

Orengedokuto and berberine improve indomethacin-induced small intestinal injury via adenosine

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Abstract

Background Recent endoscopic technology has revealed that small intestinal injury is a serious threat to patients receiving nonsteroidal anti-inflammatory drugs (NSAIDs). We previously showed that Japanese herbal medicine, Orengedokuto (OGT; Huang-Lian-Jie-Du-Tang in Chinese), protects mice from lethal indomethacin (IND)-induced enteropathy. To elucidate the mechanism of the protective effect of OGT, we performed microarray analyses and high power statistical analyses of microarray data using new bioinformatics tools.

Methods Female BALB/c mice were subcutaneously injected with IND (20 mg/kg) once a day for 2 days. OGT-treated mice received a diet containing OGT from the first IND injection until the end of the experiment. Gene expression signals of small intestine were obtained with

GeneChip[®]. Analyses for overrepresentation of Gene Ontology categories were conducted using MetaGene Profiler (MGP) and the changes were visualized by Cell Illustrator Online (CIO). Furthermore, active ingredients of OGT were investigated.

Results MGP and CIO suggested a critical role for the adenosine system, especially adenosine deaminase (ADA), a key enzyme of adenosine catabolism. Quantitative real time RT-PCR and in situ hybridization showed that OGT decreased the expression of ADA, which possibly resulted in the elevation of the anti-inflammatory nucleoside adenosine. Blockade of the adenosine A2a receptor abrogated the protective effect of OGT. Berberine, a major ingredient of OGT, suppressed ADA expression and reduced the incidence of lethality.

Conclusions OGT may prevent IND-induced enteropathy by decreasing ADA which results in the elevation of adenosine. Modulation of the adenosine system may be an efficient therapeutic strategy for NSAID-induced enteropathy.

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deaminase · Microarray

Introduction

The gastroduodenal toxicity of nonsteroidal anti-inflammatory drugs (NSAIDs) is well recognized. Meanwhile recent advances in diagnostic methods, such as video capsule endoscopy (VCE) have revealed a high prevalence (2/3) of small intestinal lesions among long-term NSAID users [1–3]. NSAID may present a variety of small intestinal lesions including ulcers [4], and may cause lethal

outcomes, such as bowel obstruction, perforation, and sepsis. Thus, prevention of NSAID-induced small intestinal lesions, even those judged “subclinical” by conventional diagnosis, may be an important issue for long-term NSAID users. However, various suggested treatments [e.g. co-administration of misoprostol (PGs) or rebamipide] [5, 6] are yet to be established as clinical strategy for NSAID-induced small intestinal lesions, and furthermore COX-2 selective agents, which were anticipated to be effective according to theories concerning the pathogenesis of NSAID-induced gastrointestinal lesions, did not reduce the occurrence of small intestinal lesions in long-term NSAID users [7].

Previously we reported that Oregedokuto (OGT; Huang-Lian-Jie-Du-Tang in Chinese), a Kampo (Japanese traditional herbal) medicine, prevents indomethacin (IND)-induced lethal enteropathy in a rodent model [8]. In Japan, Kampo medicines, which are pharmaceutical grade herbal medicines manufactured on a modern industrial scale under strict quality control, are integrated into the national medical system and are ethically used by physicians educated in modern Western medicine. OGT is one such medicine and has been used for various indications including gastric ulcers, gastritis and melena. We demonstrated that the beneficial effects of OGT in the IND-induced enteropathy model were accompanied by an increase in the number of COX-2 expressing cells in lamina propria and in the production of PGE2 by isolated lamina propria mononuclear cells. However, it is known that the pathogenetic mechanism of IND-induced enteropathy depends not only on COX-PGE2 association but also on other factors including intestinal bacteria and nitric oxide [9, 10]. Furthermore, OGT is a mixture comprising four crude herbs, each of which contains an enormous number of pharmacological compounds. The biological effect of OGT is thought to be mediated by a combination of various pharmacological actions via diverse signaling pathways driven by a number of different compounds. Thus, we anticipated that additional mechanisms might be involved in the beneficial effect of OGT on the enteropathy model. Consequently, a comprehensive analysis by “omics” technologies, such as functional genomics, proteomics and metabolomics, is an essential prerequisite for investigating such a complex system.

In the present study, we performed microarray analyses using the Affymetrix GeneChip platform. High power statistical analyses of microarray data by new bioinformatics tools MetaGene Profiler (MGP) [11] and Cell Illustrator Online (CIO) [12] suggested that the adenosine system, especially adenosine deaminase (ADA), may be involved in the effect of IND and/or OGT on the small intestine. Validation by biological experiments and investigation of active ingredients were also described.

