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Effects of hangeshashinto on butyrate-induced cell death in murine colonic epithelial cell

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Recent findings demonstrated that a significant number of *Fusobacterium varium* was identified in the mucosa of patients with ulcerative colitis (UC) (Ohkusa et al., J. Gastroenterol. Hepatol., 17, 849-853, 2002), and it has been suggested that the butyrate produced by *F. varium* is involved in the pathogenesis of UC (Ohkusa *et al.*, Gut, 52, 79-83, 2003). In the screening of Kampo formulas to protect butyrate-induced death of murine colonic epithelial cells MCE301 as an in vitro model of UC, it was found that the hangeshashinto (HST, Banxia Xiexin Tang in Chinese) and its related Kampo formulas showed the potent inhibitory activity against butyrate-induced cell death. Of the components of the formulation of HST, only decocted extract of Coptidis Rhizoma showed the potent activity. The inhibitory effect of HST extract was disappeared after removal of Coptidis Rhizoma from the formula of HST. By bioassay guided fractionation of Coptidis Rhizoma extract, and HPLC analysis of the active fraction in comparison with standard samples, berberine was identified as one of active ingredients of Coptidis Rhizoma extract. These results suggest that berberine in HST and related formulas inhibits the butyrate-induced colonic epithelial cell death. HST and its related formulations have been clinically used for the treatment of UC. The data presented here may partly explain the mechanism of clinical effectiveness of HST and its related Kampo formulas on treatment of UC.

Key words ulcerative colitis, colonic epithelial cell, butyrate, hangeshashinto, Coptidis Rhizoma, berberine.

Introduction

Ulcerative colitis (UC) is a diffuse and nonspecific inflammatory bowel disease with unknown cause, mainly affecting the mucosa and often causing severe erosion or ulcer. In 1875, UC was first described by Wilks and Moxon as a different disease from other bowel diseases with mucopurulent hematochezia.[1)] In the present day, the number of patients with UC has increased dramatically in Japan, and this increase in the number of patients has been suggested to be partly attributed to the change in eating habits due to Westernization of diet.[2)]

As a medical therapy, 5-aminosalicylic acid (5-ASA, mesalazine) has been considered as first-line therapy for active mild-moderate, left-sided or extensive UC. Clinically, mesalazine has been shown to be effective for treating active UC, as well as in maintaining remission.[3-5)] However, there are many patients with mesalazine-resistant UC.[6)] For the treatment of these mesalazine-resistant cases, adrenal corticosteroid and immunosuppressants such as cyclosporine and azathioprine can be used but these are not suitable to use at high-doses or for long-term administration because of possible adverse effects. Under these circumstances, the development of other suitable medication is required for the treatment of UC. Meanwhile, it has been reported that Kampo formulas have a certain level of therapeutic effects for the treatment of UC by clinical studies.[7,8)] Therefore, it is presumed that Kampo formulas may contain effective in gredients for the treatment of UC. Although there are several reports on the efficacy of Kampo formulas for experimental colitis animal model,[9-11)] details of the mechanism of Kampo formulas as well as active ingredient(s) for the treatment of UC are not fully elucidated.

In the mucosal area of UC patient, it is known that the excessive death of colon epithelial cells is observed in the crypt.[12)] The cell death attenuates the protective ability of the intestinal mucosa and facilitates the invasion of various antigens from the gastrointestinal tract, and is considered to be one of the factors to make the inflammation worse.[13)] A significant number of *Fusobacterium varium* has been identified in the mucosa of patients with UC,[14)] and the number of *F. varium* in the mucosa of UC patients was significantly reduced after the antibiotic combination therapy using amoxicillin, tetracycline and metronidazole, which were selected based on susceptibility tests against *F. varium.* Clinical assessment and colonoscopic and histological scores of patients also showed statistical improvement after the antibiotic combination therapy.[15,16)] Because it has been suggested that butyrate produced by *F. varium* contributes to the pathogenesis of UC,[17)] the clinical effect by the antibiotic combination therapy may partly be explained by the reduction of butyrate production in the mucosa due to the decrease in the number of *F. varium.* Recently, we demonstrated that the butyrate caused potent cytotoxicity against the normal murine colonic epithelial cells MCE301 at

