IL-1β inhibits intestinal smooth muscle proliferation in an organ culture system: involvement of COX-2 and iNOS induction in muscularis resident macrophages

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Ohama T, Hori M, Momotani E, Elorza M, Gerthoffer WT, Ozaki H. IL-1β inhibits intestinal smooth muscle proliferation in an organ culture system: involvement of COX-2 and iNOS induction in muscularis resident macrophages. Am J Physiol Gastrointest Liver Physiol 292: G1315–G1322, 2007. First published January 18, 2007; doi:10.1152/ajpgi.00487.2006.—Intestinal inflammation causes hyperplasia of smooth muscle that leads to thickening of the smooth muscle layer, resulting in dysmotility. IL-1β is a proinflammatory cytokine that plays a central role in intestinal inflammation. In this study, to evaluate the effect of IL-1β on proliferation of ileal smooth muscle cells in vivo, we utilized an organ culture system. When rat ileal smooth muscle tissue was cultured under serum-free conditions for 3 days, most smooth muscle cells maintained their arrangement and kept their contractile phenotype. When 10% FBS was added, an increased number of smooth muscle cells per unit area was observed. Moreover, immunohistochemical staining for PCNA demonstrated that FBS induced proliferation of smooth muscle cells. IL-1β inhibited the proliferative effect of FBS. Furthermore, IL-1β upregulated inducible nitric oxide (NO) synthase and cyclooxygenase-2 mRNA and protein and thus stimulated NO and PGE₂ productions. Moreover, exogenously applied NO and PGE₂ inhibited the increase of bromo-deoxyuridine-positive cells stimulated with FBS. Immunostaining revealed that the majority of cyclooxygenase-2 and inducible NO synthase was located in the dense network of macrophages resident in the muscularis, which were immunoreactive to ED2. Based on these findings, IL-1β acts as an anti-proliferative mediator, which acts indirectly through the production of PGE₂ and NO from resident macrophage within ileal smooth muscle tissue.

interleukin-1β; intestine

A transition in smooth muscle cells from contractile to proliferating phenotype results in hyperplasia (increased cell number) of smooth muscle cell, which is often observed in intestinal inflammation (2, 24). Understanding the mechanism behind this morphological change is extremely important, because it is thought to contribute to stricture formation and motility disorders.

IL-1β is a proinflammatory cytokine that plays a central role in inflammation. Elevated IL-1β levels have been measured in both the mucosa and the muscle layer during acute and chronic intestinal inflammation. This includes inflammatory lesions of patients with forms of inflammatory bowel disease, such as Crohn’s disease and ulcerative colitis (15, 16, 28, 31). IL-1β is a cytokine that induces proliferation in numerous cell types (8). As for the intestine, previous reports have reported that IL-1β directly induces the proliferation of cultured intestinal smooth muscle cells (11, 12, 26). These findings strongly suggest a role of IL-1β in inflammation-induced thickening of the intestinal smooth muscle layer; however, limited data are available regarding the effects of IL-1β on proliferation of smooth muscle under more physiological conditions.

Smooth muscle cells in culture rapidly change their phenotype (3), which may be partly due to the absence of extracellular matrix and/or cell networks (19, 21). Organ culture systems, in which the surrounding matrix and cell networks are maintained, can overcome this drawback and can be used to assess the mechanisms by which smooth muscle phenotypic changes occur. Moreover, the organ culture method makes it possible to dissociate the influence of systemic cellular and humoral immune responses, so that the direct effects of agents on proliferation and the phenotypic change of smooth muscle cells can be investigated. As for the factors affecting smooth muscle proliferation during chronic intestinal inflammation, it is attractive to consider the possible involvement of resident macrophages, which are regularly distributed with a network structure at the level of the myenteric plexus and inside the muscle layer (1, 22, 27, 30).

The present study aimed to examine the effects of IL-1β on proliferation of intestinal smooth muscle cells using an organ culture system. Unexpectedly, we found an inhibitory effect of IL-1β on proliferation of ileal smooth muscle cells and revealed that this inhibitory effect is mediated indirectly through the production of PGE₂ and nitric oxide (NO) from the resident macrophages in the muscularis.

EXPERIMENTAL PROCEDURES

Animals. Male Wistar rats (200–250 g) were purchased from Charles River Japan. Animal care and treatment were conducted in accordance with the institutional guidelines of the University of Tokyo.

