Research Communication

Regulated Spatial Distribution of Cyclooxygenases and Lipoxygenases in Crohn's Ulcer

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Background and Aims. Arachidonic acid metabolism actively participates in the initiation, climaxing, and resolution phases of inflammation, and its close connection with inflammatory bowel diseases has been only recently discovered. We aimed to clarify the role of different arachidonic pathways and the interrelationships between them in Crohn's disease. *Methods*. Seventeen specimens of Crohn's disease dated between 2003/1/1 and 2005/1/1 were collected and underwent immunohistochemical analyses with cylcooxygenase 1, cyclooxygenase 2, 5-lipoxygenase, and 15-lipoxygenase-1 antibodies. *Results*. (1) The spatial distribution of the three leading enzymes in arachidonic acid pathway—cyclooxygenase 2, 5-lipoxygenase, and 15-lipoxygenase-1—followed sequential arrangement in Crohn's ulcer: neutrophils highly expressing 5-lipoxygenase were in the utmost surface which bordered the band of cyclooxygenase-2 expression that is located just beneath it, and in the lower layers and below the granulation region were eosinophils carrying 15-lipoxygeanse-1. (2) Cyclooxygenase-2 and 15-Lipoxygenase-1-positive cells formed two barrier-like structures that possibly inhibited neutrophil infiltration. *Conclusion*. The regulated distribution indicated coordinated interplay between inflammatory cells and parenchymal cells, between arachidonic acid pathways, and between innate and adaptive immunity; and the barrier-like structures indicated protective roles for cyclooxygenase 2 and 15-Lipoxygenase-1 in Crohn's disease.

INTRODUCTION

Crohn's disease (CD) is an inflammatory bowel disease (IBD) characterized by transmural, segmental, and typically granulomatous inflammation of the gut, with alternating remitting and relapsing phases in its clinical course. It is generally believed that dysregulated immune response to luminal bacteria plays a causative role in its pathogenesis. New research is elucidating genetic factors in the development of CD, among which the discovery of the Nod2 gene is especially exciting [1, 2]. However, despite these encouraging findings and the initial success of antitumor necrosis factor-alpha therapies, definitive cure for this disease is still lacking.

In the meantime, advancement in the field of arachidonic acid metabolism and the rapidly expanding database on the versatility of cyclooxygenase (COX) and lipoxygenase (LOX) enzymes both in physiologic and pathologic conditions, and in particular the two-faced COX2 enzyme, which not only serves as a sensitive index for acute inflammation under various inflammatory circumstances and in a variety of cells and tissues, but also has prohealing and antiinflammatory functions [3], led us to suspect pivotal roles for COX2 in CD's clinical fluctuation between active and inactive phases. Besides, recent research on the lipoxygenase pathway has also

yielded interesting results, including the finding that lipoxins may serve as potent "endogenous stop signals" in inflammation in vivo [4, 5]. Along this line, some authors went on to suggest the existence of a switch from proinflammatory 5-LO pathway to proresolution 15-LO pathway in arachidonic acid metabolism as a beneficial counterregulation in inflammation [5]. Most excitingly, recent studies have clearly shown a close relationship between arachidonic acid metabolism and intestinal inflammation, in which researchers demonstrated dramatic therapeutic effects of lipoxin analogs on experimental IBD [6, 7]. All of these led to our study on expressions of cyclooxygenases and lipoxygenases in CD, an attempt to clarify specific components of arachidonic acid pathways that actually function in CD in vivo, and the interrelationships between them, in the hope that these data would eventually result in a better understanding of CD's pathogenesis and hence more effective treatments.

