Proinflammatory Th2 Cytokines Induce Production of Thymic Stromal Lymphopoietin in Human Colonic Epithelial Cells

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Abstract

Purpose Thymic stromal lymphopoietin (TSLP) is released by intestinal epithelial cells (IECs), and TSLP-conditioned dendritic cells appear to be involved in immune homeostasis of intestine and immunoglobulin A (IgA) class-switching in the physiological condition. In contrast, TSLP activates dendritic cells to induce strong T-cell proliferation and is involved in inflammatory T helper (Th) 2 responses in human allergic diseases. However, it is not clear how TSLP production by IECs is regulated in ulcerative colitis (UC), which appears to involve inflammatory Th2 responses. The aim of this study is to examine how TSLP production by IECs is regulated in ulcerative colitis.

Results We show here that expression of TSLP was enhanced in mucosal lesions from UC patients in which inflammatory Th2 cytokine production was predominant. In addition, using a human colonic epithelial cell line, we demonstrated that a combination of tumor necrosis factor-alpha (TNF-α) and interleukin-4 (IL-4) induced TSLP expression and that TSLP expression by TNF-α + IL-4 was further enhanced by either Toll-like receptor 3 ligand or interferon (IFN)-γ.

Conclusions Taken together, as in human allergic diseases, an inflammatory Th2 condition in the mucosal lesions of UC patients may trigger increased TSLP expression by IECs, resulting in exacerbation of UC.

Keywords TSLP · Ulcerative colitis · Interleukin-4 · Tumor necrosis factor-α · Interferon-γ · Toll-like receptor 3 ligand

Introduction

Intestinal epithelial cells (IECs) play a crucial role in immune homeostasis of the intestine. IECs primarily...
provide a physical barrier against commensal and pathogenic microorganisms in the intestine, whereas these microorganisms and/or their products stimulate IECs to produce a variety of cytokines and chemokines [1–3]. The stimulated IECs prime immature dendritic cells (DCs) to differentiate into functionally distinct mature DCs, resulting in qualitatively distinct responsiveness to commensal and pathogenic bacterial species [4–6]. In the physiological condition, the intestinal immune system protects against infection while steady-state DCs inhibit the development of destructive inflammatory responses to the normal microbiota. In the disease process of inflammatory bowel disease (IBD), dysregulated production of inflammatory cytokines and chemokines by epithelial cells may initiate and exacerbate IBD. However, it is not well understood how IEC-producing cytokines are regulated in the pathological condition in the disease process.

Thymic stromal lymphopoietin (TSLP) is released by IECs, and TSLP-conditioned DCs induce noninflammatory Th helper (Th) 2-type responses and produce a proliferation-inducing ligand (APRIL). This results in enhancement of IgA2 class switching by IECs in the physiological condition [7–9]. However, blood myeloid DCs activated by TSLP induce strong T-cell proliferation, and primed CD4+ T-cells differentiate into inflammatory Th2 cells. These cells produce the proallergic cytokines interleukin (IL)-4, IL-5, IL-13, and tumor necrosis factor-α (TNF-α), while downregulating IL-10 and interferon (IFN)-γ [10, 11]. Human TSLP is highly expressed by keratinocytes of atopic dermatitis and inflamed bronchial epithelial cells as well as mast cells [10, 12]. TSLP expression is increased in asthmatic airways and correlates with expression of Th2-attracting chemokines and disease severity [12, 13]. These data suggest that increased TSLP expression is involved in inflammatory Th2 responses in human allergic diseases. Although Rimoldi et al. [7] observed an alteration of TSLP production by IECs in Crohn’s disease, it is not clear how TSLP production by IECs is regulated in the pathological condition in ulcerative colitis (UC), which appears to involve inflammatory Th2 responses. In this study, we examine how TSLP production by IECs is regulated in the pathological condition in the disease process.

Materials and Methods

Colonic Mucosa Samples

This study was approved by the Institutional Review Board for Human Research of the Graduate School of Medicine, Kyoto University. Mucosal samples were obtained from inflamed mucosa in surgically resected specimens of the colon from seven patients with UC (male/female: 3/4, mean age 34.4 years, range 15–70 years). Samples of normal controls were taken from 11 patients with colonic adenocarcinoma (male/female: 9/2, mean age 66.1 years, range 51–85 years), in whom absence of inflammation was histopathologically confirmed. In the experiment for Fig. 2, we used samples of mucosa from biopsy specimens of uninfamed mucosa and of inflamed lesions from five UC patients. Samples were immediately frozen for immunohistology or RNA extraction and stored at −80°C until use.

