12 h of CTX and CTB on mRNA transcripts for IL-6, IL-8, IL-10, TGF-β1, and NGF. CTX increased IL-8 and IL-6 mRNA in a dose-dependent manner. The optimal effect was found at 100 ng/ml (P < 0.01), and the maximal effects occurred at 6 h with both cytokines (data not shown). No significant effects were found on cytokine induction by CTX and CTB and a selective induction of IL-10 by CTB.

IL-10 Selectively Enhances NGF mRNA Expression

Because CTB differentially increased IL-10 mRNA expression, we wondered what the effect of IL-10 on cytokine induction would be on epithelial cells. Different concentrations (1, 10, and 100 ng/ml) of IL-10 were incubated with T84 cells for 0.5, 1, 2, and 6 h. IL-10 had no effect on IL-6, IL-8, TGF-β1, and IL-10 (data not shown) at any point in time. However, IL-10 significantly enhanced NGF mRNA expression in a dose-dependent manner (optimal dose was 10 ng/ml). Within 0.5 h incubation, NGF mRNA transcripts increased; the maximal effect occurred at 1 h (P < 0.05) and had returned to baseline by 6 h (Fig. 3).

NGF Selectively Increases IL-10 mRNA Expressions

Because IL-10 selectively increased NGF mRNA transcripts, we examined whether NGF had any effect on cytokine induction. NGF had no effect at different concentrations (10, 100, and 1,000 ng/ml) for 1, 2, and 6 h on IL-6, IL-8, TGF-β1, or NGF at any time point. However, NGF selectively and significantly increased IL-10 mRNA transcripts in a dose-dependent fashion (Fig. 4) after 1 and 2 h of incubation. The maximal response occurred at 1 h at an optimal concentration of 100 ng/ml. These results showed that NGF and IL-10 have a reciprocal relationship, and both effects occur at early stages of stimulation.

Statistics

Experimental results are expressed as means ± SE. Statistical analyses were performed with unpaired, two-tailed Student’s t-tests, or one-way ANOVA followed by Newman-Keuls test for comparing all pairs of groups (GraphPad PRISM, version 2.0). P < 0.05 was considered statistically significant, and n represents the number of experiments performed.

RESULTS

CTX and CTB Induce Differential Patterns of Cytokine mRNA Expressions

We determined by RT-PCR the effects of different concentrations (0.1, 1, 10, 100, and 1,000 ng/ml) for 2, 6, and 12 h of CTX and CTB on mRNA transcripts for IL-6, IL-8, IL-10, TGF-β1, and NGF. CTX increased IL-8 and IL-6 mRNA in a dose-dependent manner. The optimal effect was found at 100 ng/ml (P < 0.01), and the maximal effects occurred at 6 h with both cytokines (data not shown). No significant effects were found on IL-10, TGF-β1, and NGF mRNA transcripts with CTX at any concentration or time of incubation. CTB had no apparent effects on IL-6 (Fig. 1) or IL-8, TGF-β1, or NGF (data not shown) but significantly increased the mRNA transcripts of IL-10 in a dose-dependent fashion (Fig. 2) at 6 h. These results showed differential cytokine induction by CTX and CTB and a selective induction of IL-10 by CTB.

![Fig. 1. Cholera toxin (CTX) and cholera toxin B subunit (CTB) effects on IL-6 mRNA expression in T84 cells. At a concentration of 100 ng/ml, CTX increased IL-6 mRNA expression significantly, but CTB had no effect on IL-6 mRNA compared with medium controls. Results were expressed as relative intensity of each IL-6 mRNA expression to GAPDH calculated for the respective densities. **P < 0.01 compared with media control group, n = 3.](Image 89x574 to 269x734)