MATERIALS AND METHODS

We used specimens randomly chosen from an archival tissue database in Jinling Hospital dated between 2003/1/1 to 2005/1/1 comprising of 17 Crohn's disease and 11 control

intestinal specimens, which had been fixed in 10% buffered formalin and embedded in paraffin. The diagnosis of CD was based on well-rounded consideration of history, endoscopic evaluation, histologic findings, and adjuvant laboratory tests. The CD specimens were from patients who underwent enteroctomy for CD-associated intestinal fistulas or other serious complications at the Department of General Surgery, Jinling Hospital. The anatomic localizations of the 17 Crohn's lesions were as follows: nine distal ileums, six ileum-colon anastomoses, and two colons. Among the eleven control specimens, five colon tissues were from uninvolved resection margin of colectomy in patients of colon cancer and the six ileum specimens were either obtained by biopsy of intestinal issues more than 15 centimeters upstream the ileum fistula or from uninvolved resection margin of ileuctomy in patients of intestinal fistula caused by acute mesenteric embolism. The CD patients were between 14 and 56 years of age (mean $\pm$ SD, 32.9 $\pm$ 12.6 years); 8 were males and 9 were females.

Specimens were sliced into $3\,\mu$m sections and mounted on silane-coated slides. The slides were deparaffinized in xylene and dehydrated through graded alcohols. The deparaffinized sections were autoclaved in 10 mmol/L of citrate-buffered saline solution (pH 6.0) for 20 minutes for antigen retrieval. Endogenous peroxidase was blocked by placing sections into 3% hydrogen peroxidase in methanol for 20 minutes. After a rinse in phosphate-buffered saline solution (PBS), nonspecific binding was blocked for 20 minutes with 4% fetal bovine serum, 0.1% sodium azide, 0.1% Triton X-100, 0.3 mol/L sodium chloride, and 5% nonfat skim milk. The slides were incubated overnight in a moist chamber at 4°C with primary COX1 monoclonal antibody, COX2 monoclonal antibody, 5-lipoxygenase polyclonal antiserum, and 15-lipoxygenase-1 polyclonal antiserum (Cayman Chemical, Ann Arbor, MI, USA) diluted 1 : 200, 1 : 200, 1 : 300, and 1 : 600, respectively, in Common Antibody Diluent (Bio-Genex, San Rarron, CA, USA). Selected slides were also incubated with primary CD68, Vimentin, CK-pan, monoclonal antibodies (Maixin_Bio, Fuzhou, China). After 3 rinses in PBS, the slides were incubated with biotin-conjugated second antibody (Maixin_Bio, Fuzhou, China) for 30 minutes at 37°C. After another 3 rinses, the slides were incubated with streptavidin-peroxidase (Maixin_Bio, Fuzhou, China) for 30 minutes at 37°C. 3-3'-diaininobenzidine was then applied as a chromogen. After rinse, the slides were counterstained with Mayer's hematoxylin, dehydrated, cleared, and coverslipped. Negative controls were established by replacing primary antibody with the aforementioned Common Antibody Diluent.

The immunostaining was evaluated independently by two pathologists blind to the experiment design, analyzing the intensity, area, and pattern of immunostaining. In presence of a discrepancy, a consensus was reached after further evaluation. The staining was graded on a scale of five grades: 1 = negative; 2 = suspiciously positive; 3 = weakly positive; 4 = moderately positive; 5 = strongly positive.

The SPSS software (SPSS/PC+ 10.0, Chicago, Ill, USA) and independent-samples T test were used to compare expressions of the four enzymes in CD's lesion with those in control tissues and P values of less than .05 and .01 were considered statistically significant and very significant, respectively.

RESULTS

Immunohistochemical staining with anti-COX-1, anti-COX-2, anti-5-LO, and anti-15-LO-1 antibodies

The most notable feature of cyclooxygenase expression in CD specimens was the marked immunostaining of both COX1 and COX2 in Crohn's ulcer. COX1 was mainly expressed in microvessels and some fibroblast-like cells in the surface of the ulcerated region, while COX2 expression was restricted to the fibroblast-like cells in the surface of Crohn's ulcer. Subsequent immunostaining experiments in serial slides showed that these COX2-positive cells also expressed Vimentin, but not CKpan or CD68, so they might be of the mesenchymal origin. Sporadic COX1 expressions were also seen in endocrine cells in intestinal crypts and some of the lamina propria mononuclear cells, whereas intestinal epithelia also expressed COX2 to varied degrees. The immunostaining of 5-LO was by far the strongest among the four enzymes we examined, which located mainly in infiltrating neutrophils, intestinal epithelia, and in some of the lamina propria mononuclear cells, submucosal lymphocytes, and so forth; 15-LO-1 expression was typically detected in eosinophils in intestinal lamina propria and in Crhon's ulcer as well as in granuloma.