Materials and methods

Drugs and chemicals

OGT consists of crude ingredients extracted with boiling water from the following four medicinal herbs in the ratio given in parentheses: Ogon (*Scutellariae radix*; 3.0), Oren (*Coptidis rhizoma*; 2.0), Sanshishi (*Gardeniae fructus*; 2.0) and Obaku (*Phellodendri cortex*; 1.5). Spray-dried extract powders of OGT and its constituent herbs were prepared by Tsumura & Co. (Tokyo, Japan). IND and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Treatment of mice

Female BALB/c mice (7-weeks old) were purchased from Charles River Japan, Inc. (Kanagawa, Japan). The animals were housed in an air-conditioned room with a 12-h light–dark cycle under specific pathogen-free conditions. All mice were given AIN-93M powder diet (CLEA Japan, Inc., Tokyo, Japan) and water ad libitum. Prior to each experiment the mice were fasted for 24 h and then re-fed the normal diet or diets containing OGT at concentrations of 2%, which corresponds to the effective dose established in our previous study. These diets were administered throughout the experimental period. Enteropathy was induced as described in a previous study. In brief, 1 h after re-feeding, mice were subcutaneously injected (sc) with freshly prepared IND (20 mg/kg) once a day for two days.

Tissue dissection

For preparation of tissue samples, mice were sacrificed by cervical dislocation 24 h after the second IND injection and the small intestines were immediately dissected. Tissues used for morphological studies were rinsed with saline, opened longitudinally, spread flat on filter paper and fixed in 15% formalin neutral buffer solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Tissues used for RNA extraction were washed in ice-cold PBS and then immediately frozen in liquid nitrogen. Tissues used for in situ hybridization (ISH) were dissected, cut longitudinally and fixed with Tissue Fixative (Genostaff Co., Ltd) before being embedded in paraffin. All animal experiments were conducted in accordance with the institutional guidelines for the care and use of laboratory animals for research, which conform to the guidelines of the Science Council of Japan.

Morphological studies

Using a stereoscopic microscope, we counted the number of ulcers and quantified the sum of ulceration areas in the

whole small intestines by tracing the outline of the areas with an image processing program, Image J (National Institutes of Health, Bethesda, MD).

RNA extraction

Mice were sacrificed and the dissected small intestines were used for the preparation of total RNA. Each of the frozen samples was homogenized in a 1 ml/0.1 g tissue of TRI REAGENT (Sigma-Aldrich Japan, Tokyo, Japan) with a POLYTRON tissue homogenizer (Kinematica, Littau-Lucerne, Switzerland) and incubated for 10 min at room temperature. A conventional chloroform extraction and isopropanol/ethanol precipitate technique were used to isolate RNA.

Microarray analysis

Total RNA was re-purified by RNeasy spin columns (Qiagen, Valencia, CA) according to the manufacturer's instructions. All samples were monitored using an Agilent Bioanalyzer (Agilent Biotechnologies, Boeblingen, Germany) and consistently demonstrated high-quality RNA (28S/18S ratio, ~2). GeneChip analysis using an MG-U74Av2 array (Affymetrix, Santa Clara, CA) was performed according to the Affymetrix protocol. Data were analyzed using the Affymetrix Microarray Suite (MAS) v.5.0 with all of the parameters set at default values (a global normalization was applied). Probe sets that had three present A MAS detection calls per group (3 samples) in at least one of the groups were included in the analysis.

MetaGene Profiler and Cell Illustrator Online

MetaGene Profiler has been developed to evaluate the significance of predefined sets of genes from transcriptome data (<http://metagp.ism.ac.jp/>) [11]. The method accumulates statistical evidence from a set of genes in order to build a more powerful test than can be achieved by analyzing individual genes. In the present study, we first predefined the group of genes for each gene ontology (GO) term [all three categories, i.e., biological process (BP), cellular component (CC) and molecular function (MF), were used]. The number of gene sets annotated by GO terms was over 20,000. To obtain the *P*-values for individual genes, Welch's *t* test was performed. The individual *P*-values of the genes included in the gene set were integrated to obtain "the integrated *P*-value for the gene set", as described previously (<http://metagp.ism.ac.jp/>). A gene set containing too small a number of genes is, in principle,

unsuitable for evaluation of over-represented gene sets. Preliminary examination suggested GO terms containing more than 100 genes provided relatively little information because these terms represent too broad a concept to give a foothold for further biological investigation. Therefore MGP analysis was applied to the gene sets consisting of 4–99 genes. As described in the results section, the above-mentioned analysis suggested a possible involvement of the adenosine system in the pathogenesis of IND-induced enteropathy and its amelioration by OGT. Therefore additional analysis using MGP was performed on the gene sets representing distinct facets of the adenosine system, that is, purine metabolism, adenosine metabolism and/or signaling, three types of adenosine-related apoptosis and literature analysis of ADA. CIO is a graphic platform for modeling and simulating signaling pathways (<http://www.cellillustrator.com>) [12].