![Fig. 2. CTB effect on IL-10 mRNA expression in T84 cells. Increasing concentrations of CTB (0.1–1,000 ng/ml) were added to T84 cells for 6 h. CTB dose dependently upregulated IL-10 mRNA expression. *P < 0.05, **P < 0.01 compared with media control group, #P < 0.05 compared with CTB 10 ng/ml group, n = 3.](Image 353x114 to 533x290)
Increased intracellular IL-10 staining was seen in confocal microscopy in all three cell lines after incubation with CTB and NGF under exactly the same conditions as were used and represented above for the flow cytometry experiments. Similarly, when all three cell lines were incubated with IL-10, confocal microscopy revealed increased intracellular staining for NGF, which paralleled the observations already made by the cytometry. Because these experiments were confirmatory of results obtained by flow cytometry, examples of images obtained are not presented.

**DISCUSSION**

The intestinal mucosa is in a constant state of controlled inflammation. In recent years it has come to be recognized that the epithelium is an active participant in the maintenance of the integrity of the mucosal barrier, through a variety of physiological mechanisms mediated largely by molecules that it synthesizes and secretes (3, 13, 18, 21, 29). On interaction with pathogenic organisms, epithelial cells synthesize and secrete proinflammatory cytokines such as IL-6 and IL-8 (22, 45) and upregulate chemokine receptors (14) so as to promote the immigration of neutrophils and guide their orderly progression onto the cell surface (17).

The intestinal epithelium can synthesize a large number of cytokines, which include IL-1, TNF-α, TGF-β, granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein 1 (MCP1), IL-6, and IL-8 (3), but it has generally been assumed that IL-10 is not constitutively synthesized by T84 cells (15).

Our results the cholera holotoxin caused upregulation of mRNA for IL-6 and IL-8 but had no effect on IL-10. It was therefore surprising that the recombinant CTB caused selective IL-10 upregulation with no evidence for increases in mRNA for TGF-β1, IL-6, IL-8, or, for that matter, NGF.

IL-10 is a potent anti-inflammatory agent that can also suppress both Th1 and Th2 type inflammatory

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**Fig. 3.** Time course of IL-10 effect (10 ng/ml) on nerve growth factor (NGF) mRNA expression. NGF mRNA was constitutively expressed in T84 cells at low level at all time points. NGF mRNA was upregulated by IL-10 as early as 0.5 h of incubation but restored to medium control level after 6 h. The maximal effect of IL-10 on NGF mRNA expression occurred at 1 h. Results were expressed as percentage of NGF mRNA relative intensity of IL-10 treated to medium controls. *P < 0.05 compared with 0.5-h group, n = 3.

**Fig. 4.** NGF effect on IL-10 mRNA expression in T84 cells. Different concentrations (10–1,000 ng/ml) of NGF were added to the culture for 1 h. NGF increased IL-10 mRNA transcripts in a dose-dependent manner, and 100 ng/ml was optimal for the effect. *P < 0.05, **P < 0.01 compared with media control group, #P < 0.05 compared with NGF 10 ng/ml group, n = 3.
responses (44). It has been shown to be an important determinant factor in the development of colitis in experimental models, because transgenic IL-10 knockout mice spontaneously develop colitis if housed under conventional conditions (26). Feeding of Lactococcus lactis, engineered to secrete biologically active IL-10, to transgenic IL-10 knockout animals prevented the onset of colitis (43). Recently, IL-10 has been shown to cause synthesis of chemokine decoy receptors (10). This cytokine is generally thought to be an important but not essential molecule in the mediation of so-called oral tolerance. Indeed, the two molecules thought to be most important in this respect are TGF-β and IL-10 (16). Because the most potent way of inducing oral tolerance appears to be via conjugation of protein and other antigens to the CTB (9), our observations that IL-10 is upregulated by CTB may be particularly significant especially because IL-10 promotes the growth and differentiation of a T cell regulatory subset that itself makes IL-10 (19). CTB causes its effect by binding to GM1 ganglioside on the cell surface, and it is highly relevant that other gangliosides have also been shown to induce IL-10 in human T-cells (23). It is not known whether the selective IL-10 induction shown in our experiments follows this or other pathways. However, it is known that liglation of CD1d, a nonclassical major histocompatibility complex (MHC) molecule expressed on the cell surface of intestinal epithelium, causes mRNA for IL-10 to increase in T84 cells (6). Furthermore, evidence for secretion of functionally bioactive IL-10 after CD1d ligation was also obtained. Receptors for IL-10 are expressed on epithelial cells (12), and through interaction with these receptors, IL-10 can block the effects of interferon-γ such as expression of MHC II and interruption of barrier integrity (32).