Organ culture procedure. Organ culture was performed as previously described (25). Briefly, a segment of the ileum was placed in sterile Hanks’ balanced salt solution, after which the smooth muscle layer was detached from the mucosal layer. The muscle strips were...
cultured with medium 199 supplemented with 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, 0.25 µg/ml amphotericin B, and 2 mM l-glutamine. The culture dishes were incubated at 37°C in an atmosphere of 95% air and 5% CO2. The incubation medium was replaced every day. After 1 or 3 days of culture, the tissues were used for assays.

**Morphological examination.** Isolated smooth muscle tissue was fixed in 10% neutral-buffered formalin and embedded in paraffin. Four-micrometer-thick sections were stained with hematoxylin and eosin. To perform proliferating cell nuclear antigen (PCNA) staining, sections were subjected to immunostaining as previously described (32). Deparaffinized sections were first treated with 3% hydrogen peroxide in 10% methanol, followed by 10% normal goat serum to protect against nonspecific reactions. Incubation with mouse monoclonal antibodies against PCNA (1:50 dilution) was subsequently performed. The sections were further incubated with biotinylated goat anti-mouse IgG (1:200 dilution), followed by reaction with an avidin-biotin peroxidase complex (DAKO). Immunoreactive protein was visualized with 0.05% 3,3’-diaminobenzidine tetrahydrochloride solution containing 0.01% hydrogen peroxide. The sections stained with PCNA or bromodeoxyuridine (BrDU) were counterstained with methyl green.

**RT-PCR analysis.** Rat inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), and GAPDH RT-PCR analysis was performed as previously described (13). Briefly, first-strand cDNA was synthesized using a random 9mer primer and avian myeloblastosis virus Reverse Transcriptase XL at 30°C for 10 min, followed by 55°C for 45 min, 99°C for 5 min, and 4°C for 5 min. PCR amplification was performed using the hot start method with Taq Gold (Perkin Elmer, Branchburg, NJ). Amplification was performed by initial denaturation at 95°C for 10 min, followed by 36 amplification cycles, including 40 s at 94°C, 60 s at 55°C, and 90 s at 72°C. The PCR products were then separated by electrophoresis on 2% agarose gel containing 0.1% ethidium bromide. The resultant fluorescent bands were visualized using an ultraviolet transilluminator using FAS-III (Toyobo, Tokyo, Japan), after which band density was measured using NIH Image software.

**Western blotting.** Western blot analysis was performed as previously described (25). Ileal muscle strips devoid of mucosa were homogenized in homogenizing buffer to extract protein. The homogenizing buffer contained 60 mM β-glycerophosphate, 2 mM EGTA, 0.5% Nonidet P-40, 0.2% SDS, 100 mM NaF, 1 mM Na3VO4, and 50 mM Tris-HCl at pH 8.3. Roche’s Complete protease inhibitor cocktail was added to homogenizing solution. Twenty micrograms of total protein were loaded in each lane to detect COX-2 and iNOS. PBS containing 5% powdered milk was used as a blocking buffer and treated for 30 min at room temperature. Anti-COX-2 antibody (1:2,000 dilution, Cayman) and anti-iNOS antibody (1:1,000 dilution, Transduction Laboratories) were treated as the first antibody in the blocking buffer overnight at 4°C, and biotinylated anti-rabbit IgG (for COX-2, 1:1,000 dilution, Vector Laboratories) or biotinylated anti-mouse IgG (for iNOS, 1:1,000 dilution, Vector Laboratories) as the secondary antibody for 1 h at room temperature. After that, horseradish peroxidase-streptavidin (1:1,000 dilution, Zymed Laboratories) was treated for 1 h at room temperature. COX-2 and iNOS were detected using an enhanced chemiluminescence plus Western Blotting detection system (Amersham Biosciences). Bands were visualized using an LAS-1000mini luminescence imager (FUJIFILM, Tokyo, Japan).

**PGE2 and NO productions.** The amounts of PGE2 and NO in culture medium were examined by using PGE2 Quantikine ELISA kit (R&D Systems) and nitric oxide assay kit (Calbiochem). The assay was performed as recommended by the manufacturer.

**BrdU staining.** Twenty micromolar BrDU was added to the culture medium for 24 h before fixation. Paraffin-embedded sections were treated with anti-BrdU mouse MAb (Daco) and biotin-labeled anti-mouse IgG polyclonal antibody (KPL) as the primary and secondary antibodies, respectively. Immunostaining for BrDU was performed using a Vector ABC kit (Vector Laboratories). BrDU-positive smooth muscle cells were visualized by using PGE2 Quantikine ELISA kit (R&D Systems) and nitric oxide assay kit (Calbiochem). The assay was performed as recommended by the manufacturer.

