Crypt Proliferation and Growth Factor Expression in Acute Indomethacin-Induced Small Intestinal Injury

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ABSTRACT. NSAIDs cause small intestinal mucosal injuries, but early events within the mucosa have not been clarified. The aim of this study was to characterize early changes in both crypt proliferation and the expression of growth factors in indomethacin-induced small intestinal damage. Crypt proliferation and intestinal mRNA expression of TGF-β_1, IGF-1, HGF, and NGF were investigated in rats that received 24 mg/kg of intracolonic indomethacin. Proliferation was assessed by immunohistochemical staining for 5'-bromo-2'-deoxyuridine, and mRNA expression of growth factors was measured semi-quantitatively by reverse transcription (RT)-PCR. Crypt proliferation in the mid-small intestine did not decrease at two hours after administration of indomethacin but that in the jejunum and the ileum decreased significantly. Crypt proliferation was decreased at all sites after six hours. TGF-β_1, IGF-1 and HGF mRNA expression increased until six hours, whereas NGF showed no such increase. Early mucosal damage induced by indomethacin is characterized by alteration of the crypt architecture without changes in crypt cell proliferation. NGF may play a role in the progression of this mucosal damage.

Key words: indomethacin — small intestine — proliferation — growth factors — immunohistochemistry

It has been well known that nonsteroidal anti-inflammatory drugs (NSAIDs) cause gastrointestinal damages in humans and experimental animals.[1-4] Although the mechanism of this adverse effects has not been clearly understood, recent investigations have confirmed that leukocytes play a major role for the development of mucosal injury in the small intestine[5,6] as well as in the stomach.[7-9] In particular, it has recently been proven that adherence of leukocytes to the endothelial cells via E-selectin and CD11/18[10,11] is the pivotal event in the early phase of gasrointestinal lesions.

In contrast to emphasis on the role of polynuclear cells, Anthony *et al*[12] described that early morphological changes, termed villous contractions, are the initial event in the pathogenesis of mucosal damages within the small intestine. They subsequently confirmed that this phenomenon is independent of leukocytes, and that it depends instead upon cyclooxygenase inhibition.[13] It has also been established that NSAIDs affect cell cycles of cultured epithelial cells.[14] However, correlation between crypt cell proliferation and early morphologic changes in acute small intestinal injury induced by indomethacin has not been specified. In addition, since it has been described that epithelial

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restitution accelerated by various growth factors was suppressed by NSAIDs in an in vitro study,[15] we made an attempt to investigate early morphological changes in the intestinal mucosa with respect to crypt cell proliferation and the mRNA expression of various growth factors (GFs) in rats treated with indomethacin. Transforming growth factors (TGF)-β_1, insulin-like growth factor-1 (IGF-1), and hepatocyte growth factor (HGF) were assessed, since all these GFs have been recently described to be overexpressed in intestinal inflammation.[16–18] Nerve growth factor (NGF) was also investigated, since it has been reported to be synthesized in the intestinal epithelia and[19] to promote repair of both cultured epithelial cells and experimental colitis.[20]

MATERIALS AND METHODS

Animals

Male Wistar rats (six to seven weeks of age, weighing 200-250 g) were used in this study, and the animals were housed in plastic cages with a maximum of three animals per cage. They were maintained in a room with restricted access at a controlled temperature. Standard laboratory pelleted formula and tap water were provided ad libitum. The experiments were approved by the Animal Research Committee of Kawasaki Medical School (No B015 1997) and conducted according to the "Guide for the Care and Use of Laboratory Animals" of Kawasaki Medical School.

Induction of intestinal ulcers

Intestinal ulcers were induced with indomethacin using a previously described method.[21] In brief, each animal was anesthetized with sodium amobarbital (100 mg/kg) and 24 mg/kg of indomethacin diluted in carboxymethylcellulose (CMC) solution (100 mg/ml) was subsequently administered into the colon. Crypt proliferation and mRNA expression of growth factors were investigated at two, three and six hours after administration of indomethacin. Control rats received CMC solution.

