Biochemical and Biophysical Research Communications 424 (2012) 124–129

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Rebamipide induces dendritic cell recruitment to *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG)-exposed rat gastric mucosa based on *IL-1β* upregulation

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ARTICLE INFO

Article history:
Received 5 June 2012
Available online 23 June 2012

Keywords:
Rebamipide
Gastric cancer
N-methyl-*N*′-nitro-*N*-nitrosoguanidine
Dendritic cell
IL-1β
CD68

ABSTRACT

Rebamipide is usually used for mucosal protection, healing of gastric ulcers, treatment of gastritis, etc., but its effects on gastric malignancy have not been elucidated. Using Lewis and Buffalo rat strains treated with peroral administration of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), we evaluated the effect of rebamipide on the induction of tumor-suppressive dendritic cells, which are known to be heterogeneous antigen-presenting cells of bone marrow origin and are critical for the initiation of primary T-cell responses. Using CD68 as a marker for dendritic cells, the stomach pyloric mucosae of Lewis and Buffalo rats were immunohistochemically analyzed in the presence or absence of rebamipide and MNNG. After a 14-day treatment of rebamipide alone, no significant change in number of CD68-expressing cells was detected in either rat strain. However, after concurrent exposure to MNNG for 14 days, treatment with rebamipide slightly increased CD68-positive cells in the Lewis strain, and significantly increased them in the Buffalo strain. Analysis of two chemotactic factors of dendritic cells, IL-1β and TNF-α, in the gastric cancer cells showed that expression of *IL-1β*, but not *TNF-α*, was induced by rebamipide in a dose-dependent manner. A luciferase promoter assay using gastric SH-10-TC cells demonstrated that an element mediating rebamipide action exists in the *IL-1β* gene promoter region. In conclusion, rebamipide has potential tumor-suppressive effects on gastric tumorigenesis via the recruitment of dendritic cells, based on the upregulation of the *IL-1β* gene in gastric epithelial cells.

1. Introduction

Rebamipide, an amino acid analog of 2(1H)-quinolinone, is clinically used for mucosal protection, healing of gastric ulcers, and treatment of gastritis [1,2]. The healing effects depend on the enhancement of mucosal defense, scavenging free radicals, and activation of genes such as cyclooxygenase-2 and some growth factors [2]. In the view of carcinogenesis, however, reports concerning the effects of rebamipide on gastric malignancy have been scarce. In this study, we focused on the recruitment of dendritic cells to examine whether rebamipide has a tumor-suppressive effect on gastric mucosa.

Dendritic cells are heterogeneous antigen-presenting cells of bone marrow origin that are critical for the initiation of primary T-cell responses [3,4]. They are thought to play key roles in tumor-specific immune responses via (1) cross-priming of tumor cells and dendritic cells, (2) presentation of tumor antigens through MHC class I, and (3) the generation of CD8+ cytolytic T-cells [4]. We have previously demonstrated that dendritic cells infiltrate the mesenchymal layer of rat stomach during chemical carcinogenesis induced by administration of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) [5]. In our previous study using ACI (sensitive to chemically induced carcinogenesis) and Buffalo (resistant to chemically induced carcinogenesis) rat strains, we observed

Abbreviations: MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α.

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0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.06.087

a negative correlation between the susceptibility to MNNG-induced carcinogenesis and induction of dendritic cells [5,6].

In the present study, we examined the effect of rebamipide on the recruitment of dendritic cells to the gastric mucosa, using CD68 as a dendritic cell marker [7,8]. In addition, focusing on the candidate chemotactic factors of dendritic cells, we examined the effect of rebamipide on gastric cells by analyzing the gene expression of two cytokines, IL-1β and TNF-α, involved in dendritic cell mobilization. Our study should shed light on a novel effect of rebamipide on the precancerous gastric mucosa.