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![Fig. 1](http://ajpgi.physiology.org/). The FBS-induced increase in cell number is inhibited by IL-1β treatment. Hematoxylin and eosin staining was applied to ileal smooth muscle in organ culture tissue isolated from normal rats. Ileal smooth muscle tissue was cultured under serum-free conditions (A), with 10% FBS (B), or with 10% FBS and 10 ng/ml IL-1β (C) for 3 days. D: number of cells per unit area. N = 6–8. *P < 0.05 vs. serum-free conditions. #P < 0.05 vs. FBS treatment.
Fig. 2. The FBS-induced increase in proliferating cell nuclear antigen (PCNA) positive cells is inhibited by IL-1β treatment. Immunostaining for PCNA was performed on rat ileal smooth muscle in organ culture. Ileal smooth muscle tissue was cultured under serum-free conditions (A), with 10% FBS (B), or with 10% FBS and 10 ng/ml IL-1β (C) for 3 days. D: an enlarged picture of smooth muscle tissue (circular smooth muscle layer) cultured with 10% FBS. Quantitative data are shown for percentage of PCNA positive smooth muscle cells in circular (E) and longitudinal (F) layer. N = 4–6. **P < 0.01 vs. FBS treatment.

Fig. 3. IL-1β induces cyclooxygenase-2 (COX-2) and inducible nitric oxide (NO) synthase (iNOS) mRNA and protein upregulation in ileal smooth muscle tissue. Rat ileal smooth muscle tissue was cultured with 10% FBS, or with 10% FBS and 10 ng/ml IL-1β, for 1 and 3 days. COX-2 (A) and iNOS (B) mRNA expression were examined by RT-PCR. COX-2 (C) and iNOS (D) protein expression were examined after 3 days of culture. The open and solid columns demonstrate results after treatment with FBS, and FBS and IL-1β treatment, respectively. N = 4–5. **P < 0.01 vs. FBS treatment.
muscle cells were identified under a light microscope and expressed as number of positive cells per total cell number.

**Immunohistochemistry for COX-2 and iNOS.** Immunostaining for COX-2 and iNOS was performed as previously described (13). Briefly, smooth muscle tissues were fixed with either 4% paraformaldehyde in 0.15 M phosphate buffer at pH 7.4 for iNOS and ED2 double staining or in Zamboni solution for COX-2 and ED2 double staining, and they were processed for whole mount preparations. Anti-rat resident macrophage antibody (ED2; 1:500 dilution; BMA), anti-COX-2 antibody (1:50 dilution; Cayman), and anti-iNOS antibody (1:1,000 dilution; BD) were treated in the blocking buffer overnight at 4°C as the first antibody. Then, Texas red conjugated anti-rabbit IgG (1:500 dilution; Vector) for COX-2 and iNOS, and Alexa 488 conjugated anti-mouse IgG (1:500 dilution; Molecular

**Fig. 4.** IL-1β stimulates PGE2 and NO production. Rat ileal smooth muscle tissue was cultured with 10% FBS, or with 10% FBS and 10 ng/ml IL-1β, for 3 days. PGE2 (A) and NO (B) releases were examined, as indicated in EXPERIMENTAL PROCEDURES. The open and solid columns demonstrate results after treatment with FBS, and FBS and IL-1β treatment, respectively. N = 4. *P < 0.01 vs. FBS treatment.

**Fig. 5.** The FBS-induced increase of bromodeoxyuridine (BrdU) positive cells is inhibited by IL-1β, NO, and PGE2 treatment. Immunostaining for BrdU was performed on rat ileal smooth muscle organ culture. Ileal smooth muscle tissue was cultured under serum-free conditions (A), with 10% FBS (B), with 10% FBS and 10 ng/ml IL-1β (C), with 10% FBS and 1 μM PGE2 (D), or with 10% FBS and 1 mM NOC-18 (equivalent to 1–5 μM NO) (E) for 3 days. F: number of BrdU positive cell per area. N = 4–6. **P < 0.01 vs. serum-free conditions. ##P < 0.01 vs. FBS treatment.
Probes) were treated for 1 h at room temperature as secondary antibody. Colocalization was analyzed using a confocal laser scanning microscope (LSM510; Zeiss).

Statistical analyses. Results are expressed as means ± SE. Comparisons between the control and test groups were performed by one-way ANOVA, followed by Dunnett’s multiple-comparison test. For all evaluations, P values <0.05 were considered statistically significant.