Caco2 Cell Culture

Upon 80% confluence of the human colon cancer cell line passage 20–30, Caco-2, the cells were trypsinized (Trypsin-EDTA, Gibco, Tastrup, Denmark), reseeded at 6.0 × 10⁵ cells per well in 12-well plates, and maintained in Dulbecco’s modified Eagle medium (Gibco BRL, Grand Island, NY) supplemented with 50 mM HEPES, 10% (v/v) heat-inactivated fetal calf serum (Sigma, St. Louis, MO), penicillin G, and streptomycin (Gibco). Six hours after seeding, cells were stimulated for 48 h in the presence of TNF-α (10–100 ng/ml, R&D Systems, Minneapolis, MN), with or without IL-4 (10–100 ng/ml, R&D), IFN-γ (10–100 ng/ml, R&D), IL-17 (10–100 ng/ml, R&D), 25 µg/ml of poly(I:C) (InvivoGen, San Diego, CA), 1 µg/ml lipopolysaccharide (LPS) (Sigma, St Louis, MO), 10 ng/ml flagellin (InvivoGen), 5 µg/ml E. coli RNA/LyoVec (InvivoGen) or 15 µg/ml CpG oligonucleotide, ODN1826 (InvivoGen).

Real-Time Quantitative Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

Real-time quantitative RT-PCR was performed as described previously [14]. Colonic mucosal samples and Caco2 cells were frozen in RNAlater (Qiagen, Valencia, CA) and stored at −80°C until use. Total RNA was extracted using an RNeasy mini kit (Qiagen) and treated with DNase I (Qiagen) according to the manufacturer’s instructions. Single-strand complementary DNA (cDNA) was synthesized with SuperScript™ II reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time quantitative reactions were performed with an ABI Prism 7300 detection system (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Values are expressed as arbitrary units relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The following primers were used: GAPDH: 5′-CCACATCGGTCAGACACCAT-3′ and 5′-GGCAACAATATCCACCTTTACCAGAGT-3′; IL-4: 5′-ACACAGACCTGGAAGAACGCG-3′ and 5′-ATATCGGCTGGTTTCCC-3′; IL-8: 5′-GGCAAGCTTCCATGATTGTT-3′ and 5′-CTTGGCAAAACTGCACCTTCA-3′; IL-10: 5′-CTACGGCGCTTGCATGATT-3′ and 5′-CACGGCTTGGCTTGGTTTTT-3′;
IFN-γ: 5′-GAAAAGCCTCAATTACGTGGATGAG-3′ and 5′-GCAACGCTCAATGTTTGCAAGTG-3′; TNF-α: 5′-ATCTGTCACCCCACTGGAG-3′ and 5′-GGGTTTGTCATCTGTGAG-3′; TSLP: 5′-CCAGGCTACTAATTATTCGGTAACTG-3′ and 5′-GTTCAGCCATCACTTGGATGAG-3′; TLR3: 5′-TGACTGAACTCCATCTCATGTCC-3′ and 5′-GCCACAATCCTTTGTAATGTG-3′; TLR4: 5′-CTGCAGGGTACCATGTTGGA-3′ and 5′-CCAACTCTCCTTGGAG-3′; TLR5: 5′-TCGTCAGGATTGTGCTGATG-3′ and 5′-TGCGCTAACCACCTTGAAGAGA-3′; TLR9: 5′-CACCCTCAACTTCACCTTGGA-3′ and 5′-GTCACGGTCACCAGGTGTG-3′.

Immunohistochemistry

Immunohistological staining was performed as described previously [11, 14, 15]. In brief, 6-μm sections were cut from tissue blocks of frozen colonic mucosal samples and mounted onto glass slides. In the case of Caco2 cells, we cultured cells on camber glass slides, washed three times to remove culture media. The sections were air-dried for 30 min, fixed in acetone for 5 min, and blocked with phosphate-buffered saline containing 10% nonfat dried milk for 30 min. After using an Avidin/Biotin Blocking kit (Vector Laboratories, Burlingame, CA), the sections were stained with anti-human TSLP Abs for 1 h, followed by staining using a Vectastain Elite ABC kit (Vector Laboratories) according to the manufacturer’s instructions. After the final wash, the slides were mounted by Vectashield (Vector Laboratories, Burlingame, CA) and examined under fluorescence microscope.