physiological concentrations, and the cell death induced by butyrate might be useful in vitro new bioassay system for the screening of effective drugs of UC.[18] Therefore, inhibitory substances of the butyrate-induced colonic epithelial cell death are expected to have the effectiveness to relieve such symptoms of UC caused by the butyrate-induced excessive cell death in mucosa.

In the present study, we screened the Kampo formulas which can inhibit butyrate-induced colonic epithelial cell death, and found that several Kampo formulas had the potent inhibitory activity. We now report on the inhibitory effect of Kampo formulas against butyrate-induced colonic epithelial cell death and its active constituent in order to evaluate the clinical effects of Kampo formulas on UC.

Materials and Methods

Materials. Murine normal colonic epithelial cells MCE301 were cultured in Dulbecco's modified Eagle medium (DMEM)/Ham F-12 (1:1) medium (Sigma, St. Lous, MO, USA) supplemented with 5% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA), 10 μg/ml insulin, 5.5 μg/ml transferrin, 2 μg/ml ethanolamine, 5 μg/ml sodium selenite, and 10 ng/ml epidermal growth factor at 37℃ in a humidified atmosphere of 5% CO_2 in air. After reaching confluence, the cells were cultured at 39℃ in DMEM/Ham F-12 alone for 3 days, and then subjected to experiment. All experiments using MCE301 cells were carried out at 39 ℃. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was obtained from Dojindo (Kumamoto, Japan). Berberine hydrochloride was purchased from Nakarai Chemical (Kyoto, Japan).

Preparation of Kampo formulas. All crude drugs used in this study were deposited at the Herbarium of Oriental Medicine Research Center of the Kitasato Institute (Tokyo, Japan), and the Kampo medicines (formulas) were prepared according to the prescription book of the Oriental Medicine Research Center. The extract of Kampo formulas were prepared as follows: The mixture of the crude drugs was decocted with 600 ml of boiling water for 40 min to half volume. The extracted solution was centrifuged at 6,000 rpm for 20 min, and then the supernatant was filtered and lyophilized.

Fractionation of hot water extract of Coptidis Rhizoma. The roots of *Coptis japonica* Makino var. dissecta Nakai (10 g) were decocted with water (200 ml) to half volume, and then the extract was lyophilized. A part of the extract (150 mg) was re-suspended in 20 ml water and then partitioned twice with the same volume of *n*-hexane. The resulting aqueous layer was partitioned 3 times with the same volume of AcOEt. The aqueous layer was further successively partitioned with $CHCl_3$ and then *n*-BuOH. Each fraction was concentrated to give an n-hexane fr. (0.84 mg), AcOEt fr. (1.61 mg), $CHCl_3$ fr. (26.84 mg), *n*-BuOH fr. (13.52 mg), and H_2O fr. (94.60 mg), respectively.

HPLC analysis. The HPLC analysis was performed using a HP 1100 liquid chromatograph system equipped with a diode-array detector (Aligent Technologies, Palo Alto, CA, USA). Separation was performed on a reversed-phase column Pegasil ODS (4.6 x 250 mm i.d., Senshu Co., Tokyo, Japan). Mobile phase; 10 mM sodium dodecyl sulfate: acetonitrile (45:55). Flow rate: 1.0 ml/min.

Measurement of Cell Viability. The viability of cultured cells was evaluated using MTT assay.[19] In brief, the cells were grown on 96-well culture plates with 200 μl of medium. After treatment with tested samples for a period of time, 20 μl of MTT solution (5 mg/ml) was added and incubated for 4 h. The reaction was stopped by the aspiration of the MTT-containing medium. The formed formazan crystals were dissolved in 100 μl of isopropanol containing 40 mM HCl and then diluted with an equal volume of water. Absorbance was measured in a microplate reader (Bio-Rad M-450) at 570 nm with a reference filter at 405 nm.