2. Materials and methods

2.1. Animals

Forty male 6-week-old Lewis rats (LEW/Crj; Charles River Japan, Inc., Yokohama, Japan) and 40 male 6-week-old Buffalo rats (BUF/NacJCl; Nihon Clea, Tokyo, Japan) were used. Based upon the combination of drinking water and food (CRF1, Oriental. Yeast Co., Ltd., Tokyo, Japan) provided, the rats were divided into four groups, with 10 rats in each group, as follows: (1) water with 120 mg/l of MNNG (Sigma–Aldrich, St. Louis, MO) and food with 0.25% w/w rebamipide (Sigma–Aldrich), (2) water with 120 mg/l of MNNG and normal food, (3) normal water and food with 0.25% w/w rebamipide, and (4) normal water and normal food. Treatment of animals used in this study adhered to the Declaration of Helsinki.

2.2. Immunohistochemistry

Deparaffinization, endogenous peroxidase inactivation, and antigen retrieval of rat stomach tissues were performed as described previously [9,10]. Immunostaining with anti-CD68 mouse monoclonal antibody (Clone: KP1; DAKO, Tokyo, Japan) at a 1:200 dilution was performed, followed by visualization with 20 mg/dl 3,3′-diaminobenzidine tetrahydrochloride (DAKO) solution containing 0.006% hydrogen peroxide. The immunostained sections were evaluated independently by two pathologists along with hematoxylin and eosin-stained sections from the same lesions.

2.3. Cell cultures

Twenty gastric cancer cell lines (NUGC-4, AZ521, KATO-III, SH-10-TC, MKN-7, H-III-TC, MKN-1, MKN-45, MKN-74, TGBC11TKB, KE-39, KE-97, GCIY, HGC-27, HuG1-PI, HuG1-N, AGS, NCI-N87, ECC-10, ECC-12), 10 colorectal cancer cell lines (WiDr, DLD-1, SW480, COLO320DM, HCT116, HCT-15, SW620, LS174T, LOVO, HT-29), and two non-gastrointestinal cancer cell lines (HeLa-S3 and MDA-MB435) were maintained in DMEM with 10% fetal calf serum (Gibco/Invitrogen, Carlsbad, CA) at 37 °C, as previously reported [11]. Rebamipide at concentrations of 6, 2, 0.7, and 0 mM in culture medium (DMEM with 10% fetal calf serum) was used for treatment.

2.4. RT-PCR

Total cellular RNAs were prepared using the Isogen RNA isolation reagent (Wako Pure Chemical Industries, Osaka, Japan) as previously reported [12]. Semi-quantitative RT-PCR was performed via a Superscript One-Step reaction using the Platinum Taq (Invitrogen). The primer pairs used to detect the transcripts of *TNF-α*, *IL-1β*, and *GAPDH* were as follows: 5′-gtgcttgttcctcagcctct-3′ and 5′-ttgatggcagagaggaggtt-3′ for *TNF-α*, 5′-tccagggacaggatatggag-3′ and 5′-ccctagggattgagtccaca-3′ for *IL-1β*, and 5′-accacagtccatgccatcac-3′ and 5′-tccaccaccctgttgctgta-3′ for *GAPDH*. RNA was reverse-transcribed for 30 min at 50 °C, and after an initial denaturation at 94 °C for 3 min, cDNA amplification procedures were performed as follows: for *TNF-α* and *IL-1β*, 40 cycles of 94 °C for 30 s, 58 °C for 1 min, and 72 °C for 1 min; for *GAPDH*, 24 cycles of 94 °C for 30 s, 58 °C for 1 min, and 72 °C for 1 min.

2.5. Luciferase promoter assay

SH-10-TC cells were seeded in 96-well (0.32 cm^2) plates in a semi-confluent manner in the presence or absence of 6 mM rebamipide in medium for 24 h. They were then transfected with 6.5 ng of Renilla luciferase control vector pGL4.74 (Promega, Madison, WI), and either 200 ng of firefly luciferase experimental vector pGL4.12 (Promega, negative control), pGL4.12_IL-1β-P1 (upstream 1062 bp from the exon 1 of *IL-1β*), pGL4.12_IL-1β-P2 (upstream 513 bp), pGL4.12_IL-1β-P3 (upstream 131 bp), or pGL4.12-TK (positive control). All transfections were performed with Lipofectamine Reagent and Lipofectamine PLUS (Invitrogen). Luciferase activities were measured at 48 h post-transfection using the Dual Luciferase Reporter Assay System (Promega).