Statistical Analysis

Statistical significance (P < 0.05) between groups was determined by unpaired Student’s t-test. All data are shown as the mean ± standard deviation (SD).

Results

Expression of TSLP Is Enhanced in Mucosal Lesions from UC Patients but Not in Normal Colonic Mucosa

To evaluate the expression of TSLP in mucosal lesions from UC patients, we first measured the expression levels of messenger RNA (mRNA) encoding TSLP. We found increased expression of TSLP in mucosal lesions from UC patients compared with those from normal subjects (Fig. 1a). To confirm this finding, frozen sections of mucosal lesions from UC patients were stained with anti-TSLP antibodies. As shown in Fig. 1b, immunoglobulin isotype control antibodies did not produce any positive staining (Fig. 1b i and ii), and there was no detectable immunostaining for TSLP in normal colonic mucosa (Fig. 1b iii). However, we found strong anti-TSLP staining of epithelial cells in the inflamed colonic mucosa from UC patients (Fig. 1b iv). Interestingly, in comparison with un-inflamed colonic mucosa from UC patients, TSLP expression was strongly increased in inflamed mucosal lesions.
from the same patients (Fig. 2b). These data suggest that expression of TSLP is enhanced only in inflamed mucosal lesions of UC patients, implying that inflammation of mucosal lesions in UC patients may trigger enhanced TSLP expression.

Human TSLP was highly expressed by inflamed bronchial epithelial cells in asthmatic lung [12]. In addition, using an in vitro culture system, TSLP expression was significantly upregulated by stimulation with proinflammatory cytokines and Th2 cytokines in human bronchial epithelial cells [16–18]. To investigate the contributions of cytokines to increased expression of TSLP in mucosal lesions from UC patients, we first performed real-time quantitative RT-PCR analysis of five cytokines: IL-4, IL-8, IL-10, IFN-γ, and TNF-α. We found that expression of a Th2 cytokine, IL-4, but not IL-10 was increased in mucosal lesions from UC patients (Fig. 3). Unlike the inflammatory cytokines IL-8 or IFN-γ, expression of TNF-α was significantly increased in mucosal lesions from UC patients compared with normal subjects (Fig. 3). Thus, IL-4 and TNF-α may contribute to the enhanced expression of TSLP in inflamed colonic mucosa of the UC patients.

A Combination of TNF-α and IL-4 Induces TSLP Expression in Human Colonic Epithelial Cells

To test whether IL-4, TNF-α or the combination of IL-4 and TNF-α can induce enhanced expression of TSLP in mucosal epithelial cells, a human colonic epithelial cell line comprised of Caco2 cells was cultured for 48 h with various concentrations of TNF-α, IL-4, IFN-γ, Th17 cytokines or a combination of these cytokines. Expression of a nuclear factor kappa-B (NF-κB)-dependent inflammatory cytokine, IL-8, was induced by TNF-α. Induced expression by TNF-α was further upregulated with a combination of IFN-γ but not IL-4 (Fig. 4a–c lower panels). Although expression of TSLP appears to depend on NF-κB activation, TSLP expression was strongly induced only by a combination of TNF-α and IL-4 (Fig. 4a–c). In contrast, the expression of TSLP was not upregulated by combinations of TNF-α + IL-17 or TNF-α + IL-22, or IL-4 alone (Fig. 4d and data not shown). These data suggest that TNF-α with a combination of IL-4 but not IFN-γ or Th17 cytokines induces TSLP production in human colonic epithelial cells and that regulation of TSLP expression is different from that of the inflammatory cytokine IL-8.
IFN-\(\gamma\) Enhances TNF-\(\alpha\) + IL-4-Induced TSLP Production in Human Colonic Epithelial Cells