Scanning electron microscopy

After sacrificing animals by amobarbital overdose, the small intestine was fixed by perfusion-fixation method using 10% formol-saline as described by Anthony *et al.*[12] The middle small intestine of the rats was immediately fixed in phosphate buffered 1% osmium tetroxide for two hours, 1% tannic acid for overnight, and again in 1% osmium tetroxide for an hour. The specimens were dehydrated through a graded ethanol series and they were dried in a critical point dryer. After being coated with platinum-palladium, they were observed by a scanning electron microscopy (Hitachi S-570).

Crypt cell proliferation

The proliferating cells within the crypts were labeled with 5'-bromo-2'-deoxyuridine (BrdU).[22] BrdU (Sigma Corporation, St. Louis, MO, USA) was diluted with purified water to a concentration of 20 mg/ml. The rats were intraperitoneally administered 100 mg/kg body weight of BrdU. Exactly 60 minutes after this procedure, they were sacrificed by a sodium amobarbital overdose, and specimens were immediately obtained from the small intestine.

Whole-thickness specimens (approximately 1 cm in longitudinal length) were obtained from the jejunum (10 cm from the pylorus), from the middle small intestine, and from the ileum (10 cm from the ileocecal junction). These specimens were opened by a longitudinal incision and fixed in 70% ethyl alcohol solution for 48 hours.

The specimens were then embedded in paraffin and cut into histological sections (4 μm thick). These sections were deparaffinized to distilled water in a xylene and ethyl alcohol series, and kept in a methyl alcohol solution with 1% H_2O_2 for 30 minutes to block intrinsic peroxidase activity. The sections were then immersed in 2N HCl for 10 minutes at 37℃ to denature the DNA; rinsed in 0.1M sodium tetraborate for one minutes; rinsed three times in 0.01M phosphate-buffered saline (PBS); and then applied with 10% rabbit serum (Histofine SAB-PO(M) kit, Nichirei Corporation, Tokyo, Japan) for 10 minutes. After these procedures, the sections were incubated in anti-BrdU monoclonal antibody (1 : 100) (Mouse IgG, Becton-Dickson, Mt. View, CA, USA). Following overnight incubation, they were rinsed three times, for five minutes each time, in PBS, and then a biotinated second antibody (anti-mouse IgG, A, and M, Histofine SAB-PO(M) kit) was applied for 20 minutes. Following three rinses in PBS (10 minutes each), the sections were incubated in streptoavidin-peroxidase conjugate (Histofine SAB-PO(M) kit) at room temperature for 20 minutes. After three rinses in PBS (five minutes each), they were incubated in diaminobenzidine tetrahydrochloride (DAB, Histofine DAB kit, Nichirei Corporation) at room temperature for two minutes. They were further rinsed in PBS, and counterstained with hematoxyline.

Under light microscopy with a ×400 high-power view, 20 crypts which had been sectioned completely from the bottom to the top of their architecture were evaluated. The mucosal proliferative activity at each site of the small intestine was expressed as a labelling index which was calculated as the ratio of the number of positive BrdU cells against the total number of cells present in the crypts.

Villous height and crypt depth

Villous height and crypt depth were measured in H & E stained samples. The vertical length of 20 completely sectioned villi and crypts were measured, and the average value was regarded as the villous height and crypt depth in each specimen.

Expression of growth factor mRNA

Total RNA was extracted from 500 mg weight tissue of the middle small intestine by the acid guanidium thiocyanate/phenol/chloroform method,[23] and mRNA was subsequently extracted by poly(A) $^+$RNA by oligo(dT)-cellulose chromatography. The mRNA extraction was incubated at 65℃ for 10 minutes, and then chilled on ice. 20 microlitters of the solution was reverse transcribed at 37℃ for 60 minutes in 13 μl buffer containing 45 mM Tris (pH 8.3), 68 mM KCl, 15 mM DTT, 9 mM $MgCl_2$, 0.08 mg/ml BSA, 1.8 mM each of dATP, dCTP, dGTP, and dTTP, 1 μg of random pd$(N)_6$ primer, 20 U RNasin ribonuclease inhibitor, and 20 UM-MLV reverse transcriptase (Pharmacia Biotech Inc). cDNA products were subsequently amplified by PCR in 50 μl of 10 mM Tris pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each of dATP,