The quantitative comparison between expression grades for the four enzymes in CD's ulcer, CD's epithelia, and lamina propria and those in control specimens is illustrated by Figure 1.

Spatial distributions of COX2, 5-LO, and 15-LO-1 expressions in Crohn's ulcer

The expressions of the three major enzymes in arachidonic acid pathway followed special spatial arrangement. The 5-LO positive neutrophils were in the utmost surface of the ulcer, which formed a dark band with clear boundary. Just beneath it, COX2 positive cells aligned, again forming a band with clear boundary. Beneath this COX2 band, there was a relatively staining-negative region—granulation tissues consisting of numerous microvessels and lymphocytes, and below it, a band formed by eosinophils that highly expressed 15-LO-1 was seen. As shown in Figure 2, the expressions of the three enzymes roughly formed concentric rings surrounding the U-shaped ulcer, with 5-LO in the innermost, followed by COX2, and 15-LO-1 in the outermost ring.

There are several details that are worth noting. Firstly, both the 5-LO-expressing neutrophil band and COX2 band had clear boundaries, and the former ended exactly where the latter started, which strongly suggested an inhibitory effect of COX2 on neutrophil migration to the ulcer surface, as we could only see thinly scattered neutrophils below the COX2 band, and almost none within the COX2 band. Moreover, COX2 band located right between the surface neutrophils and the microvessel-rich granulation region. As
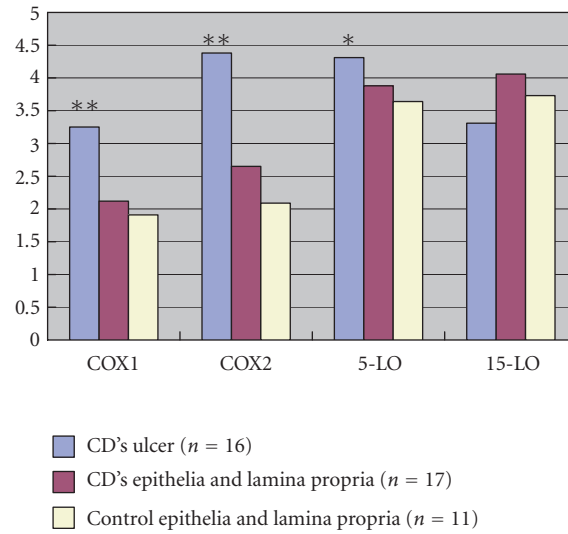
FIGURE 1: Expression grades for COX1, COX2, 5-LO, and 15-LO-1 in Crohn's ulcer, Crohn's epithelia, and lamina propria and control specimens. (** $P < .01$ compared to control; * $P < .05$ compared to control).
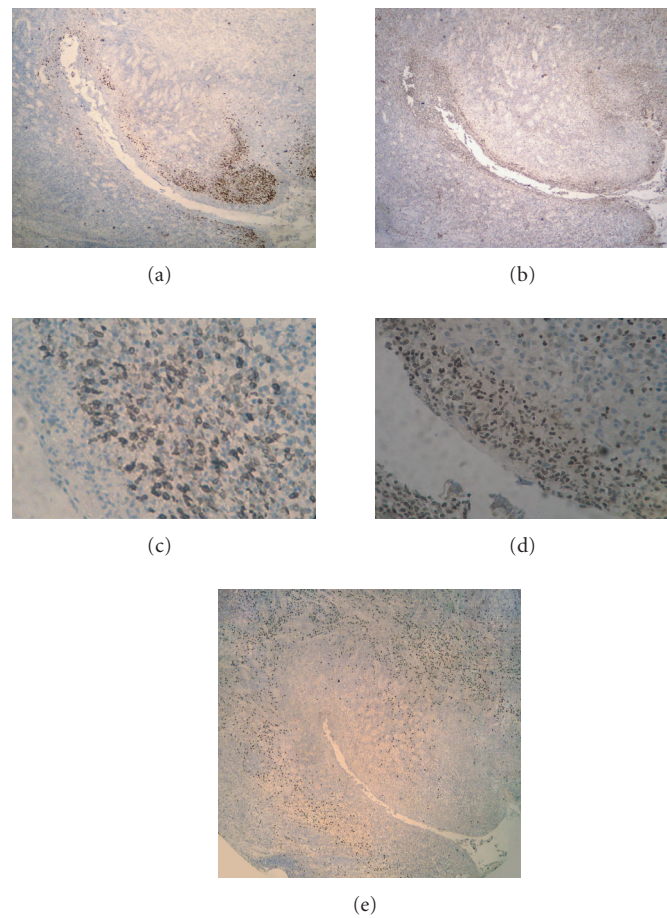