Ozawa et al. [19] recently reported that IFN-\(\gamma\) inhibited LPS- and poly(I:C)-induced TSLP production in synovial fibroblasts, whereas Bogiatzi et al. [20] showed that IFN-\(\gamma\) did not have any effect on TNF-\(\alpha\) + IL-4-induced TSLP production in human skin explants. Because the expression of IFN-\(\gamma\) was not significantly, but only slightly increased in mucosal lesions from UC patients in comparison with those from normal subjects (Fig. 3), we examined whether IFN-\(\gamma\) had an effect on TNF-\(\alpha\)+IL-4-induced TSLP production in human colonic epithelial cells. Caco2 cells were cultured with various concentrations of IFN-\(\gamma\) with a combination of IL-4 and TNF-\(\alpha\). Interestingly, enhancement of TSLP expression by a combination of IL-4 and TNF-\(\alpha\) was detectable when we used 0.1 ng/ml of IFN-\(\gamma\); it reached a maximal level at 1.0 ng/ml of IFN-\(\gamma\) and was reduced at 10 ng/ml (Fig. 5a). These data suggest that enhancement of TSLP expression depends on the concentration of IFN-\(\gamma\) and may occur only within a narrow window of IFN-\(\gamma\) concentration.

To confirm increased expression of TSLP at the protein level, fixed Caco2 cells were stained with anti-TSLP antibodies. As shown in Fig. 5b, immunoglobulin isotype control antibodies did not produce any positive staining (Fig. 5b\(i\) and \(ii\)), and there was no immunostaining for TSLP in nonstimulated Caco2 cells (Fig. 5b\(iii\)). However, we found increased staining of Caco2 cells stimulated with IL-4, TNF-\(\alpha\), and IFN-\(\gamma\) (Fig. 5b\(iv\)). These data suggest that expression of TSLP is enhanced at the protein level in colonic epithelial cells.

Toll-Like Receptor 3-Ligand (TLR3-L) also Enhances TSLP Expression by TNF-\(\alpha\) + IL-4 in Human Colonic Epithelial Cells

Bronchial epithelial cells express several Toll-like receptors (TLRs); virus-derived double-stranded RNA activates bronchial epithelial cells through TLR3 to produce proinflammatory mediators, including TSLP [17]. We further examined whether TLR ligand stimulation had an effect on TNF-\(\alpha\)+IL-4-induced TSLP production in human colonic epithelial cells. Caco2 cells were cultured with various

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**Fig. 4** Expressions of TSLP and IL-8 in colonic epithelial cells. A human colonic epithelial cell line comprised of Caco2 cells was cultured for 48 h at various concentrations (10, 33, 100 ng/ml) of TNF-\(\alpha\) (a) or combinations of TNF-\(\alpha\) with IL-4 (b), IFN-\(\gamma\) (c) or IL-17 (d), or medium alone. Cells were used for real-time quantitative RT-PCR analyses for TSLP and IL-8 mRNA expressions. Data represent one of five independent experiments.
TLR ligands with or without a combination of IL-4 and TNF-α. Using real-time quantitative RT-PCR, we found that TLR3, TLR4, TLR5, TLR7/8, and TLR9 ligand agonist poly(I:C), LPS, flagellin, LyoVec or CpGODN induced the increase of expression of their own receptor genes, respectively. Moreover, although the stimulation of IL-4 + TNF-α did not affect any TLR gene expressions, it only enhanced poly(I:C)-induced TLR3 gene expression in colonic epithelial cells (Fig. 6). As shown in bronchial epithelial cells, TLR3-ligand agonist poly(I:C) induced enhanced TSLP expression in human colonic epithelial cells (Fig. 7). TSLP expression was also significantly enhanced by a combination of IL-4 and TNF-α. In addition, IL-4 + TNF-α-induced TSLP gene expression in colonic epithelial cells was further enhanced by stimulation with poly(I:C), but not with LPS, flagellin, LyoVec or CpGODN (Fig. 7). These data suggest that IL-4 + TNF-α had synergistic enhancing action only with TLR3 ligand on expression of their own receptor genes and that this action resulted in enhancement of TSLP gene expression in colonic epithelial cells.

Discussion

In the present study, we showed that expression of TSLP is enhanced in mucosal lesions from UC patients. In addition, using a human colonic epithelial cell line, we demonstrated that a combination of TNF-α and IL-4 induced TSLP expression and that the TSLP expression by TNF-α + IL-4 was further enhanced by either TLR3-L or IFN-γ.