RESULTS

First, we examined phenotypic change of smooth muscle cells in organ culture study. Most of the smooth muscle cells maintained their elongated shape in the muscularis when cultured under serum-free conditions (Fig. 1A), as previously reported (25). When 10% FBS was added to the incubation medium, the number of smooth muscle cells per millimeter squared was increased (Fig. 1B and D). The thickness of the longitudinal and circular smooth muscle layer was not significantly changed 3 days after FBS treatment (serum-free 104.3 ± 16.8 μm vs. with FBS 108.3 ± 9.0 μm, n = 6–8).

Interestingly, IL-1β attenuated the proliferative response of smooth muscle cells to FBS (Fig. 1C and D).

To confirm the somewhat surprising anti-proliferative effect of IL-1β, we next performed immunostaining for PCNA in organ cultured tissue. As shown in Fig. 2A, only a few smooth muscle cells were positive for PCNA in an ileal smooth muscle tissue cultured without serum. In contrast, FBS treatment markedly induced smooth muscle proliferation, as indicated by numerous PCNA positive cells in Fig. 2B. Treatment with IL-1β inhibited the proliferation induced by FBS (Fig. 2C). An enlarged picture of FBS-treated tissues (Fig. 2D) shows that PCNA positive cells were spindle shaped and had flat-shaped (cigar-like) nucleus characterizing smooth muscle cells. Figure 2E and F, shows the quantitative data for the percentage of proliferative smooth muscle cells in circular and longitudinal...
layers. The degree of increase in number was not different between circular and longitudinal smooth muscle layers.

PGE2 and NO are well-known mediators that prevent smooth muscle cell proliferation (20, 23, 29). Therefore, we next examined whether IL-1β might induce the expression of COX-2 and iNOS as part of the anti-proliferative effect. As shown in Fig. 3, IL-1β upregulated COX-2 mRNA at 1 day and iNOS mRNAs at both 1 and 3 days (A and B). COX-2 and iNOS protein level were both upregulated by IL-1β by 3 days (C and D). Moreover, we found increasing levels of PGE2 and NO released into culture medium after 3 days (Fig. 4). To determine whether PGE2 and NO were affecting smooth muscle proliferation, we assayed BrdU labeling of newly synthesized DNA. In the tissues cultured without serum, we observed a small number of smooth muscle cells that were BrdU positive. We found that FBS increased the number of BrdU positive cells in ileal smooth muscle organ culture system, and that IL-1β, PGE2, and NOC-18 (slow-releasing NO donor) inhibited this increase (Fig. 5).

We then examined the source of PGE2 and NO by immunohistochemistry. As shown in Fig. 6A, COX-2 immunopositive cells appeared after the IL-1β treatment, and they also showed immunoreactivity to antimacrophage antibody ED2. In addition, iNOS-expressing cells could be detected after the IL-1β treatment, and they were also immunoreactive for ED2 (Fig. 6B). COX-2 and iNOS immunopositive cells were not detectable in the absence of IL-1β in FBS-treated tissues.

**DISCUSSION**

It is well known that a transition in smooth muscle cells from contractile to proliferating phenotype results in hyperplasia of smooth muscle cell. Increased number of smooth muscle cells results in the thickening of the smooth muscle layer, which is often observed in intestinal inflammation (2, 24). We also observed an increase in the number of PCNA positive ileal smooth muscle cells at 2 days after trinitrobenzene sulfonic acid (TNBS)-induced ileitis (our unpublished data). IL-1β is a recognized proinflammatory cytokine that induces proliferation in numerous cell types (8), including intestinal smooth muscle cells (11, 12, 26). We confirmed the proliferative effect of IL-1β using primary rat ileal smooth muscle cells (our unpublished data).

In the present study, we examined the effect of IL-1β on intestinal smooth muscle proliferation by using an ileal smooth muscle organ culture system. Unexpectedly, we observed an inhibition of ileal smooth muscle cell proliferation by IL-1β in organ culture. Smooth muscle cells maintained the contractile phenotype in this culture system with well-maintained α-smooth muscle actin expression (25). Few smooth muscle cells were in the proliferate state during 3 days in culture (Figs. 2 and 5). When ileal smooth muscle tissue was treated with 10% FBS, smooth muscle cells adopted a proliferative phenotype and increased cell number. In the present study, we also observed increased numbers of PCNA and BrdU positive smooth muscle cells.