(a)

(b)

(c)

(d)

(e)

FIGURE 2: Spatial distributions of COX2, 5-LO, and 15-LO-1 in Crohn's ulcer. (a) COX2 immunoreactivity in ulcer surface. Note that the utmost superficial layer was negative for COX2 (magnifications: ×40). (c) COX2 immunoreactivity in ulcer in higher magnification: ×400. (b) 5-LO immunoreactivity in ulcer surface. Note the clear border of surface 5-LO-positive neutrophils (magnifications: ×40). (d) 5-LO immunoreactivity in higher magnification: ×400. (e) 15-LO-1 immunoreacivity in Crohn's ulcer, which formed a cuff-like structure around the U-shaped ulcer. Note that these cells surrounded the granulation region (magnifications: ×40) representative of similar eight.

these microvessels were the essential sites for neutrophils recruitment, the COX2 band seemed to have formed a barrier that effectively prevent neutrophil migration from blood vessels to the site of acute inflammation.

Secondly, below the COX2 band, there were scattered distributions of 5-LO-positive neutrophils in the vessel-rich granulation region and regions below, where they joined 15-LO-1-positive eosinophils. At the same time, 15-LO-1-positive eosinophils aggregated right below the granulation region, forming a half-circle surrounding the U-shaped granulation region (Figure 2(e)). These eosinophils seemed to form another barrier to prevent neutrophils of the peripheral circulation from infiltrating further into submucosal and subsequent layers of the intestine.

Thirdly, the aforementioned staining-negative region was rich in blood vessels and lymphocytes. The existence of numerous lymphocytes in the very adjacency of acute inflammation indicates this to be a site for priming of adaptive immunity and crosstalk between innate and adaptive immunity.

DISCUSSION

The spatially sequential distribution of 5-LO, COX2, and 15-LO-1 expressions from the ulcer surface to layers beneath coincides with the reported lipid mediator class switching during acute inflammation; in which the authors demonstrated temporally sequential display of LTB4, PGE2, and LXA4 in the inflammatory exudates in a murine dorsal air pouch model [5]. As 5-LO, COX2, and 15-LO-1 are the chief enzymes responsible for LTB4, PGE2, and LXA4 syntheses, respectively, the spatial sequence of these enzymes could be translated into spatial sequence of the relevant arachidonic acid metabolites. But how does this spatial distribution correlate with the temporal sequence? As we know, intestinal mucosa is the major site where the body encounters foreign antigens in the form of food or luminal bacteria, and so forth, so it is the site where inflammation initiates and the ulcer surface represents the initial phase of acute inflammation, where abundant acute inflammatory cells—neutrophils—are accumulated. Beneath, however, the general tendency of localizing the inflammation and healing become manifest, and represents the chronic and resolution phases of inflammation. And apparently, it takes more time for lipid mediators from layers beneath to secrete into the lumen as exudates. Hence, findings from this study in human CD lesions not only confirmed results of the previous work in murine air pouch model, suggesting that lipid mediator class switching be a universal mechanism in inflammation, in rodents or human, in intestine or subdermal tissues, but pinpointed the cell types that generated these lipid mediators as well. In addition, the much shorter the interval between maximal LTB4 and PGE2 formation and the longer the interval between maximal PGE2 and LXA4 formation in exudates juxtaposes perfectly with the spatial distances between corresponding enzymes in this study.