We showed that expression of TSLP was enhanced in mucosal lesions from UC patients in which inflammatory Th2 cytokine production was predominant. Although it is premature to call UC a Th2-T-cell-mediated disease [21, 22], several recent findings suggest that excessive production of Th2 cytokines (including IL-4, IL-5, and IL-13) is associated with inflammation of UC lesions [23–25]. Excessive production of Th2 cytokines is also associated with production of an inflammatory cytokine, TNF-α, in inflamed mucosa [25]. Therefore, it is likely that excessive inflammatory Th2 cytokines affect cytokine production of IECs in UC patients. In addition, epithelial-cell-producing cytokines play a crucial role not only in maintaining the immune homeostasis of the mucosa but also in the disease process of chronic inflammation of the mucosa [1–3]. Thus, not only in human allergic diseases of the mucosa and skin, but also in inflamed mucosa from UC patients, increased levels of TSLP induced by inflammatory Th2 cytokines may be involved in exacerbating inflammation.

Although in this study, expression of IFN-γ was not significantly but slightly increased in mucosal lesions from UC patients in comparison with those from normal subjects (Fig. 3), a recent study has shown that, like TNF-α, IFN-γ production is also increased in inflamed mucosa of UC patients [25]. Emerging evidence suggests that commensal microorganisms drive mucosal inflammation of inflammatory bowel diseases [21, 22] and that IECs are stimulated by commensal microorganisms and/or their products to produce a variety of cytokines, resulting in predominant Th1 responses, including IFN-γ production [1–3]. Thus, microorganism-derived stimulation mediates IFN-γ...
production in the mucosa, and IFN-γ production may drive further production of TSLP in UC lesions in which inflammatory Th2 responses are predominant.

In this study, we showed that TSLP expression by TNF-α + IL-4 is enhanced by TLR3-L. In the lung, exacerbation of asthmatic inflammation is often triggered by infection of airway-targeting viruses, such as rhinovirus, coronavirus, influenza virus, respiratory syncytial virus, and adenovirus [26–28]. Mucosal epithelial cells express several TLRs and virus-derived double-stranded RNA, and TLR3-L activates epithelial cells to produce TSLP [17, 29, 30]. In addition, cytomegalovirus infection is an important exacerbating factor in patients with UC [31–33]. Thus, miscellaneous unknown viruses may be further involved in exacerbation of UC through excessive TSLP production.

TNF-α induces NF-κB activation, and induction of both IL-8 and TSLP depends on NF-κB [16, 34]. In this study, TNF-α + IL-4 induced enhanced TSLP expression but not IL-8, whereas TNF-α + IFN-γ induced enhancement of TNF-α-induced IL-8 expression but not TSLP in colonic epithelial cells (Fig. 4). TLR3-L induces TSLP expression in airway epithelial cells through NF-κB activation [17]. IL-4 enhances TLR3-L-dependent TSLP expression through STAT6 activation [17]. In addition, previous studies have demonstrated synergy between NF-κB and STAT6 in the induction of CCL11 (eotaxin-1) in response to TNF-α, and IL-4/IL-13 mediated by a composite response element in the eotaxin promoter [35]. Because TSLP promoter also contains NF-κB binding sites and putative STAT6 binding sites [16, 17], these composite response elements in the promoter of TSLP but not IL-8 may only trigger the enhancement of TSLP expression by TNF-α and IL-4.
In this study, IFN-γ and TLR3-L further enhanced TSLP expression induced by TNF-α and IL-4 (Figs. 5, 7). Because TNF-α and TLR3-L induce NF-κB activation [34], TLR3-L-induced enhancement of TSLP by TNF-α + IL-4 in colonic epithelial cells may be mediated by further activation of NF-κB. However, it is still unclear how IFN-γ enhances TSLP expression induced by TNF-α and IL-4. Ozawa et al. [19] recently reported that IFN-γ inhibited LPS- and poly(I:C)-induced TSLP production in synovial fibroblasts, whereas Bogiatzi et al. [20] showed that IFN-γ did not have any effect on TNF-α + IL-4-induced TSLP production in human skin explants. Regulation of TSLP expression by IFN-γ may differ in different cell types.

In conclusion, we demonstrated that TSLP is enhanced in mucosal lesions from UC patients and that a combination of inflammatory cytokine and Th2 cytokine induces enhancement of TSLP expression of IECs. Moreover, IFN-γ and TLR3-L further enhance TSLP expression. Chronic inflammation in the mucosal lesions of UC patients, which contains a mixed Th2 and Th1 condition and is often complicated with viral infections, may synergistically trigger disease exacerbation of UC through enhanced TSLP expression.

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