Real-time RT-PCR

Real time RT-PCR was performed in two laboratory sites where different PCR methodologies were used: the TaqMan[®] Gold RT-PCR Kit without controls (Applied Biosystems, Foster City, CA), and the QuantiFast[™] SYBR Green PCR kit (Qiagen) according to the manufacturer's instructions. These two assays gave essentially the same results. For TaqMan[®] assay, combinations of probes and primers of Mm01247822_m1 for ADA and Mm00607939_s1 for beta-actin were used. Real time PCR analysis was performed using an ABI Prism 7900HT (Applied Biosystems) with the following thermal cycling conditions: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. All samples were run in triplicate. Data was normalized against beta-actin. For the QuantiFast[™] assay, the primers for ADA (forward: 5'-GAGCTGCGCAACATTATCG-3', reverse: 5'-GCCTTCATCTCCACAAACTC-3') and GAPDH (forward: 5'-AGGAAGCTCACTGGCATGG-3', reverse: 5'-CCTGCTTCACCACCTTCTTG-3') were used. The cycle parameters involved an initial activation step at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 10 s then annealing and extension at 60°C for 30 s. After amplification, samples were kept at 55°C for 1 min and the temperature was gradually raised by 0.5°C every 10 s to perform the melt-curve analysis. All procedures of real time PCR were performed on the iCycler iQ[™] Real-Time PCR Detection System (Bio-Rad Laboratories, Tokyo, Japan) All reactions were performed in triplicate. The threshold cycles (Ct) were used to quantify the mRNA expression levels of samples using GAPDH for normalization.

In situ hybridization

In situ hybridization was performed at Genostaff Co., Ltd. Detailed protocols are described in Supplementary Materials.

Effects of adenosine receptor blockade on IND-induced enteropathy with OGT

We wanted to test the role of the adenosine system on the effect of OGT during IND-induced enteropathy. Thus, we examined the effect of adenosine receptor antagonists on the lethality induced by IND with or without OGT. An adenosine receptor A2a antagonist 8-(3-chlorostyryl) caffeine (CSC; 10 mg/kg) or an equal volume of vehicle were administered intraperitoneally (i.p.) for five consecutive days from one day before the first IND treatment.

Results

Effect of Oregedokuto on indomethacin-induced enteropathy

As previously reported [8], IND induced lethal small intestinal ulceration and OGT prevented the ulcerations and the lethality. OGT rescued mice from IND-induced death (Fig. 1a). OGT reduced the number and area of IND-induced ulcerations (Fig. 1b, c).

Gene expression in the murine small intestine

For the gene expression studies, the mice used for this experiment were divided into four groups: N (normal), I (IND-treated), N/O (OGT-treated) and I/O (IND- and OGT-treated). Preparation of tissue samples was performed 48 h after the first injection of IND when more than 90% of mice still survived even in group I. Three comparison sets were made, that is, EXP_IND: N versus I (effect of IND), EXP_OGT: N versus N/O (effect of OGT), EXP_OGT_IND: I versus I/O (effect of OGT in IND-enteropathy model), and *P*-value for each probe set was calculated by Welch's *t* test. A list of genes that displayed increased or decreased levels of expression in each comparison set is shown in Supplementary Table S1–S3. For nine genes, the changes in gene expression induced by IND were partially abrogated by OGT (highlighted in bold font in Table S1, S2). OGT decreased ADA expression both in EXP_OGT and EXP_OGT_IND (highlighted in bold font in Table S2, S3).