Another important point is the shared clear border of 5-LO-positive neutrophil band and COX2-positive

mesenchymal band, and the barrier-like alignment of COX2-positive cells between neutrophils and the blood-vessel-rich region below, which strongly indicated the inhibitory effect of COX2 on neutrophil transendothelial migration from peripheral blood. While many authors concluded that COX2-derived PGE2 amplified acute inflammation [3, 5], our study supported the antiinflammatory roles of COX2 and its metabolites. As could be seen in Figure 2, contrary to results from ex vivo studies using peripheral neutrophils in milieu of various agonists, by immunohistochemical methods, we showed that neutrophils actively participating in acute inflammation did not express COX2. Given the established fact that PGE2 is the dominant metabolite of COX2-mediated pathway, especially in the intestine [8], it is likely that the observed COX2-positive cells in CD lesion produced PGE2, which exerted inhibitory effects on migration and other functions of neutrophils.

There is much evidence supporting COX2's antiinflammatory properties. Gilroy and coworkers showed that COX2, by generating PGE2, might be proinflammatory during the early phase of inflammation, but may aid resolution at the later phase by generating 15deoxyΔ [9–11] PGJ2 [3]. While these authors translated their findings into proinflammatory roles of COX2 and PGE2 in the early phase of inflammation, others pointed to antiinflammatory and immunosuppressive roles of PGE2 [9, 10, 12–14]. Of special interest is that COX2 inhibition increased leukocyte adherence to gastric vascular endothelium and COX1 inhibition reduced gastric blood flow [11], which is in agreement with our results. In an H pylori-induced gastritis murine model, COX1 inhibitor SC-560 augmented MPO activity and epithelial cell apoptosis with reduced PGE2 production, whereas COX2 inhibitor NS-398 had the same effects without affecting PGE2 production [15]. In other studies, COX2 inhibition or gene disruption exacerbated inflammation-associated colonic injury in experimental colitis [16, 17], and COX2 inhibition had only limited antiinflammatory efficacy in carrageenan-airpouch inflammation [18]. COX2 inhibition can also induce enteropathy [19] and NSAIDs delay gastrointestinal ulcer healing [20, 21]. In this study, COX2 expression was detected solely in the superficial region in Crohn's ulcer with negligible expression in other intestinal tissues. Together, these data urge us to see COX2 in a new light: the induced expression of COX2 in inflammation should not be interpreted as its proinflammatory nature; on the contrary, its induction may be a part of a universal mechanism to counterregulate inflammation and promote healing.

The exact mechanism of this inhibitory role of COX2 on neutrophil recruitment is elusive. Serhan and coworkers showed that PGE2 inhibits LTB4 but mobilizes lipoxin A4 (LXA4) in peripheral blood human PMNs [5], and LXA4 blocks neutrophil migration across postcapillary venules and inhibits neutrophil entry into inflamed tissues in animal models [22]. Thus it is possible that those COX2-positive cells produced abundant PGE2, and the latter not only suppressed LTB4 production but induced LXA4 production in neutrophils, and in turn LXA4, with its well-appreciated antiinflammatory role [4, 22–25], inhibits neutrophil