2.6. Constructions

To construct the vectors for reporter assays, a 1500-bp upstream sequence from the 5′-end of exon 1 of the *IL-1β* gene was first amplified from genomic DNA derived from SH-10-TC cells using the primers 5′-atacttgcacagaggctcac-3′ and 5′-gattggctgaagagaatccc-3′. The amplified products were cloned into pT7blue T-vector cloning vectors (TAKARA Bio Inc., Shiga, Japan) to generate pT7Blue-IL-1β-P0. The 1.6-kb DNA BamHI/SpeI fragment was blunted with Klenow fragment (TAKARA) and inserted into the EcoRV site of pGL4.12 (Promega) to generate pGL4.12-IL-1β-P0. After the 5.5-kb XhoI/SmaI fragment derived from pGL4.12-IL-1β-P0 was blunted with Klenow fragment, self-ligation was performed to generate pGL4.12-IL-1β-P1 (upstream 1062 bp). The 5.0-kb SacI fragment derived from pGL4.12-IL-1β-P0 was also self-ligated to generate pGL4.12-IL-1β-P2 (upstream 513 bp). The 0.15-kbp HindIII/BamHI fragment derived from pT7Blue-IL-1β-P0 was inserted into EcoRV site of pGL4.12 to generate pGL4.12-IL-1β-P3 (upstream 131 bp). For positive control, the 0.8 kb HindIII/KpnI fragment (human simplex virus thymidine kinase gene promoter) derived from pGL4.74 (Promega) was inserted into HindIII/KpnI site of pGL4.12 to generate pGL4.12-TK.

3. Results

3.1. Treatment with rebamipide increases the number of dendritic cells in MNNG-exposed rat gastric mucosa

To evaluate the effect of rebamipide on the gastric mucosa, we analyzed the expression of CD68-positive cells in the stomach pyloric mucosa of Lewis and Buffalo rat strains. CD68 is an established marker of dendritic cells, and CD68-positive cells in the gastric mucosa can be considered as dendritic cells [7,8,13]. After the 14-day treatment of rebamipide alone, an obvious microscopic change of the gastric mucosa could not be detected in both Lewis and Buffalo strains. There were also no significant differences in the number of CD68-positive cells with or without the 14-day treatment of rebamipide (Fig. 1A and 1B).

We next analyzed the effect of rebamipide on the recruitment of CD68-positive cells in the stomach pyloric mucosa of the MNNG-exposed Lewis and Buffalo rats. Consistent with previous reports [14,15], neither intestinal metaplasia nor adenocarcinoma could be observed in the gastric mucosa after the treatment of MNNG for only 14 days. We have already reported that the number of dendritic cells in the rat stomach pyloric mucosa increase after