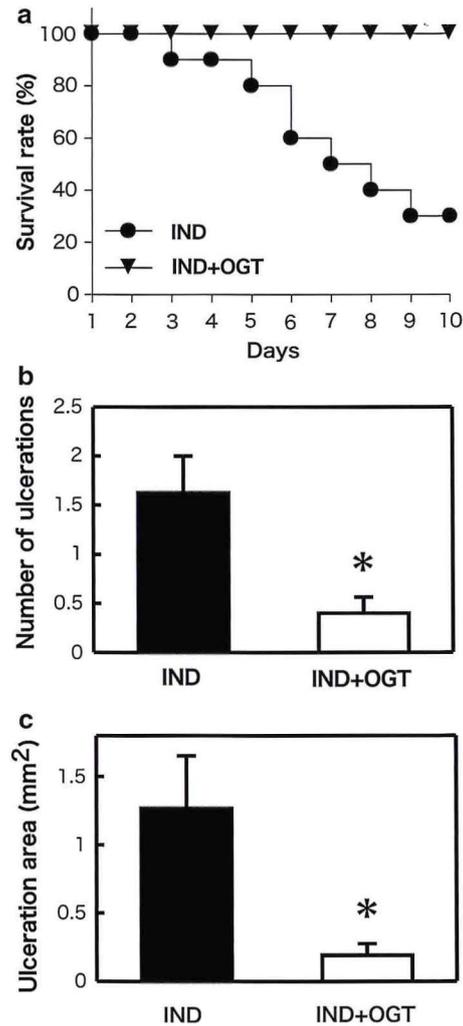


Fig. 1 a Oregedokuto (OGT) rescued mice from indomethacin (IND)-induced death. Survival curves were drawn using the Kaplan–Meier method. The difference in survival ratio at day 10 was analyzed by Fisher’s exact test, **P* < 0.01 (*n* = 10 per group) b, c OGT reduced IND-induced small intestinal lesions. Small intestines were harvested at 24 h after the second injection of IND. The number and areas of ulcerations were calculated macroscopically. **P* < 0.01, data represent the means ± SEM (*n* = 10 per group)

Gene set analyses by MetaGene Profiler on GO terms

Filtering genes by setting the threshold for expression level, fold-changes, statistical significance, etc. is not ideal because the setting of the threshold cannot eliminate the problem of arbitrary selection. Furthermore, single-gene analysis has been known to have a number of major limitations. MGP, a recent developed method to evaluate microarray data at the level of gene sets, gives the “integrated *P*-value” for a given gene set by integrating *P*-values of all probe sets contained in the gene set. MGP facilitates an evaluation of the statistical significance of the given gene set. Initial analysis was performed using the

gene sets defined by the GO consortium. The “integrated *P*-values” for all possible gene sets defined by all GO terms (>20,000 terms) were calculated and the top 20 GO terms in MF, BP and cell component (CC) categories for EXP_IND are shown in Table 1 and Supplementary Table S4. A detailed discussion of these results is given in the Supplementary Materials. As shown in Table 1, among the GO terms denoting relatively broad and general concepts, those containing a small number of genes specific for particular biological activity, such as “rhodopsin-like receptor activity” and “ADA activity”, were ranked. The gene list of “rhodopsin-like receptor activity” contains 4 probe sets for adenosine receptors among 19 probe sets in total. The results of MGP analysis for EXP_IND_OGT are shown in Table 2 and Supplementary Table S5. For the MF category, “deaminase activity” whose major members are ADAs, “rhodopsin-like receptor activity” and “ADA activity” were ranked. These MGP analyses prompted us to hypothesize a possible involvement of the adenosine system in the enteropathy and recovery by IND and OGT, respectively. Because the spectrum of adenosine functions is extremely wide, we defined several subsets of adenosine system-related genes. Further MGP analyses were then performed on the newly defined gene sets. This secondary MGP analyses suggested that the genes involved in adenosine metabolism were the most affected gene set by IND and/or OGT treatment (Table 3; details have been described in Supplementary Materials). MGP analysis for EXP_OGT gave only a few GO terms (7 GO terms in 3 categories with integrated *P*-value <0.05) suggesting that OGT has little or no effect on the expression profile of normal small intestines (data not shown).

Biological investigation of a possible involvement of the adenosine system in the effects by IND and/or OGT

In the next step, the possible involvement of the adenosine system was examined biologically. Firstly, ISH was performed to clarify the localization of ADA, which was most clearly affected by IND/OGT treatment (Fig. 2). ADA expression was observed specifically at the luminal surface of intestinal villous tips. It was increased in IND-treated mice, which was reduced by co-treatment with OGT. ADA signals were absent in the site of ulceration or perforation where villi had been destroyed because of the exclusive localization of ADA to villous tips (data not shown). Next, we performed real time RT-PCR to confirm the change of ADA gene expression of the small intestine. In accordance with microarray and ISH data, IND increased the expression of ADA mRNA in the small intestine whereas OGT decreased the expression level of ADA mRNA (Fig. 3). Administration of OGT alone also decreased ADA mRNA

Table 1 MGP analysis on EXP_IND: effect of IND

MF (molecular function)		
GO ID	GO term	Integrated P
0003779	Actin binding	1.48E-14
0008565	Protein transporter activity	1.65E-11
0004930	G-protein coupled receptor activity	2.40E-11
0004842	Ubiquitin-protein ligase activity	9.41E-11
0008234	Cysteine-type peptidase activity	1.76E-10
0003682	Chromatin binding	4.00E-10
0004197	Cysteine-type endopeptidase activity	5.36E-10
0030145	Manganese ion binding	1.33E-09
0005200	Structural constituent of cytoskeleton	1.98E-09
0008289	Lipid binding	5.90E-09
0005529	Sugar binding	1.76E-08
0046983	Protein dimerization activity	2.82E-08
0008026	ATP-dependent helicase activity	4.28E-08
0001584	<i>Rhodopsin-like receptor activity</i>	4.45E-08
0004000	<i>Adenosine deaminase activity</i>	5.04E-08
0030528	Transcription regulator activity	5.48E-08
0008083	Growth factor activity	5.65E-08
0004386	Helicase activity	5.93E-08
0042802	Identical protein binding	8.37E-08
0046982	Protein heterodimerization activity	1.24E-07