dCTP, dGTP, and dTTP, and 5.0 U Taq polymerase (Takara, Otsu, Japan) in the presence of 10 pmol each of 5' and 3' primer. The specific primers and the targeted sites for each growth factor are indicated in Table 1. The temperature profile of the amplification consisted of 30 seconds denaturation at 94℃, 30 seconds annealing at 55℃, and 1 min extension at 72℃. The reaction was subsequently followed by 7 min of final extension at 72℃. All the PCR procedures were performed using a GeneAmp 2400 (Perkin Elmer Inc). Each sample was initially amplified by 25 cycles, and the amplified products were electrophoresed on 2% agarose gels (SeKem ME, FMC BioProducts, ME, USA), and stained with ethidium bromide. The PCR was repeatedly performed by step-increase in cycles for each GF, until any band could be visualized, and PCR products were blotted on Hybond-N+positively charged nylon membrane (Amersham Life Science). PCR product was crosslinked and fixed to the membrane under transilluminator for two to three minutes 312 nm wavelength UV (Stratalinker, Stratagene), and dried for one hour. Then the membrane was hybridized with specific oligonucleotid probes under 50℃ for 12 hours. Each oligonucleotid probe was labeled 5'-end with digoxigenin (Boehringer Mannheim). The hybrid molecules of the hybridized membrane were visualized by the chemiluminescent reagent CSPD on a conventional X-ray film (CSPD: Disodium 3-4-methoxyspiro {1, 2-dioxetane-3, 2'-(5'-chloro) tricyclo [3. 2. 1. 1] decan} -4-yl phenyl phosphate dilution is a registered trademark of Tropix Inc., Bedford, MA, USA). Signal intensity was measured with a computerized image system (National Institute of Health Image Version 1.55, by Dr. Wayne Rasband). Analyzed data revealed integrated optical density units (IOD). The data were expressed as a ratio of IOD to control rats.

TABLE 1. Oligonucleotide primer and probe sequences

Growth factor name		5'-3'	Amplified product (bp)
Primer for RT-PCR			
TGF-β_1	sense	5'-TTTGGCTTCTAGTGCTGACGCC-3'	866
	antisense	5'-TTTTGGAGCTGAAGCAGTAGTTG-3'	
IGF-1	sense	5'-TTTTCTCTTCTACCTGGCACTCTGC-3'	876
	antisense	5'-TTTAAGTAGTTGGTGGATATTTG-3'	
HGF	sense	5'-TTTCATTGCCTTGCAGTAAGAACCG-3'	977
	antisense	5'-TTTGTAACGGAACGTCATTCTTGG-3'	
NGF	sense	5'-TCCAACAGGACTCACAGGAGC-3'	768
	antisense	5'-TCCGGAATGTAGACCAGAGC-3'	
Probe for RT-PCR product			
TGF-β_1		5'-GAGTACATTATCTTTGCTGTCACAAGAGCAGTGAGC-3'	
IGF-1		5'-GGAGGCTCCTCCTACATTCTGTAGGTCTTGTTTCCT-3'	
HGF		5'-ATGGTATTGGTGGTTCCCCTGTAACCTTCTCCTTGG-3'	
NGF		5'-GTGTGAGTCGTGGTGCAGTATGAGTTCCAGTGCTTG-5'	

Statistical analyses

The raw data were expressed as the mean±SEM. When comparing values between the groups, Student's t-test were used. Probabilities <0.05 were considered significant.

RESULTS

Scanning electron microscopy

In rates with intracolonic indomethacin, the villi in the mid-small intestine was distorted with tortuous fashion two hours after indomethacin treatment. These changes were predominantly observed in the mesenteric side within the intestinal lumen. The villous surface was swollen, and fine structure was distorted. These tortuous alteration in villous architecture was observed in all animals treated by indomethacin, whereas it was not obvious in vehicle-treated rats (Fig 1A, 1B). At six hours after indomethacin treatment, the villous component became obscure.