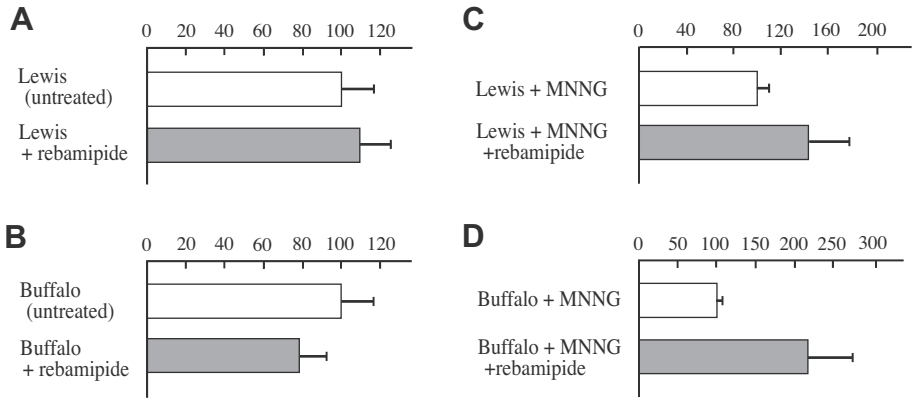

Fig. 1. Numbers of CD68-positive cells in the gastric pyloric mucosa of Lewis and Buffalo rat strains in a single microscopic view (200×). Data were obtained from the eight groups of 10 rats as follows: (A) Lewis untreated (control) and Lewis treated with rebamipide for 14 days; (B) Buffalo untreated (control) and Buffalo treated with rebamipide for 14 days; (C) Lewis treated with MNNG alone for 14 days and Lewis treated with a combination of MNNG and rebamipide for 14 days; and (D) Buffalo treated with MNNG alone for 14 days and Buffalo treated with a combination of MNNG and rebamipide for 14 days. In each panel, the number of CD68-positive cells was standardized to that of the rats without rebamipide treatment. About 120 mg/l of MNNG in drinking water and 0.25% w/w of rebamipide in food were used as treatments. The error bars indicate the standard errors based on the data obtained from corresponding 10 rats.