(Fig. 3). To determine whether adenosine signaling is involved in the effects elicited by IND and/or OGT, we administered an adenosine A2a receptor antagonist, CSC, to IND-treated mice with or without OGT and observed lethality. Treatment with CSC alone gave no significant effect on IND-treated/untreated mice (data not shown) but abrogated the OGT’s preventive effect on IND-induced enteropathy (Fig. 4).

Investigation of the active ingredients of OGT for IND-induced death

To gain an insight into the active ingredients responsible for OGT’s beneficial effect, we prepared three fractions consisting mainly of (a) alkaloids, (b) high molecular weight substances (e.g. polysaccharides) or (c) the residues, whose contents were equivalent to those contained in 2% OGT. Mice treated with the alkaloid fraction survived IND-induced death. By contrast, the high molecular weight fraction and the residue fraction had no preventive effect and only a modest preventive effect, respectively (Fig. 5a). Therefore we next tested the effects of four major alkaloids contained in OGT on the lethality induced by IND. Diets containing each alkaloid at twice the concentration corresponding to that in 2% OGT (2%OGT includes 0.0703% berberine, 0.00706%

Table 2 MGP analysis on EXP_IND_OGT: effect of OGT in IND_enteropathy

MF (molecular function)		
GO ID	GO term	Integrated P
0003924	GTPase activity	2.94E-05
0046873	Metal ion transporter activity	6.23E-04
0019239	<i>Deaminase activity</i>	2.74E-03
0004445	Inositol-polyphosphate 5-phosphatase activity	3.05E-03
0004601	Peroxidase activity	5.46E-03
0004907	Interleukin receptor activity	6.01E-03
0003847	1-Alkyl-2-acetyl-glycerophosphocholine esterase activity	9.05E-03
0001584	<i>Rhodopsin-like receptor activity</i>	1.31E-02
0005044	Scavenger receptor activity	1.56E-02
0004252	Serine-type endopeptidase activity	1.68E-02
0004000	<i>Adenosine deaminase activity</i>	1.81E-02
0043022	Ribosome binding	1.89E-02
0004930	G-protein coupled receptor activity	2.10E-02
0016772	Transferase activity, transferring phosphorus-containing groups	2.34E-02
0003779	Actin binding	2.39E-02
0005375	Copper ion transporter activity	2.47E-02
0005385	Zinc ion transporter activity	2.52E-02
0005200	Structural constituent of cytoskeleton	2.76E-02
0000049	tRNA binding	2.96E-02
0004263	Chymotrypsin activity	3.12E-02

Table 3 MGP analysis on selected gene sets of adenosine system

Gene set	Description	Effect of IND	Effect of OGT in IND-model	Effect of OGT
1	Purine metabolism	2.896E-02	1.019E-02	9.341E-01
2	Adenosine metabolism	1.935E-06	1.860E-02	6.567E-01
3	Adenosine signaling	2.977E-02	2.829E-01	8.654E-01
4	Adenosine metabolism/signaling	1.307E-05	5.245E-02	8.718E-01
5	T cell apoptosis subset	3.513E-01	4.211E-02	7.906E-01
6	Other cell apoptosis subset	1.824E-04	6.813E-03	9.530E-01
7	AdoHcy-mediated apoptosis	6.306E-01	1.630E-01	9.086E-01
8	Literature analysis	1.182E-07	8.794E-07	5.397E-01

Definition of gene sets (Table S6 in supplementary material)

coptisine, 0.00129% palmatine and 0.00176% magnoflorine) were administered from the first injection of IND until 10 days after the first IND injection ($n = 10$ per group). Our results showed that berberine and coptisine, but not palmatine and magnoflorine, possibly reduced the incidence of IND-induced death (data not shown). Then we focused our attention on berberine, which has been ethically used. Berberine suppressed IND-induced lethality in a dose-dependent manner (Fig. 5b). Real time RT-PCR showed that berberine treatment reduced IND-induced increase in ADA mRNA expression in the small intestine (Fig. 6).