Butyrate-induced cell death of MCE301 cells. MCE301 cells were cultured for 72 h with 8 mM butyrate in the presence or absence of a 30-min pretreatment with various concentrations of Kampo formulas, and then the cell survivals were measured by MTT assay. Protective activity against butyrate induced cell death was calculated as follows:
Inhibition (%) = {(sample + butyrate) $A_{570\ nm}$ - butyrate $A_{570\ nm}$} / (control $A_{570\ nm}$- butyrate $A_{570\ nm}$) x 100.

Statistical analysis. Data were expressed as mean ± s.d., and differences between groups were analyzed by ANOVA followed by *post hock* analysis using Scheffe's test using a personal computer with the StatView-J program for Macintosh (SAS Institute Inc., Cary, NC, USA).

Result and Discussion

In the screening of Kampo formulas to inhibit butyrate-induced cell death, it was found that five kinds of Kampo formulas out of the tested forty-four kinds of them showed the potent inhibitory activity (Table 1). The active Kampo formulas were hangeshashinto (HST), kanzoshashinto (KST), shokyoshashinto (SST), orento (OT), and saikanto (SKT). HST, KST, SST and OT have clinically been used for the treatment of UC. Constitution of the formulas was shown in Table 2. It is considered that the HST is the basic formulation of KST, SST, OT and SKT. In brief, KST consisted of an increased amount of the Glycyrrhizae Radix than HST, and SST is composed by the addition of Zingiberis Rhizoma to HST. OT contains Cinnamomi Cortex instead of Scutellariae Radix in formula of HST. SKT is formulated by the elimination of Zingiberis Siccatum Rhizoma from formula of HST, and the addition of Zingiberis Rhizoma, Trichosanthis Semen and Bupleuri Radix to formula of HST. Therefore, the effect of HST on butyric acid-induced cell death was examined in the following experiment.

HST reduced butyrate-induced cell death in a dose dependent manner (Fig. 1). In order to know which crude drug in HST formula is important for the prevention of the cell death induced by butyrate, the effect of decocted extract of component crude drugs of HST was compared. The cell death inhibitory activity of seven crude drugs which composed of the HST formula was summarized in Fig. 2. Of the

Table 1. Inhibitory effect of Kampo formulas against butyrate-induced cell death in murine colonic epithelial cells MCE301

Kampo formulas	Inhibition (%)	Kampo formulas	Inhibition (%)
Anchusan	<5	Koshayoito	<5
Bukuryoin	5.3 ± 2.6	Kyukichoketsuin daiichikagen	<5
Goshakusan	<5	Makyoyokukanto	<5
Hangeshashinto	18.5 ± 2.3	Maoto	<5
Hainoto	<5	Ninjinto	<5
Heiisan	<5	Orento	13.3 ± 0.9
Hochuekkito	<5	Otsujito	<5
Hokikenchuto	6.3 ± 2.5	Rikkunshito	<5
Ifuto	<5	Saikanto	23.2 ± 1.9
Ireito	<5	Saikokeishito	<5
Juzentaihoto	<5	Saireito	<5
Kakkonto	<5	Senkinnaitakusan	<5
Kakkoshokisan	<5	Senshibyakujutsusan	<5
Kanzoshashinto	18.9 ± 3.5	Shikunshito	<5
Keishikakakkonto	<5	Shinbuto	<5
Keishikashakuyakuto	<5	Shokyoshashinto	19.6 ± 1.9
Kigikenchuto	<5	Shosaikoto	<5
Keihito	<5	Takurishodokuin	<5
Keishininjinto	<5	Tokato	<5
Kobokushokyohangekanzoninjinto	<5	Tokishakuyakusan	<5
Koshaheiisan	<5	Tooto	<5
Kosharikkunshito	<5	Yokuininto	<5

MCE301 cells were cultured for 72 h with 8 mM butyrate in the presence or absence of Kampo formulas (100 μg/mL). Data are expressed as mean ± s.d. (n=4). Experiment was repeated twice, and similar result was observed.