migration and exerts other proresolution effects. Indeed, we observed that the 5-LO expression pattern in surface neutrophils was slightly different from that in scattered neutrophils below the COX2 band—the staining was weaker and the lobed nuclei were somewhat indistinct in the surface neutrophils. We did not observe noticeable 15-LO-1 expression in these cells; this, however, does not rule out the possibility that the eicosanoid profile was undergoing a switch from the powerful chemoattractant leukotrienes to resolution-promoting lipoxins. It is interesting to mention that we did find 15-LO-1 expression in the necrotized neutrophils in the intestinal lumen right above the COX2 expression layer. PGE2 may also inhibit neutrophil migration by other immunosuppressive routes, and other COX2 metabolites, especially PGD2 [26], and even metabolites-independent mechanisms might have a role. But the well-appreciated antiinflammatory 15deoxy Δ [9–11] PGJ2 may not have participated in this process, because of its lack of effects on neutrophil adhesion and neutrophil-selective chemokine expression [27, 28].

There was a second barrier-like structure formed by 15-LO-1-expressing eosinophils, located just below the blood-vessel-rich region, which probably had prevented neutrophils from further infiltration into intestinal tissues beneath. The coexistence of 15-LO-1-expressing eosinophils and scattered 5-LO-expressing neutrophils strongly indicated interactions of these two cell types in transcellular biosynthesis of lipoxins, which eventually formed a barricade against neutrophil infiltration. These results showed that the classical transcellular biosynthetic pathway of lipoxins could actually take place in Crohn's ulcers in vivo, and lipoxins thus formed could be the key factor in limiting neutrophils-associated injuries in intestines. Recently, evidence of the dramatic proresolution effect of lipoxin analogs and resolvins is gathering in IBD models, suggesting an excitingly new solution to the challenging problem of IBD treatment [6, 7].

To improve the picture of Crohn's Ulcer, this piece of information is added: LTD4 and 5-oxo-ETE, two 5-LO metabolites, are eosinophil chemoattractants [29]. Thus, the typical proinflammatory 5-LO pathway has its own negative feedback: by recruiting eosinophils from peripheral blood and the intestinal submucosa to the inflammatory lesion, transcellular biosynthesis of lipoxins and consequently counterregulation of inflammation become possible. In addition, one of the 15-LO products, 15-hydroxyeicosatetraenoic acid directly inhibits 5-LO [30].

Lastly, the lymphocyte and blood-vessel-rich granulation region in Crohn's ulcer could be the site for intimate crosstalk between innate immunity and adaptive immunity. COX-derived eicosanoids, like PGE2, and possibly lipoxygenase-derived eicosanoids, could modulate lymphocyte's functions in a number of ways [31], and thus innate immunity is reshaping adaptive immunity. Vice versa, cytokines secreted by T cells, such as IL-1β, IL-4, IL-10, IFN, TNFα, could up- or down-regulate COX2 expression [32, 33], and IL-4 and IL-13 could induce 15-LO expression in macrophages as well [34–36]. Therefore, it is highly possible that crosstalk between the two immune systems is ongoing in the granulation region beneath Crohn's ulcer surface. PGE2 has a tendency

of biasing the immune system towards Th2 and away from Th1 respolnses [31], thus dysregulated arachidonic acid metabolism and suboptimal COX2 induction in inflammation would lead to suppression of Th2 responses and overreaction of Th1 responses, which might result in CD.

To sum up, this study demonstrated spatial interrelationships between the three leading enzymes in arachidonic acid metabolism—COX2, 5-LO, and 15-LO-1 in Crohn's ulcer; and by combining data from previous works, we described interactions between different inflammatory cells and arachidonic acid pathways, all of which are part of the multistage inflammatory events recognized as Crohn's ulcer. Our results indicated protective roles for COX2 and 15-LO-1 in CD and a new hope for the treatment of CD, namely, by enhancing COX and 15-lipoxygenase-1-mediated pathways and inhibiting 5-lipoxygenase-mediated pathway.

ABBREVIATIONS

CD	Crohn's disease
COX	Cyclooxygenase
IBD	Inflammatory bowel disease
LOX	Lipoxygenase
LX	Lipoxin
PG	Prostaglandin

ACKNOWLEDGMENT

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