Schematic representation of changes in gene expression of adenosine system by CIO (<http://www.cellillustrator.com>)

We represent the changes in gene expression of the adenosine system by a visualization method using CIO (Fig. 7). The genes involved in the adenosine system were classified into three categories; intracellular adenosine metabolism, extracellular adenosine metabolism and adenosine signal transduction. Intracellular adenosine is provided by SAH hydrolase (Ahcy) from *S*-adenosylhomocysteine and degraded by ADA or adenosine kinase (ADK). In the

Fig. 2 In situ hybridization for ADA mRNA. Small intestines were harvested 24 h after the second injection of IND

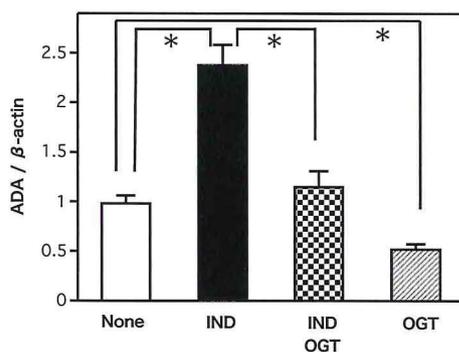
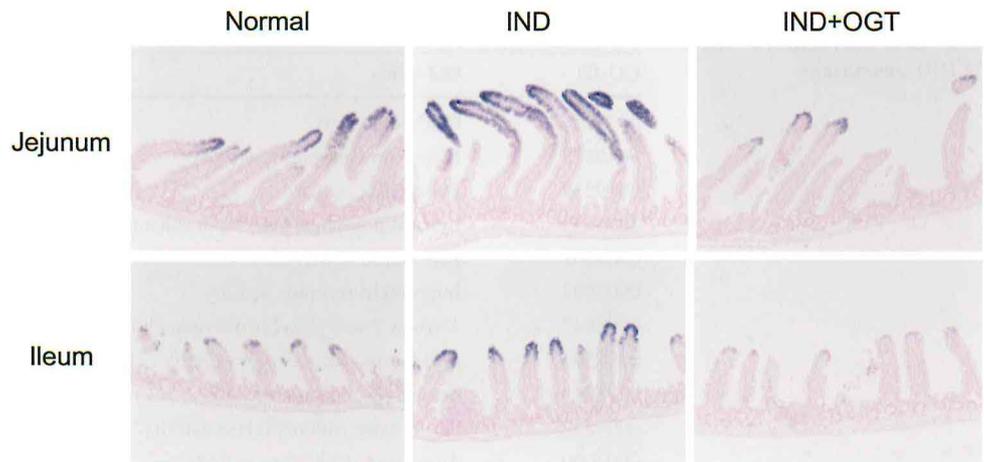


Fig. 3 OGT reduced ADA mRNA expression in the small intestines of both IND-treated and untreated mice. Small intestines were harvested at 24 h after the second injection of IND. * $P < 0.05$, t test with Bonferroni correction. Data represent the means \pm SEM ($n = 3$ –5 per group)

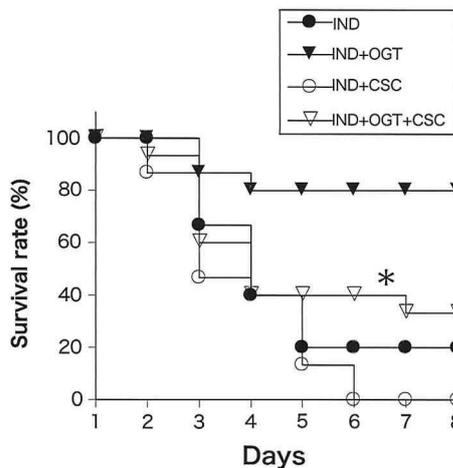


Fig. 4 Adenosine A2a receptor antagonist abrogates the effect of OGT. Adenosine A2a receptor antagonist (CSC; 10 mg/kg i.p.) was injected for 5 consecutive days prior to the first injection of IND. Survival curves were drawn using the Kaplan–Meier method and statistical analysis was done by the logrank test. * $P < 0.02$ versus IND + OGT ($n = 15$ per group)

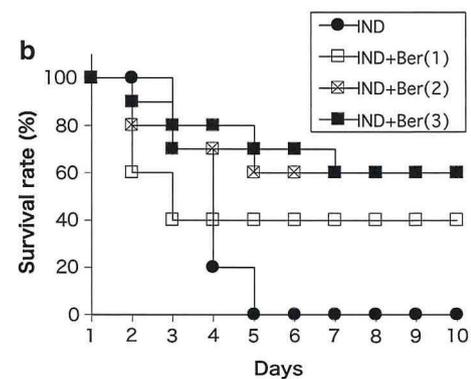
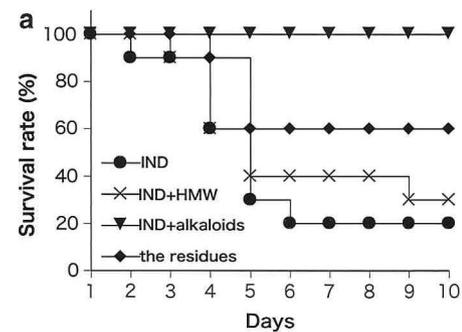