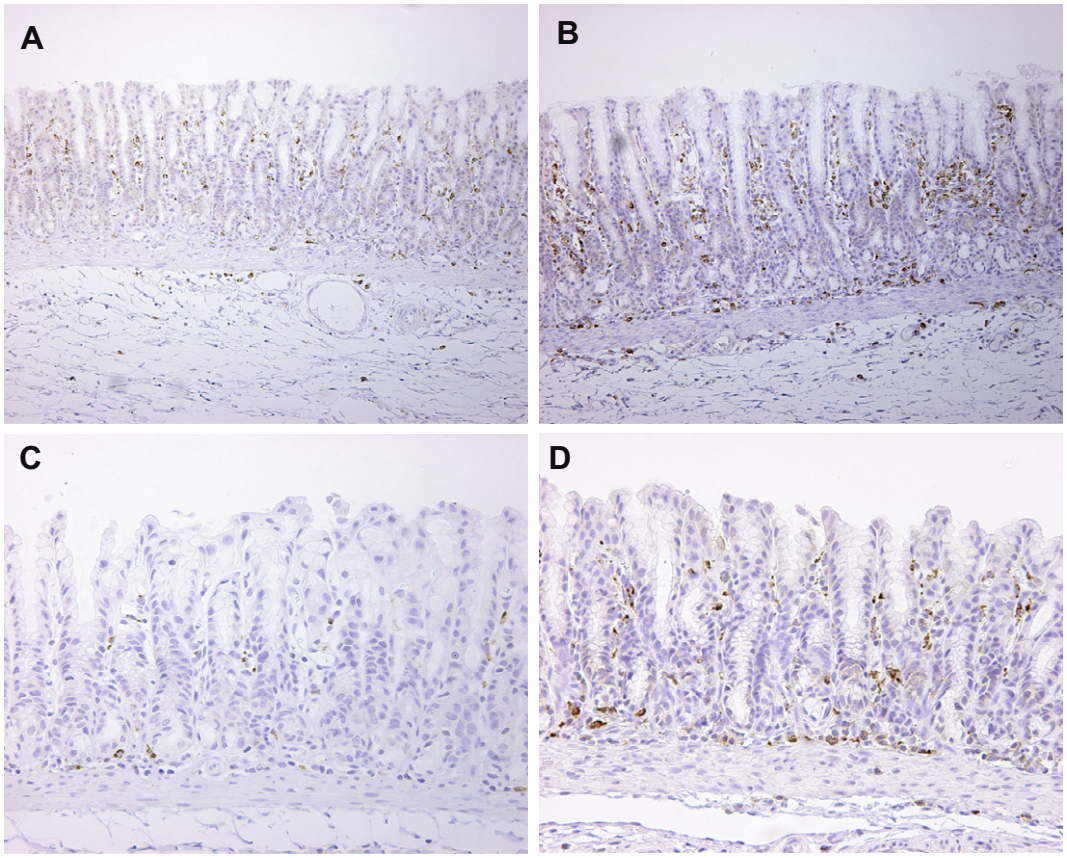

Fig. 2. Immunohistochemical staining of CD68 in gastric pyloric tissue sections obtained from Lewis and Buffalo rats. Magnification 200×. (A) Lewis strain rats treated with MNNG alone for 14 days. (B) Lewis strain rats treated with a combination of MNNG and rebamipide for 14 days. (C) Buffalo strain rats treated with MNNG alone for 14 days. (D) Buffalo strain rats treated with a combination of MNNG and rebamipide for 14 days. About 120 mg/l of MNNG in drinking water and 0.25% w/w of rebamipide in food were used as treatments.

exposure to MNNG [5]. In the Lewis strain, induction of CD68-positive cells seemed to be stronger with rebamipide treatment (Fig. 2A and 2B), although statistical significance could not be obtained (Fig. 1C). As for the Buffalo strain, a significant increase in CD68-positive dendritic cells was detected with the rebamipide treatment (Figs. 2C, 2D and 1D). From these results, we concluded that oral rebamipide intake has the potential to induce the migration of dendritic cells to the gastric mucosa. Rebamipide and MNNG possibly have an addictive or multiplier effect on the dendritic cell migration.

3.2. Treatment with rebamipide activates the transcription of IL-1β, a potent chemotactic factor for dendritic cells, in various gastric cell lines

It is well established that dendritic cells are mobilized in response to a large variety of chemical, physical, or biological stimuli.

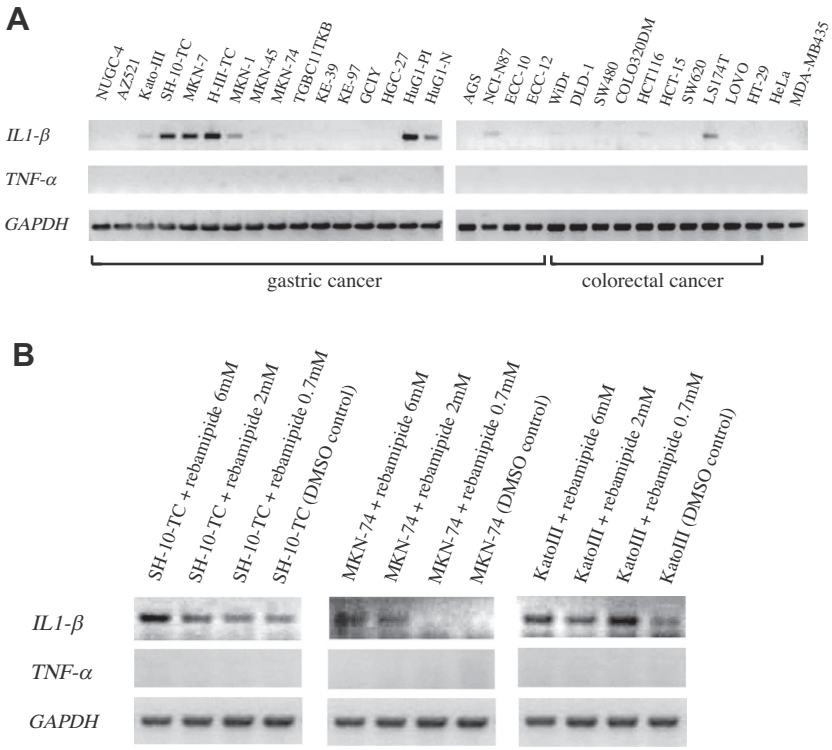

Fig. 3. (A) Expression patterns of *IL-1β*, *TNF-α*, and *GAPDH* mRNAs in a panel of 32 human cancer cell lines. Twenty gastric cancer cell lines, 10 colorectal cell lines, and two non-gastrointestinal cell lines (HeLa-S3 and MDA-MB435) were analyzed by RT-PCR. (B) Expression of *IL-1β*, *TNF-α*, and *GAPDH* after a 2-day treatment of rebamipide at various concentration (6, 2, 0.7, or 0 mM). Three gastric cancer cell lines (SH-10-TC, MKN-74, and KATO-III) were analyzed by RT-PCR.