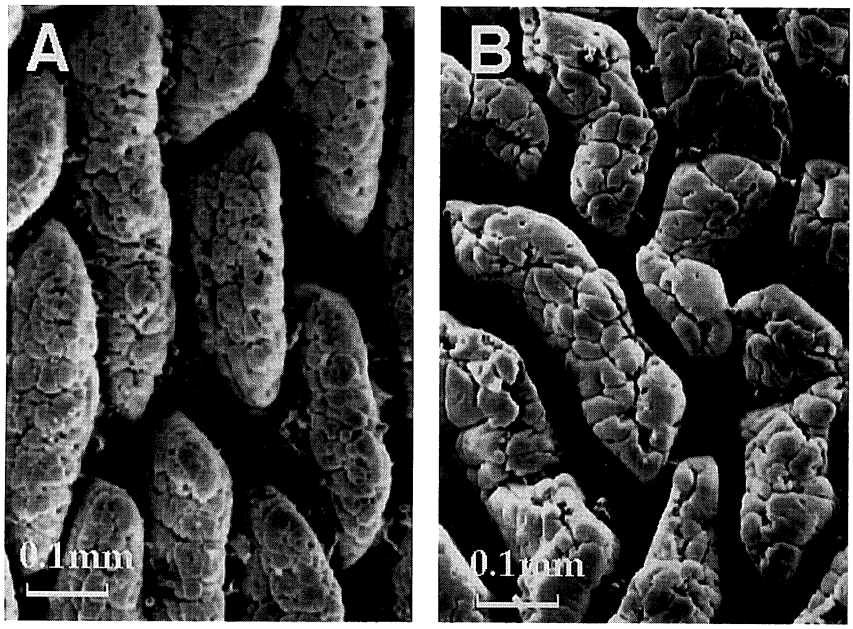

Fig 1. Scanning microscopy obtained two hours after treatment of intracolonic vehicle (A) and indomethacin (B). The villous components are distorted in shape with tortuous configuration and the villi is swollen in a rat with indomethacin (B). There are no such alteration in villous architecture in a rat treated by vehicle (A).

Mucosal measurements and crypt proliferation

The villous height and crypt depth at two and six hours after indomethacin induction or vehicle treatment are indicated in Table 2. At two hours after the treatment, both the villous height and crypt depth values at three segments within the small intestine were lower in the indomethacin-treated group than in the controls treated with the vehicle. In particular, the crypt depth of the mid-small intestine in indomethacin-treated group (13.4 ± 0.2 μm) was significantly shorter than that in the control group (15.2 ± 0.6 μm, $p < 0.05$), while the differences were not statistically significant either in the jejunum or in the ileum. Measurements of villous height and crypt depth at six hours after indomethacin treatment were still smaller in the indomethacin-treated group than in the controls, but the differences were not significant at any site within the small intestine.

Comparison of the labelling indices of the crypt cells is shown in Fig 2A and 2B. At two hours after indomethacin treatment, there were significant

TABLE 2. Comparison of villous height and crypt depth between control and indomethacin-treated rats

	Control rats		Indomethacin-treated rats	
	Villous height (μm)	Crypt depth (μm)	Villous height (μm)	Crypt depth (μm)
2 hour	(n=6)		(n=6)	
Jejunum	42.7±1.6	15.4±1.4	40.6±1.6	13.5±0.8
Mid-small intestine	40.3±3.7	15.2±0.6	34.5±1.7	13.4±0.2*
Ileum	25.0±0.6	16.1±0.5	22.8±1.3	14.7±0.8
6 hour	(n=6)		(n=6)	
Jejunum	48.5±1.9	13.8±0.3	44.6±1.2	13.2±0.4
Mid-small intestine	36.7±0.5	13.7±0.6	36.3±1.3	14.0±0.4
Ileum	23.6±1.0	14.3±0.8	27.1±0.7	14.7±0.2

*Statistically different when compared to controls.