Table 2. Constituents of the hangeshashinto and its related Kampo formulas

Crude drug (Latin name)	Amount of crude drug (g)				
	HST	KST	SST	OT	SKT
Pinelliae Tuber	5.0	5.0	5.0	6.0	5.0
Coptidis Rhizoma	1.0	1.0	1.0	3.0	1.5
Scutellariae Radix	2.5	2.5	2.5	-	3.0
Ginseng Radix	2.5	2.5	2.5	3.0	2.0
Zingiberis Siccatum Rhizoma	1.0	1.0	0.5	1.0	-
Zizyphi fructus	2.5	2.5	2.5	3.0	3.0
Glycyrrhizae Radix	2.0	3.5	2.0	2.0	1.5
Zingiberis Rhizoma	-	-	4.0	-	0.5
Cinnamomi Cortex	-	-	-	3.0	-
Trichosanthis Semen	-	-	-	-	3.0
Bupleuri Radix	-	-	-	-	5.0

Hangeshashinto; HST, kanzoshashinto; KST, shokyoshashinto; SST, orento; OT, and saikanto; SKT. The amount of crude drugs of the formulas was listed in the prescription book of the Oriental Medicine Research Center of the Kitasato Institute.

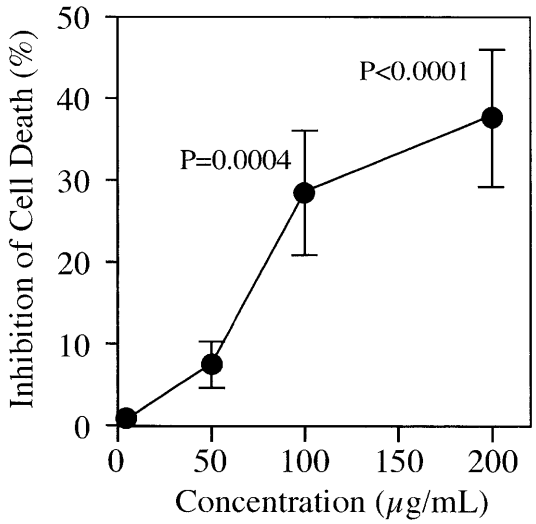

Fig. 1 Effect of hangeshashinto on butyrate-induced cell death in murine colonic epithelial cells MCE301. MCE301 cells were cultured for 72 h with 8 mM butyrate in the presence of various concentration of hangeshashinto. Data are expressed as mean ± s.d. (n=4).

components of the formulation of HST, only decocted extract of Coptidis Rhizoma showed the potent inhibitory activity, and the activity of the extract was 54.9% at a concentration of 10 μg/ml (Fig. 2). To confirm whether Cotidis Rhizoma contributes to expression of the activity, the effect of the removal of Coptidis Rhizoma from HST on the activity was examined. When Coptidis Rhizoma was removed from the formula of HST, the inhibitory activity disappeared (Fig. 3). These results suggest that Coptidis Rhizoma is the most important crude drug in formula of HST for the expression of inhibitory activity against butyrate-induced cell death.