Despite their diversity, most of the mobilization signals appear to exert their activity through a pair of intermediate messenger cytokines, IL-1β and TNF-α [16]. IL-1β and TNF-α are not only required but are also sufficient for dendritic cell mobilization, as subcutaneous administration of either cytokines alone promotes rapid migration of dendritic cells to lymph nodes in the absence of other stimuli [17,18].

To elucidate the mechanism of rebamipide-induced dendritic cell recruitment, expression patterns of both cytokines were analyzed using twenty gastric cell lines and 10 colorectal cell lines. *TNF-α* mRNA was barely detected in these gastrointestinal tumor cells (Fig. 3A), suggesting that the expression of TNF-α is strongly suppressed in cells of the alimentary tract. In contrast, *IL-1β* gene transcript was detected in several gastric cancer cells (Fig. 3A); we speculate that IL-1β may be a major inducer of dendritic cells during gastric canceration.

Next, we analyzed the expression of *IL-1β* and *TNF-α* in the presence of rebamipide at various concentrations using three gastric cancer cell lines: SH-10-TC, MKN-74, and KATO-III. As was expected, marked upregulation of *IL-1β* expression, but not *TNF-α* expression, was detected in an approximately dose-dependent manner (Fig. 3B). From these results, we concluded that rebamipide treatment activates transcription of *IL-1β* in gastrointestinal cells.

3.3. The element mediating rebamipide action in gastric cells exists in the promoter region of IL-1β gene

To elucidate the mechanism of rebamipide-induced *IL-1β* upregulation, we designed a series of reporter constructs using the upstream sequence of *IL-1β* gene and performed a luciferase assay in SH-10-TC cells (Fig. 4). The upstream 131 bp (P3) alone did not express the reporter luciferase gene, whereas the upstream 513 bp region (P2) had an obvious promoter activity (Fig. 4B). These results suggest that the sequence between −513 and −131 of the *IL-1β* promoter has promoter activity in the gastric cells, although no difference was seen between the rebamipide-treated cells and rebamipide-untreated cells (Fig. 4B). The upstream 1062 bp region (P1) also had an evident promoter activity, which got stronger in the presence of rebamipide.

As was expected, the three reporter constructs (P1, P2, and P3) did not show any transcriptional activity in IL-1β-deficient MDA-MB435 cells originated from breast cancer (Fig. 4B). From these results, we concluded that an element mediating rebamipide action, capable of increasing transcriptional activity in gastric cells, exists in the *IL-1β* promoter region from −1062 to −513. This result reinforces our speculation that rebamipide-induced dendritic cell recruitment to MNNG-exposed rat gastric mucosa should be based upon the *IL-1β* upregulation.

4. Discussion

It is well established that *Helicobacter pylori*-associated chronic gastritis is a precancerous condition of gastric malignancy [19,20]. Although the effects of rebamipide on gastric malignancy have been barely elucidated, there have been some reports concerning its tumor-suppressive effects via an influence on gastritis or gastric cancer cells. For instance, it was reported that rebamipide inhibits the production of neutrophil chemokines (CINC/KC) and TNF-α, and consequently prevents the development of chronic gastritis [21]. In the gastric cancer mouse model induced by *N*-methyl-*N*-nitrosourea (NMU) treatment and *Helicobacter pylori* infection, it was also reported that long-term rebamipide administration inhibits its upregulation of oncogenic proteins and down-regulation of anti-oncogenic proteins in gastric cells [22]. In addition, it was recently reported that rebamipide treatment inhibits gastric cancer