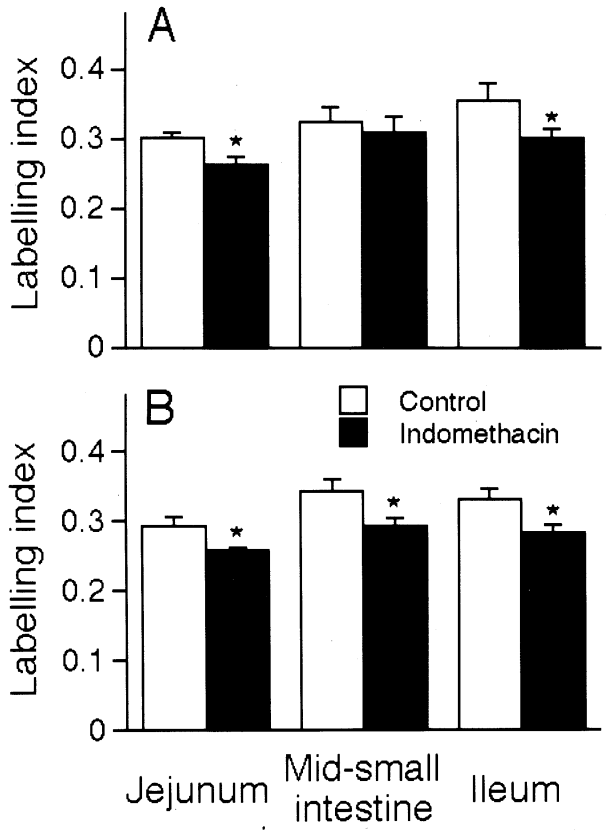

Fig 2. Comparison of the labelling index of crypt cells within the small intestine in rats at two hours (A) and six hours (B) after indomethacin or vehicle treatment. Each group consisted of six animals. At two hours after induction, the labelling indices of the jejunum and the ileum had significantly decreased as compared with those of the vehicle-treated controls. The value had not decreased at the mid-small intestine (A). The labelling index was significantly decreased at all sites within the small intestine at six hours after indomethacin treatment (B).

decreases in the labelling index in the jejunum and the ileum when compared with controls (0.26±0.011 vs 0.30±0.009, p=0.026 for the jejunum, and 0.30± 0.013 vs 0.36±0.025, p=0.041 for the ileum) (Fig 2A). However, there was no difference in the labelling index of the mid-small intestine between the indomethacin-treated rats and controls (0.31±0.023 vs 0.32±0.020, p=0.636). At six hours after the treatment, the labelling index was significantly lower in the indomethacin-treated group than in the controls at all sites (Fig 2B).

mRNA expression of growth factors

Amplified products were checked by agarose minigel electrophoresis and visualized by ethidium bromide staining (Fig 3). Individual results of dot blot analysis (Fig 4) and semiquantitative comparison of each GF mRNA (Fig 5) are indicated in the figures. TGF-β_1, IGF-1 and HGF increased in a time-dependent manner. TGF-β_1 expression was two-fold greater at six hours (three hours : 1.61±0.50, six hours : 2.02±0.49). IGF-1 was also increased at six

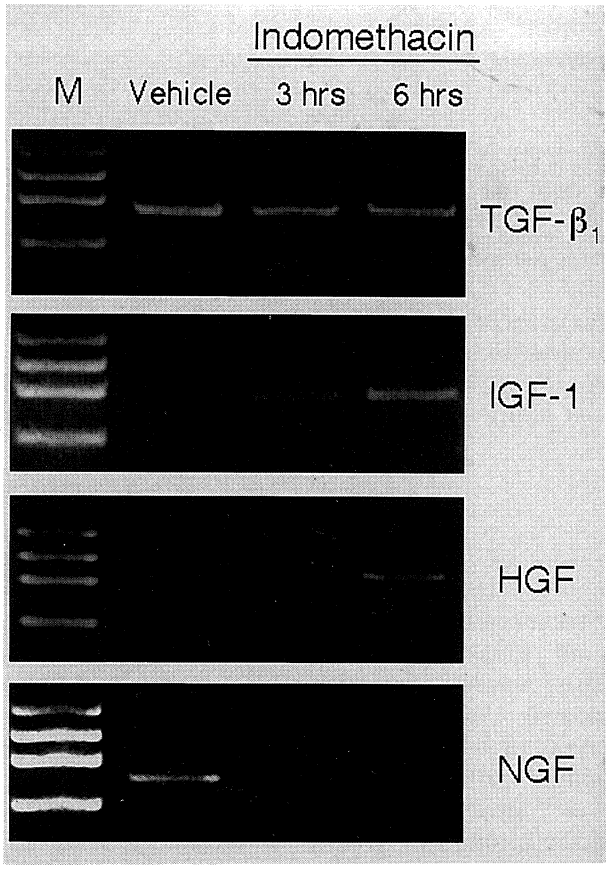

Fig 3. RT-PCR detection of mRNA expression of growth factors in the mid-small intestine in a vehicle-treated control rat and an indomethacin-treated rat. Lane M indicates the molecular weight band (the molecular weight marker used was DRIgestIII : Pharmacia Biotech). TGF-β_1 could be detected in the control and indomethacin-treated rats. IGF-1 and HGF were expressed in the indomethacin-treated rats, and the expressions of these GFs were more obvious at six hours after indomethacin treatment. In contrast, the expression of NGF was obscure after indomethacin administration.