We were surprised to find that IL-10 in a dose-dependent fashion caused upregulation and synthesis of NGF. Neither holotoxin nor CTB caused this effect. The IL-10 effect itself was selective, because it did not cause upregulation of TGF-β1, IL-6, IL-8, or IL-10 itself. Additionally, NGF caused selective upregulation of IL-10 with no effect on TGF-β1, IL-6, IL-8, or NGF. It is pertinent, then, that astrocytes incubated with IL-10 upregulated the synthesis of NGF (4).

Very little information exists as to the production of NGF by intestinal epithelium (49). In our experiments, NGF was constitutively synthesized in low amounts by human T84 cells. NGF is essential for the growth and differentiation of many nerves, both in the peripheral and central nervous system (31). It is made by a variety of structural cell types such as keratinocytes, fibroblasts, and glial cells and also by a host of inflamma-
ory and immune cells that include mast cells (30), eosinophils (42), dendritic and Langerhans cells (46), and T-cells (27), especially Th2. It is found in large amounts in external secretions, especially those of the submandibular glands. There is no information as to the extent of degradation of NGF that occurs in transit through the intestine.

NGF has both pro- and anti-inflammatory properties (11). It is a potent mast cell degranulator in association with lysophosphatidylserine (38), has a significant mastopoitetic effect (34), promotes colony growth of basophils and eosinophils (35), and synergizes with IL-5 and GM-CSF (47) in this function. It has a potent anti-apoptotic effect on mast cells (25), eosinophils (20), and neutrophils (24). On the other hand, NGF has significant protective effects in a variety of situations and enhances repair processes. Thus it causes healing of human corneal ulceration (28), is a potent promoter of skin wound healing in both normal and diabetic animals (36), and has a highly anti-inflammatory effect in inhibiting the onset of experimental autoimmune encephalomyelitis (40). It has also been shown to prevent carrageenan-induced inflammation (1). Perhaps more importantly for the present study, it is involved in protection against the inflammation induced by trinitrobenzene sulfonic acid in a murine model of colitis (41). We have previously shown (33) that while NGF promotes the upregulation of synthesis of IL-6 by mast cells, it downregulates production of the proinflammatory cytokine TNF-α through increase in synthesis and secretion of PGE2. This may explain one of the mechanisms of its actions as an anti-inflammatory agent, the other being the upregulation of IL-10.

Bush et al. (5) using transgenic mice, which expressed the herpes simplex virus thymidine kinase gene coupled to a glial promoter, showed that administration of the antiviral drug ganciclovir caused selective enteroglial ablation in the jejunum and ileum. Consequently, there was patchy degeneration of neurons in this region, loss of integrity of the mucosa, and severe inflammatory bowel disease. Glial cells are a major local contributor of NGF to nerves and surrounding tissue. Thus there is evidence for the role of nerves in regulation of the integrity of the mucosa, and it is a high likelihood that neurotrophins are involved in that process.

In conclusion, while CTX did not affect NGF or IL-10 synthesis by epithelial cells, CTB selectively upregulated IL-10 (Fig. 6). NGF, a component of normal saliva, and itself synthesized by intestinal epithelial cells, selectively upregulated the synthesis of IL-10 and was in turn selectively upregulated by IL-10. Thus NGF may directly promote local downregulation of inflammation or act indirectly through upregulation of IL-10. This autacoid system may be worthy of further study with respect to innate mechanisms of homeostasis and defense.

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