COX-2 and iNOS are expressed under inflammatory conditions and produce mediators that regulate growth of smooth muscle cells. In cultured vascular smooth muscle cells, it has been reported that IL-1 possesses anti-proliferative effects through the induction of COX-2/iNOS (14). Furthermore, Libby et al. (20) observed that IL-1β was able to induce vascular smooth muscle proliferation when the cells were treated with COX-2 inhibitor. As for the airway smooth muscle cells, De et al. (7) also reported that the presence of COX-2 inhibitor in the culture medium was essential to demonstrate the proliferative effect of IL-1β in airway smooth muscle cells. These reports suggest that IL-1β possesses both direct proliferative and indirect (through COX-2/iNOS expression) anti-proliferative roles in vascular and airway smooth muscle cells. In contrast to results in vascular and airway smooth muscles, IL-1β directly stimulates proliferation of cultured human ileal smooth muscle cells (26) and human and mouse primary smooth muscle cells (11, 12). The difference in the response to IL-1β may be due to cell-specific difference in the ability to express COX-2 and iNOS.

Different responses to IL-1β in the studies in cultured cells and organ-cultured tissues suggest that some other cells containing smooth muscle tissues might participate in the anti-proliferative action on smooth muscle cells. Upregulation of COX-2 and iNOS in the resident macrophage was previously

![Fig. 7. Proposed mechanism for parallel and dual-signaling pathways in response to IL-1β that mediate proliferation of intestinal smooth muscle cells. IL-1β directly induces ileal smooth muscle proliferation, and simultaneously IL-1β induces COX-2 and iNOS expression in muscularis resident macrophage that leads to PGE2 and NO production. PGE2 and/or NO inhibit ileal smooth muscle proliferation.](http://ajpgi.physiology.org/)

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Importantly, COX-2 and iNOS were highly expressed in the observed in rat intestinal smooth muscle stimulated with LPS in vitro (13) and in vivo (10). In the present study in an ileal smooth muscle organ culture system, we consistently observed IL-1β-induced upregulation of COX-2 and iNOS expression. Importantly, COX-2 and iNOS were highly expressed in the resident macrophages distributed at the myenteric plexus region. Moreover, we confirmed the production of PGE₂ and NO and the inhibitory effects of exogenously applied PGE₂ and NO on smooth muscle proliferation. Based on our data and previous work of other laboratories, we hypothesized that proliferation of intestinal smooth muscle cells might be regulated via parallel and dual-signaling pathways in response to IL-1β. IL-1β has a direct proliferative effect on smooth muscle cells, as well as an indirect and antagonistic inhibitory effect through resident macrophages (Fig. 7).

It is known that IL-1β is expressed under physiological conditions, indicating that IL-1β may regulate the proliferation of smooth muscle cells under physiological conditions. It is also known that upregulation of IL-1β was observed in vivo intestinal inflammation. However, we previously reported that this upregulation of IL-1β diminished after a few days in the colon smooth muscle in the TNBS-induced colitis model (17). We also confirmed this in ileal inflammation; i.e., expression of IL-1β protein peaked at day 2 and returned toward baseline level after TNBS treatment (our unpublished data). These observations suggest that intestinal smooth muscle tissues may be exposed to increased IL-1β during only the initial phase of intestinal inflammation, and IL-1β may dynamically modulate the smooth muscle functions during exacerbation and remission.

Regulation of cytokines and chemokines is an effective strategy for the treatment of inflammation. However, because cytokines are part of complex cell-cell signaling networks, inhibition of cytokine expression sometimes has unintended consequences. IL-1 is an important mediator of local and systemic inflammation. Blockade of IL-1 is an effective strategy for the treatment of inflammatory diseases, such as rheumatoid arthritis (9). As for the intestinal inflammation, Cominelli’s group (4, 5) first reported the beneficial effects of IL-1 receptor antagonists on rabbit colitis. However, inhibition of IL-1 activity is not always beneficial; i.e., Cominelli’s group (6) also reported that a low dose of IL-1 can inhibit rabbit colitis. Moreover, Kojouharoff et al. (18) observed that the blockade of IL-1 results in exacerbation of dextran sulfate sodium-induced mouse colitis. Consistent with these findings, we observed severe pathology, including weight loss and decreasing of survival rate in IL-1α/β-deficient mice after the treatment with TNBS (our unpublished observation). Therefore, it is suggested that IL-1β possesses both proinflammatory and anti-inflammatory roles. Because the excess thickening of smooth muscle layer leads to intestinal constriction, the anti-proliferative effects on smooth muscle cells revealed by the present study may play a role in antagonizing the motility disorder frequently observed in inflammatory bowel disease.

In summary, IL-1β acts as an anti-proliferative mediator on intestinal smooth muscle cells, which acts indirectly through the production of PGE₂ and NO from resident macrophage within ileal smooth muscle tissue. These findings enhance our understanding of the role of IL-1β in intestinal motility disorders and stricture formation.

IL-1β INHIBITS INTESTINAL SMOOTH MUSCLE PROLIFERATION

GRANTS

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