To identify the active principle, the decocted extract of Coptidis Rhizoma was fractionated. When inhibitory activity against butyrate-induced cell death was compared among the fractions, $CHCl_3$ extracted fraction showed the

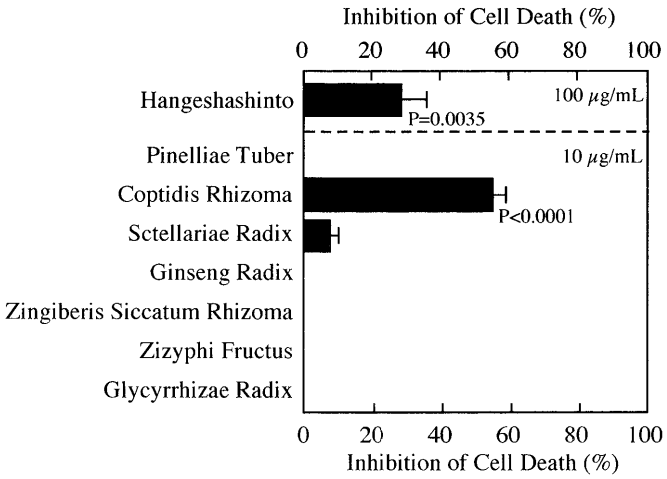

Fig. 2. Effect of decocted extract of component crude drug of hangeshashinto on butyrate-induced cell death in MCE301 cells. MCE301 cells were cultured for 72 h with 8 mM butyrate in the presence of each decocted extract of component crude drugs (10 μg/ml). Data are expressed as mean ± s.d. (n=4).

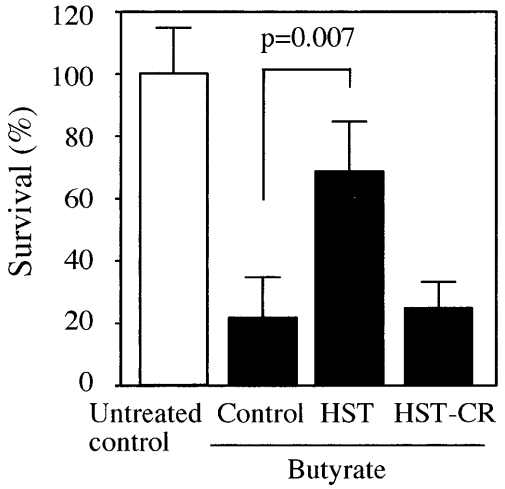

Fig. 3. Effect of the removal of Coptidis Rhizoma from hangeshashinto formula on the cell death inhibitory activity of hangeshashinto. Data are expressed as percent of cell survival. MCE301 cells were cultured for 72 h with 8 mM butyrate in the presence of each extract of formula (100 μg/ml). HST; hangeshashinto, HST-CR; eliminated Coptidis Rhizoma from hangeshashinto. Mean ± s.d. (n=4).

significant inhibitory activity (Fig. 4). At a concentration of 5 μg/ml, the cell death inhibitory activity of n-hexane fr., AcOEt fr., $CHCl_3$ fr. *n*-BuOH fr., and H_2O fr. were 0%, 4.1%, 41.1%, 0% and 12.1%, respectively (Fig. 4). When the $CHCl_3$ extracted fraction was subjected to HPLC analysis, a peak at retention time 13.9 min was observed with several minor peaks (Fig. 5A). The main peak was identified as berberine by comparison of the retention time and UV spectrum with those of the standard sample (Fig. 5B). Berberine reduced butyrate-induced cell death in a dose dependent manner up to 1 μg/ml (2.5 μM) (Fig. 6). These results suggest that berberine is one of the active ingredient(s) of Coptidis Rhizoma extract. The cell death inhibitory activity of 200 μg/ml HST seemed to be comparable to 10 μg/ml Coptidis Rhizoma (Fig. 1 and 2). By the HPLC analysis, berberine contents in the decocted extract of HST and Coptidis Rhizoma were 0.8% and 17.8%, respectively. Thus, 200 μg/ml HST and 10 μg/ml Coptidis Rhizoma were equivalent to 1.6 μg/ml and 1.78 μg/ml berberine, respectively. In addition, the cell death inhibitory activity of berberine (1.0 μg/ml) showed nearly comparable in activity to those of HST (200 μg/ml) and Coptidis Rhizoma (10 μg/ml) (Fig. 6). From these results, it is presumed that the cell death inhibitory activity of HST and Coptidis Rhizoma extract is attributed mainly to berberine. However, we cannot rule out the possibility of the presence of the combina-