Fig. 5 a Effect of OGT fractions on IND-induced lethality. Diets containing each fraction at the concentration relevant to that in 2% OGT were administered from the first injection of IND until the end of the experiments. HMW: high molecular weight fraction. The alkaloid fraction had the strongest protective effect. Survival curves were drawn using the Kaplan–Meier method ($n = 10$ per group). **b** Berberine suppressed IND-induced lethality in a dose-dependent manner. Diets containing berberine at 1 \times [Ber(1)], 2 \times [Ber(2)] or 3 \times [Ber(3)] concentration corresponding to that in 2% OGT were administered from the first IND injection until the end of the experiment. Survival curves were drawn using the Kaplan–Meier method ($n = 10$ per group)

inflammatory state, large amounts of extracellular adenosine are produced from ATP via the successive conversion by CD73/ectonucleotidase (Nt5e) and CD39 (Entpd2) and degraded by ectopically expressed ADA. Extracellular

adenosine binds to adenosine receptors (A2a, A2b) and transduces signals to MAPK1 and 3 through relevant G proteins (Gna11, Gnai2, Gnai3, Gnao1 and Gnas3). HIF1A is a master transcription factor which regulates the large portion of genes involved in the adenosine system. The expression of other relevant genes such as endonucleotidase (Nt5c2), nucleotide transporters (Slc28 family and Slc29 family), adenosine A1 and A3 receptors (Adora1, Adora3) were not detected nor assessed in the present study. The fold change and *P*-value for each gene provided by GeneChip analysis are represented as the size of icon and the line thickness of the wraparound frame, respectively. The figure clearly shows IND predominantly

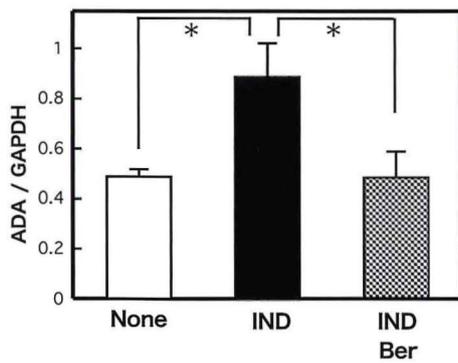


Fig. 6 Berberine reduced ADA mRNA expression in the small intestine. Small intestines were harvested at 24 h after the second injection of IND. Diet containing berberine at twice the concentration corresponding to that in 2% OGT was administered from the first IND injection until the end of experiment. **P* < 0.05, *t* test with Bonferroni correction. Data represent the means ± SEM (*n* = 5 per group)

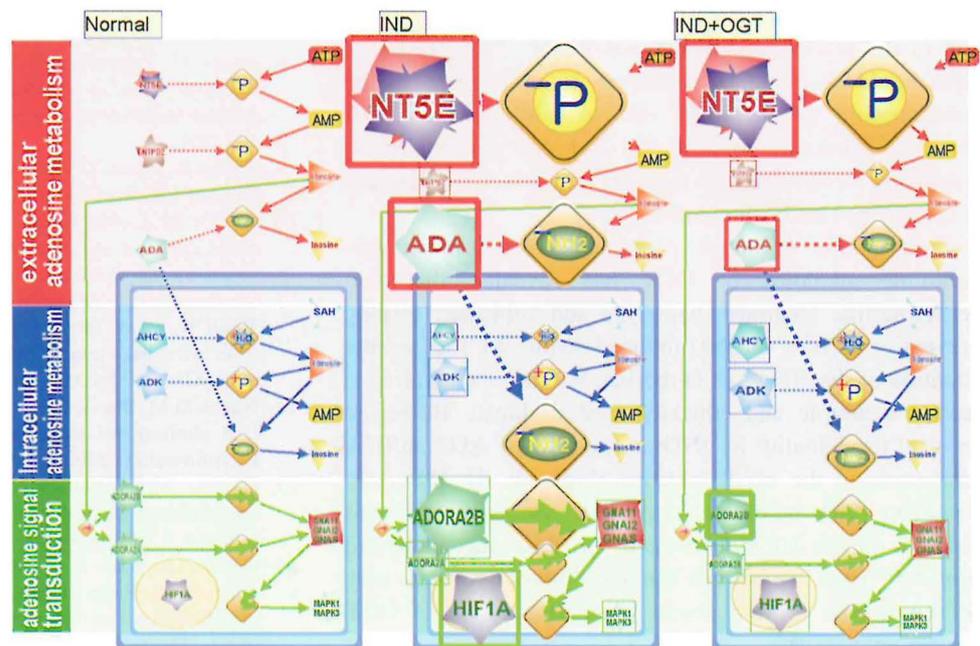
activates gene expression involved in extracellular adenosine metabolism. This suggests that in IND-treated small intestines a large amount of adenosine is synthesized and then degraded resulting in activation of the adenosine signal transduction pathway. OGT normalizes the changes induced by IND treatment.

