Competition in the Metabolism of Glycyrrhizin with Glycyrrhetic Acid Mono-Glucuronide by Mixed *Eubacterium* sp. GLH and *Ruminococcus* sp. PO1-3

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Eubacterium sp. GLH possessing glycyrrhizin (GL) and glycyrrhetic acid mono-glucuronide (GAMG) β -D-glucuronidases, *Ruminococcus* sp. PO1-3 possessing GL and GAMG β -D-glucuronidases and 3β -hydroxysteroid dehydrogenase and these mixed bacteria were cultured in GAM medium with and without GL, GAMG or both. GL added to *Eubacterium* sp. GLH accelerated the peaks of enhanced GL β -D-glucuronidase activity and suppressed GAMG β -D-glucuronidase activity, and GAMG delayed the peaks of the enhanced growth with GL and GAMG β -D-glucuronidase activities. GL added to *Ruminococcus* sp. PO1-3 enhanced gradually the growth with GL and GAMG β -D-glucuronidase activities, and GAMG enhanced slowly GL β -D-glucuronidase activity and rapidly the growth with GAMG β -D-glucuronidase activity. The metabolite glycyrrhetic acid (GA) was produced by *Eubacterium* sp. GLH and *Ruminococcus* sp. PO1-3 in larger amounts and faster from GAMG than from GL. GL (1.0 mM) and 1.0 mM GAMG added to these mixed bacteria enhanced the growth with GL and GAMG β -D-glucuronidase activities and were metabolized almost completely to GA in culture of 2 d and 1 d, respectively. It was found that the metabolism of GAMG was faster than that of GL.

GL with GAMG added to mixed *Eubacterium* sp. GLH and *Ruminococcus* sp. PO1-3 cultured for 0 and 1 d led to a lower level of these enzyme activities and the consumption of GAMG more quickly, not GL. Low GAMG β -D-glucuronidase had the ability to hydrolyze GAMG well.

Key words glycyrrhizin; glycyrrhetic acid mono-glucuronide; β -D-glucuronidase; intestinal bacteria

Liquorice is a Herbal medicine contained in almost all Kampo medicine. Its main component is glycyrrhizin (GL) and glycyrrhetic acid (GA). GL administered intravenously is used as an anti-inflammatory medicine.¹⁾ Its aglicone, GA possesses anti-inflammatory action.²⁾

The metabolism of GL by rat liver and human intestinal bacteria is shown in Chart 1.^{3–5)} GL is hydrolyzed to glycyrrhetic acid mono-glucuronide (GAMG) through GA and to GA directly by GL, GL I, GL II and GAMG β -D-glucuronidases.^{3,5–7)} The reversible reaction between GA and 3-oxo-glycyrrhetic acid (3-oxo-GA) is catalyzed by 3β -hydroxysteroid dehydrogenase (3β -hydroxyGA) and GA dehydrogenase.^{8,9)} Moreover, the reversible reaction between 3-oxo-GA and 3α -hydroxyglycyrrhetic acid (3α -hydroxyGA) is catalyzed by 3α -hydroxyGA dehydrogenase.¹⁰⁾

GL administered orally to human is detected as unchanged GL and GA in plasma.¹¹⁾ GL is metabolized largely to GAMG by rat internal organs.⁵⁾ Moreover, about 80% of GL is excreted to bile-duct.¹²⁾ These results show that GL and its metabolite GAMG are excreted to the duodenum at the same time. The hydrolysis of GL and GAMG is greater in GL β -D-glucuronidase and GAMG β -D-glucuronidase, respectively, of intestinal flora and *Eubacterium* sp. GLH than in that of internal organs.^{3,5,7)} Thus, the metabolism of GL and GAMG by intestinal flora is interesting. The effect of GL with GAMG on bacterial growth and the competition in its metabolism were examined by using *Eubacterium* sp. GLH and *Ruminococcus* sp. PO1-3 possessing the enzymes involved in the metabolism of GL.

MATERIALS AND METHODS

Chemicals GL monoammonium salt, GAMG and GA were purchased from Tokyo Kasei Kogyo Co., Tokyo, Wako

Pure Chemical Industries, Ltd., Osaka, and Nacalai Tesque Inc., Kyoto, respectively. These components were purified to one spot by using TLC (Merck, silica gel 60 F-254, layer thickness 0.25 mm). 3-Oxo-GA was prepared as described.⁸⁾ GAM broth (a trade name) was a product of Nissui Seiyaku Co., Tokyo. All other reagents were of the highest commercial quality available.

Culture Method *Eubacterium* sp. GLH and *Ruminococcus* sp. PO1-3 isolated from human feces were precultured as described.^{4,6,8)} Each preculture and the mixture of their equal volumes were added to 9 volumes of GAM medium with or without 1.0 mM GL, 1.0 mM GAMG, both or 1.67 mM GL with 1.67 mM GAMG. The bacteria were then cultured in triplicate for 3 or 4 d at 37°C under anaerobic conditions. Bacterial growth was monitored by the transmission of 650 nm.