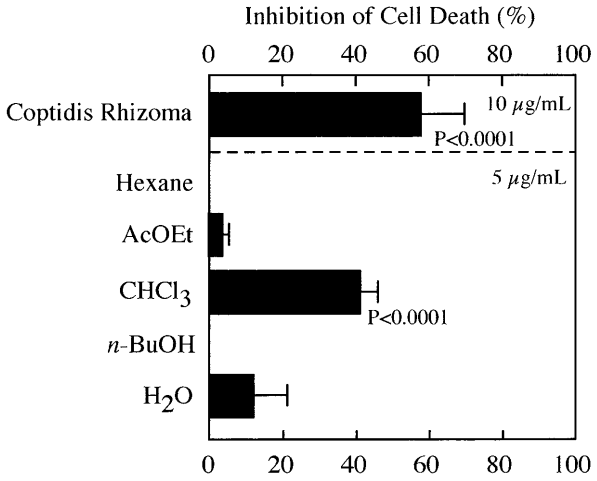

Fig. 4. Effect of fractions from decocted extract of Coptidis Rhizoma on butyrate-induced cell death in MCE301 cells. MCE301 cells were cultured for 72 h with 8 mM butyrate in the presence of each fraction (5 μg/ml). Data are expressed as mean ± s.d. (n=4).

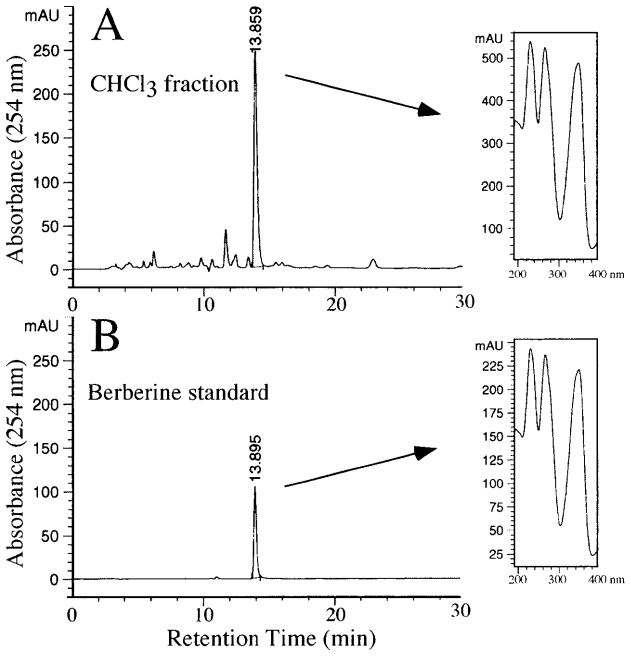

Fig. 5. High-performance liquid chromatography (HPLC) patterns of $CHCl_3$ fraction from decocted extract of Coptidis Rhizoma and standard sample of berberine. Data are expressed as mean ± s.d. (n=4).

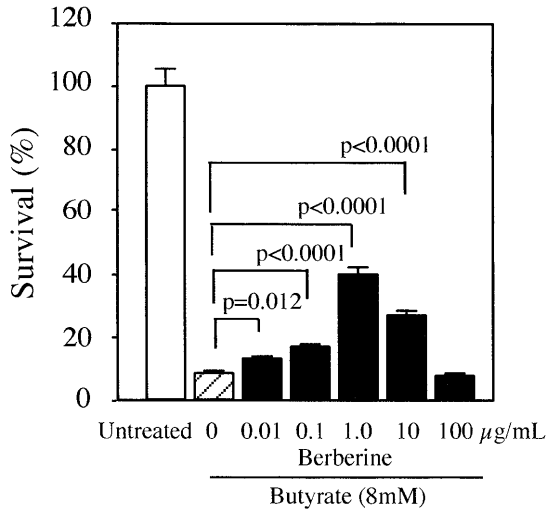

Fig. 6. Effect of berberine on butyrate-induced cell death in MCE301 cells. MCE301 cells were cultured for 72 h with 8 mM butyrate in the presence of various concentration of berberine hydrochloride. Data are expressed as percent of cell survival. Mean ± s.d. (n=4).

tion effect of berberine and other constituents. Further studies are required.