Discussion

Several pathogenic factors of IND-induced enteropathy have been suggested [13, 14], which were PGs depletion after COX inhibition [15], increase of intestinal permeability after mitochondrial damage [16], exposure of luminal bacteria to mucosa [9], oxidative stress [17], nitric oxide [10], TNF- α [18], neutrophil infiltration [19] and microcirculatory changes [20–22].

In the present study, we demonstrated that the adenosine system, including ADA, plays an important role in IND-induced enteropathy and its amelioration by OGT, which indicates that the adenosine system is another option of the mechanism and therapeutic target of IND-induced enteropathy. Adenosine is a ubiquitous endogenous purine nucleoside, which exerts its various biological actions in many organs via cell surface adenosine receptors (A1, A2a, A2b, A3) [23]. Normally the extracellular adenosine concentration is strictly controlled by a set of synthetic, transport and metabolic molecules. However, in response to cellular damage (e.g. inflammation or ischemia), adenosine concentration is quickly elevated, which plays a prominent role in modulating inflammation and helps to maintain tissue integrity [24]. The cytoprotective functions

Fig. 7 Schematic representation of changes in gene expression of the adenosine system using Cell Illustrator Online (CIO). *NT5E* CD73/ectonucleotidase, *ADA* adenosine deaminase, *ADORA* adenosine receptor, *HIF1A* hypoxia inducible factor 1A, *GNA* G protein, *ADK* adenosine kinase



of adenosine have been suggested in several experimental colitis models by modulation of adenosine signaling. Indeed, adenosine has been proposed to form the basis of a novel therapeutic option for inflammatory bowel diseases (IBDs) [25, 26]. Furthermore, the prominent role of A2a receptors suggested by the present results is in good accordance with previous reports on both acute and chronic colitis models [27, 28].

Here, we showed that OGT decreased ADA mRNA expression which was increased by injection of IND. ADA is an enzyme that is largely responsible for the degradation of adenosine. The cellular concentration of adenosine is determined by the net balance of adenosine synthesis, transport and catabolism. Therefore, it is possible that OGT may increase the adenosine concentration by decreasing the amount of ADA. Inhibition of ADA as a possible therapeutic option for enteropathy was also suggested in a report by Antonioli et al. [29] where it was shown that an ADA inhibitor attenuates DNBS colitis. Survey and analysis of the results of microarray data (Fig. 7; Table 3) suggested that the genes involved in extracellular adenosine metabolism are most profoundly affected, which support the hypothesis that the effect of IND/OGT is closely related to the extracellular concentration of adenosine. Furthermore, because OGT decreased ADA mRNA in normal mice, the decrease of ADA may not be the result of a reduction in inflammation but an independent action executed by OGT.

Pouliot et al. [30] showed that adenosine upregulated COX-2 enzyme in human granulocytes, and suggested that adenosine might promote a self-limiting regulatory process through the increase of PGE2 generation. In our previous study, we showed that OGT elicited an increase in the number of COX-2 expressing cells in the lamina propria and in the production of mucosal PGE2. These observations could be explained in terms of an increase in the concentration of adenosine due to inhibition of ADA activity. However, because we did not determine the adenosine concentration, further extensive studies are necessary to clarify this point.

Investigation of active components using major isoquinoline alkaloids of OGT has revealed that two berberine-like alkaloids, berberine and coptisine, display preventive activity on IND-induced death. We focused the attention of the effects of berberine, because the alkaloid is readily available and ethically used in Japan. Berberine reduced the lethality by IND and decreased ADA mRNA expression in the small intestine (Figs. 5b, 6). However, treatment with berberine alone did not have sufficient efficacy on the lethality even if the dose was increased (Fig. 5b). Thus, we believe that additional ingredients may be responsible for the potent preventive effect of OGT against enteropathy.

In conclusion, guided by a transcriptome approach using MGP analysis, we have demonstrated the importance of the adenosine system in IND-induced enteropathy and its amelioration by OGT/berberine. This study addresses the possible efficacy of OGT as a treatment option for the adverse effect of NSAIDs on the small intestine. We believe that modulation of the adenosine system may provide an effective therapeutic strategy for NSAID-induced enteropathy.

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