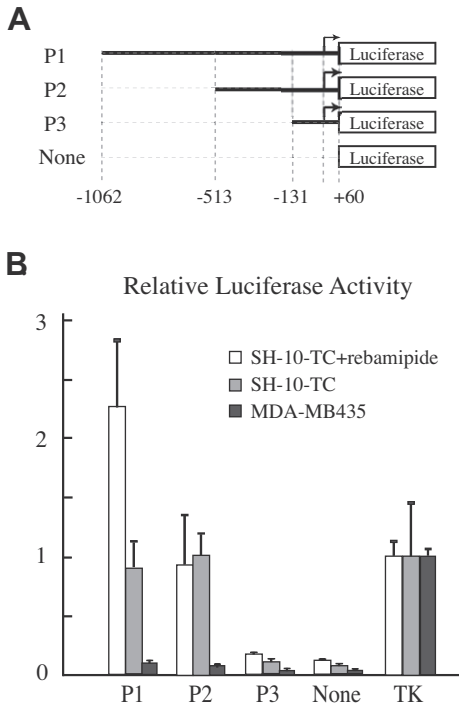

Fig. 4. (A) Schematic representation of the human *IL-1β* gene reporter constructs used in this study. The numbers represent base pairs measured from the 5′-end of the first exon of the *IL-1β* gene (+1), which is denoted as a rectangular arrow in each construct. (B) Transcriptional activities of a series of *IL-1β* promoter constructs in gastric SH-10-TC cells in the presence or absence of rebamipide. As a positive control, pGL4.12-TK (TK) harboring the human simplex virus thymidine kinase gene promoter was used. The promoter activities of MDA-MB435 cells deficient in *IL-1β* expression are also shown. Luciferase activities were measured 24 h after transfection, and the data shown are the mean values of triplicate experiments with the error bars corresponding to the standard errors.

cell growth [23,24]. These results suggest that rebamipide has some tumor-suppressive effects on gastric canceration.

In our present study, we discovered another possible anti-oncogenic effect of rebamipide upon gastric tumorigenesis, through the recruitment of dendritic cells capable of inducing tumor-specific immune responses [4]. There is a strong correlation between infiltration of dendritic cells to the tumor lesion and better prognosis in various cancers, including lung cancer [25], breast cancer [26], and hepatocellular carcinoma [27]. As for gastric cancer, there have also been several reports showing a strong correlation between the recruitment of dendritic cells and better prognosis [28–30]. In the MNNG-induced gastric cancer model rats treated with rebamipide, we detected a significant increase in dendritic cells in the precancerous gastric mucosa, which suggests a tumor-suppressive effect of the rebamipide administration.

We speculate that *IL-1β* transcriptional activation in gastric cells is one of the key mechanisms of the rebamipide-induced dendritic cell migration, but the precise mechanism of rebamipide on *IL-1β* gene promoter in gastric cells has not been elucidated. Contrary to our results, it was previously reported that rebamipide suppresses *IL-1β* expression in blood mononuclear cells [31]. The opposing results suggest that the transcriptional regulation of *IL-1β* gene in gastric epithelial cells is different from that of bone marrow-derived mononuclear cells. Considering the high-dose and long latent period required for the transcriptional activation of *IL-1β* gene by rebamipide, we presume that rebamipide-induced transactivation of *IL-1β* gene in gastric cells is due to an indirect effect of rebamipide via inducing some other transcription factors to the promoter region from −1062 to −513. Including validation of the possibility for direct binding of rebamipide to the *IL-1β* promoter region, the detailed mechanism of rebamipide on *IL-1β* gene transactivation needs to be resolved in the future. The variation of *IL-1β* gene regulation in different cell types is also a major problem that should be concurrently elucidated.

MNNG-treated rat stomachs and chronically *Helicobacter pylori*-infected human stomachs are quite different, but gastric mucosae in both cases are in a precancerous condition with chronic inflammation and upregulation of various cytokines [32]. We are planning to conduct a prospective cohort study of patients with chronic *Helicobacter pylori* infection, who will be treated with rebamipide or a placebo. Evaluation of dendritic cell migration in the presence or absence of rebamipide should be informative and suggestive for predicting its preventive effect on gastric tumorigenesis. Long-term follow-up focusing on gastric cancer incidence will finally provide clear evidence for our hypothesis of the tumor-suppressive effect of rebamipide.

Acknowledgment

This work was supported in part by a Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), and also by a grant from the Sato Memorial Foundation for Cancer Research.

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