Berberine reduced butyrate-induced cell death, and optimal inhibition occurs at approximately 1.0 μg/ml (2.5 μM) (Fig. 6). Because it is well known that berberine induced the apoptosis of several cell lines such as human leukemia cell line HL-60, human solid tumor HeLa and mouse leukemia cell line L1210 cells,[20-22] it was assumed that the induction of apoptosis might participate in the decrease of the cell viability at higher concentrations than the optimal concentration. Indeed, when MCE301 cells were incubated with berberine, significant decrease of cell viability was observed over 10 μg/ml (data not shown). However, whether berberine induces apoptosis in MCE301 cells, it is not now known.

Until now we have not known the mechanism of the inhibitory or protective effect of berberine against butyrate-induced cell death. It is known that the presence of opening structures called permeability transition pore (PTP) is observed in contact sites between the inner and outer mitochondrial membrane.[23] When the PTP is opened, hydrogen ion flows into the mitochondria, the mitochondrial membrane potential is discharged, and then the mitochondria is swollen due to the decreased membrane potential.[24] Recently, we reported that the swollen mitochondria and marked decrease in the mitochondrial membrane potential were observed in butyrate-treated MCE301 cells,[18] and these observations suggested that butyrate induced discharge of membrane potential. A major factor on the opening of the PTP is known to be Ca^{2+}.[25] It has been reported that berberine is a potent inhibitor of Ca^{2+} channel.[26,27] Therefore, the inhibition of Ca^{2+} channel by berberine may involve in the inhibitory effect against butyrate-induced cell death. To understand the detailed mechanism of the inhibition by berberine against butyrate-induced cell death, further experiments are required.

Clinically, HST has widely been used for the treatment of UC patient, and it has been reported that HST was effective in patients with UC.[28] In this study, butyrate-induced cell death in MCE301 cells was partially but significantly reduced by HST, and berberine was identified as the active ingredient. Berberine has been reported to have a broad spectrum of antibacterial activity.[29,30] It has been reported that *F. varium* may contribute to the pathogenesis of UC as a butyric acid producing bacteria.[14,15] Therefore it is presumed that both the inhibitory activity against butyrate-induced cell death and the antibacterial activity of berberine may explain a part of the clinically observed curative effect of HST in patients with UC. In order to clarify the relationship between the clinical improvement and the number of mucosa-associated *F. varium* before and after the treatment of Kampo formulas, clinical study is currently underway, and it must await further study. Elucidation of further action of HST on butyrate-mediated pathogenesis of UC are also now underway by *in vitro* and *in vivo* study.

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Japanese abstract

酪酸で誘発されるマウス結腸上皮細胞株 MCE301 細胞の細胞死を in vitro の潰瘍性大腸炎モデルとして用いて検討を行った。44種類の漢方薬を対象にスクリーニングを行った結果，半夏瀉心湯およびその関連処方 4 種が酪酸による細胞死を抑制することを見いだした。活性の観察された漢方方剤の多くは臨床的に潰瘍性大腸炎の治療に応用される漢方薬であった。半夏瀉心湯エキスの活性は，構成生薬である黄連を除くことにより消失したことから，黄連が活性発現に重要な構成生薬であると考えられた。黄連エキス中の活性物質について検討した結果，活性物質はベルベリンであることが明らかとなった。潰瘍性大腸炎に対する半夏瀉心湯の臨床的な効果には，酪酸によって誘発される結腸上皮細胞死に対するベルベリンの抑制作用が関与する可能性が示唆された。

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