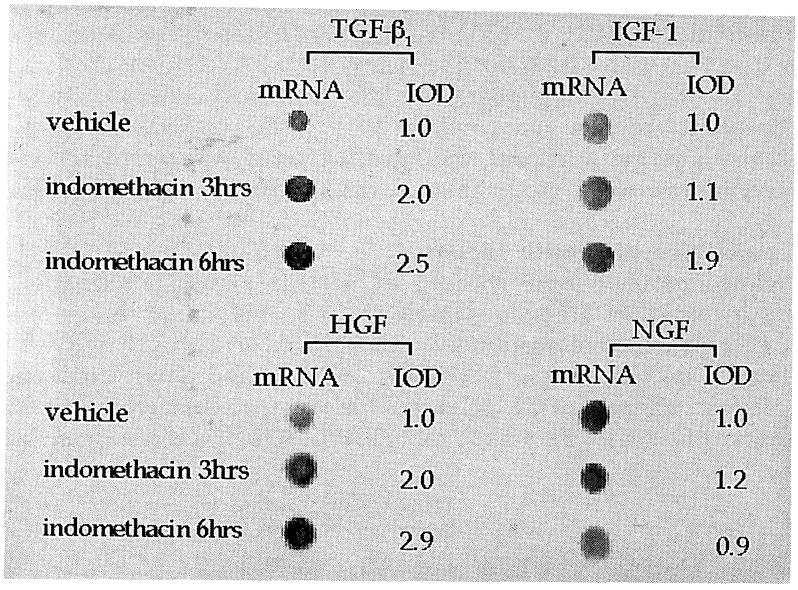

Fig 4. This figure shows representative dot blot analysis data from RT-PCR products. This blot analysis used the southern blotting method, and mRNA blotting samples revealed complemental DNA for each GF RT-PCR product. The hours indicate the time progression after intracolonically indomethacin administration, and the vehicle was the control rat data, which established semi-quantitative standards. IOD are optical density units used for measurements in image analysis methods.

hours after indomethacin treatment (three hours : 1.40 ± 0.27, six hours : 1.56 ± 0.24). HGF expression increased most rapidly during the early phase after treatment (three hours : 1.57 ± 0.26, six hours : 2.53 ± 0.833). NGF slightly increased at three hours (1.74 ± 0.68), but NGF did not increase at six hours (1.67 ± 0.66).

DISCUSSION

The mechanisms of the pathogenesis of NSAIDs-induced enteropathy in experimental animals have been explained from two points of view, one being the enhanced leukocyte-endothelial interactions[5,6,10] and the other early microvascular and smooth muscle involvement.[13] In those experiments, however, the early mucosal alterations induced by NSAIDs have not been sufficiently elucidated. In the present study, we could identify alterations in villous and crypt architectures which occur early in the phase after indomethacin administration.

Nygård *et al*[13] described that vertical alignment of the villous smooth muscle cells is the prominent feature at the early phase of indomethacin enteropathy. The initial decrease in crypt depth at the mid-small intestine, but not in the other sites within the small intestine, in indomethacin-treated rats suggests that alteration in both villous and crypt architectures is one of the initial features of this enteropathy, since the route of indomethacin administration employed for the present study results in severe damage in the mid-small intestine.[21,24] Furthermore, since scanning microscopy could identify

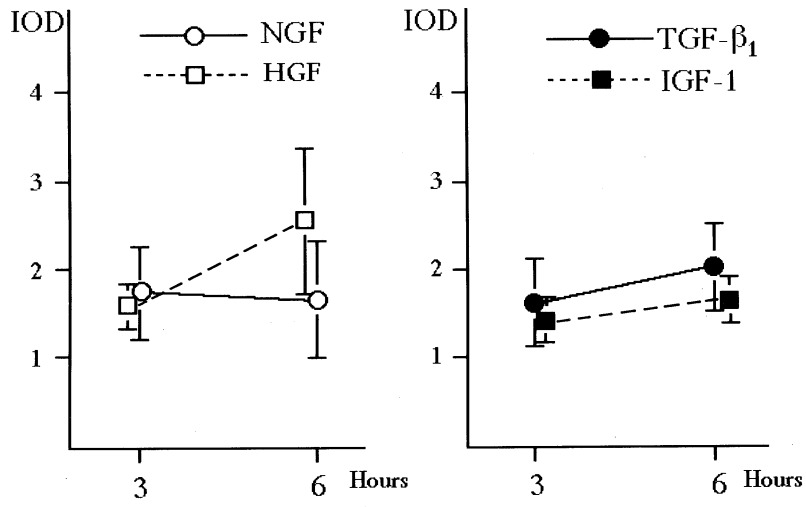

Fig 5. The vertical axis indicates IOD measurements which were used for the blotting results of GFs after RT-PCR amplification products. The horizontal axis indicates the time course after indomethacin administration (three, six hours). The figures are the mean SEM of five sample IOD measurements. (Final IOD data=sample IOD/control IOD) (open circle NGF, open square HGF, closed circle TGF-β_1, closed square IGF-1)

tortuous change in the villous structures in the present study, as well as in our pevious investigation,[25] it can be assumed that smooth muscle involvement also causes horizontal alignment of the villous structure.

Indomethacin and other NSAIDs have been known to arrest cell cycles.[14] Since intestinal crypts are composed of cells which are characterized by rapid cell proliferations, the crypts are prone to be easily affected by indomethacin. Our observations confirmed that indomethacin decreases crypt cell proliferation within six hours after indomethacin treatment. However, the decrease in proliferation occurred regardless of early morphological changes in the crypts and villi, thus suggesting a proliferation-independent pathway in the pathogenesis of the early morphologic changes of indomethacin enteropathy. There remains, however, a possibility that the decrease in crypt proliferation takes part in the subsequent progression of the mucosal damage, because the suppression of cell proliferation results in enhanced cell losses from the mucosa.[26]

Since NSAIDs suppress accelerated intestinal cell restitution induced by TGF-β_1 and HGF,[16] it seems possible that endogenous eicosanoids regulate expression of GFs. The extracellular matrix may play an important role in wound repair. In particular, TGF-β_1 promotes increases in the extracellular matrix from fibroblast and epithelial cell populations. Furthermore, TGF-β_1 stimulates migration and restitution of experimental intestinal epithelial monolayers.[16] HGF accelerates epithelial cell restitution[27] and epithelial cells are the source of HGF in rats treated with indomethacin.[28] HGF plays an important role in informational transfer between the epithelium and the submucosa, and migration of the epithelium.[27] IGF-1 has been overexpressed

in a rat model of chronic granulomatous enterocolitis,[18] and it increases crypt cell proliferation.[29] It has been recently confirmed that the binding site of IGF increased within the muscularis propria in a rat model of chronic colitis.[30] Thus, the enhanced expression of GFs in the present study can be regarded as an extremely rapid response against acute intestinal damage. Inflammatory cells can also be the source of TGF-β_1, HGF and IGF-1, because indomethacin induces neutrophil infiltration 6 hours after administration.[13]

The GF profiles detected in the present study identified that the pattern of NGF mRNA expression was different from those of other GFs at the early phase. NGF has been initially reported as a neurotrophic factor, but subsequent investigation has confirmed that NGF also binds to muscle cells.[31] Thus, our observation raises the issue that the expression of NGF may rather correlate with an extremely early event, such as the smooth muscle contraction, in indomethacin enteropathy. Furthermore, since this GF has anti-inflammatory properties in experimental inflammation,[20] insufficient expression of NGF may contribute rather adversely in this model of enteropathy at an early phase.

Our findings indicated that early morphologic damage in indomethacin-enteropathy is characterized by alteration in villous architecture and shortenings in crypt depth, and that these phenomena were independent of decrease in crypt cell proliferation. While other GFs were overexpressed immediately after the treatment, NGF mRNA expression showed a different pattern. Although other factors within the intestinal lumen, such as bacteria[31,33] food content[24,34,35] and bile acid[36] are presumed to play important roles in the development of pronounced mucosal injuries, studies of regulation in GFs and cytokines in this early damage seem to provide a clue to an understanding of the pathogenesis of indomethacin enteropathy.

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