Enzyme Assay and Determination of Metabolites Cultured GAM medium took 10 ml in each sampling time. The bacteria were collected by centrifugation of 6000 $g \times 10$ min at 4°C, were washed with ethanol to remove GL, GAMG and their metabolites and were washed by 20 mM potassium phosphate buffer (pH 7.2) to remove ethanol. Bacteria sonicated for 1 min on ice bath were used as the enzyme solution. GL and GAMG hydrolyzing activities, GA oxidizing activity and the metabolites were measured as described^{3,6)} and the mean number measured in triplicate was used. The reaction of assay mixture and cultured GAM medium was stopped by the addition of 1 M HCl. The products in each solution were extracted with ethyl acetate and were detected on a TLC plate, which was first chromatographed for GA and 3-oxo-GA with a solvent system of chloroform–petroleum ether–acetic acid (5 : 5 : 1, v/v) and secondarily for GL and GAMG with a solvent system of acetic acid–*n*-butanol–1,2-dichloroethane–H₂O (4 : 1 : 4 : 1, v/v). The quantity of product

Chart 1. The Metabolism of GL by Rat Liver and Human Intestinal Bacteria

was measured with a TLC scanner.⁸⁾

RESULTS

Effects of GL and GAMG on the Growth of *Eubacterium* sp. GLH, *Ruminococcus* sp. PO1-3 or a Mixture of Both Bacteria *Eubacterium* sp. GLH possessing GL and GAMG β -D-glucuronidases took over 24 h to reach the maximal stage in GAM medium with or without 1.0 mM GL and 1.0 mM GAMG. GL led rapidly to the increase of this bacterial growth with enhanced GL β -D-glucuronidase activity and suppressed GAMG β -D-glucuronidase activity. Also, GL accelerated the peak of GL β -D-glucuronidase activity and led to the intersection of these activities in over 12 h of culture at a similar level. With 1.0 mM GAMG, the peak of GL and GAMG β -D-glucuronidase activities and bacterial growth coincided well in the pattern changed to 84 h from 36 h of culture and 48 h from 12 h of culture, respectively (Fig. 1A). GAMG enhanced rapidly the bacterial growth from culture of 12 h and led to the lower level of these enzyme activities till culture of 24 h. Between culture of 24 and 48 h, GL β -D-glucuronidase activity and GAMG β -D-glucuronidase activity were increased slowly and rapidly, respectively. This bacterium received each different influence by the exposure of each drug.

The growth of *Ruminococcus* sp. PO1-3 reached the maxi-

mal stage between culture of 6 and 12 h and was enhanced with GL and GAMG β -D-glucuronidase activities gradually during culture of 3 d with and without 1.0 mM GL and with GAMG β -D-glucuronidase activity rapidly at culture of 12 h by 1.0 mM GAMG. This bacterial growth was greater in GAMG than with GL. Also, GL β -D-glucuronidase activity showed little increase from culture of 24 h by 1.0 mM GAMG. 3β -HSD activity showed no change at a lower level by 1.0 mM GL and 1.0 mM GAMG (Fig. 1B). This bacterium received the action enhancing the growth by these drugs.

The mixture of *Eubacterium* sp. GLH and *Ruminococcus* sp. PO1-3 as the intestinal flora including various bacteria indicating the difference in the growth and possessing both GL and GAMG β -D-glucuronidase and indexed 3β -HSD activity made for the examination. The growth of these mixed bacteria reached to the maximal stage at between culture of 6 and 12 h appeared in the characteristic of *Ruminococcus* sp. PO1-3 from low GL and GAMG β -D-glucuronidase activities and were enhanced about 2-fold at culture of 24 h by both 1.0 mM GL and 1.0 mM GAMG. GL led to the increase of GL β -D-glucuronidase activity gradually during culture of 36 h and was enhanced rapidly GAMG β -D-glucuronidase activity at culture of 12 h, and GAMG enhanced rapidly GL and GAMG β -D-glucuronidase activities at culture of 12 h. 3β -HSD activity showed almost no change at a lower level with or without 1.0 mM GL and 1.0 mM GAMG (Fig. 1C). The

Fig. 1. Effects of GL and GAMG on the Growth of *Eubacterium sp. GLH*, *Ruminococcus sp. PO1-3* and a Mixture of Both Bacteria

Each bacteria was cultured in GAM medium with and without 1.0 mM GL or 1.0 mM GAMG for 3 or 4 d at 37 °C under anaerobic conditions. Samples were taken at the indicated times. Bacterial growth, GL and GAMG β -D-glucuronidase activities and β -HSD activity were measured as described in Materials and Methods.

characteristic of these mixed bacteria appeared in strong character of *Ruminococcus sp. PO1-3* from GL β -D-glucuronidase activity against added substrate GL and GL and GAMG β -D-glucuronidase activity against added substrate GAMG. GL and GAMG were different in number of glucuronide and showed really two different effects.

The Metabolism of GL and GAMG by *Eubacterium sp. GLH*, *Ruminococcus sp. PO1-3* or a Mixture of Both Bacteria GL (1.0 mM) and 1.0 mM GAMG were converted to

about 70 to 85% of metabolite GA by *Eubacterium sp. GLH* at culture of 24 h (Fig. 2A). However, GAMG β -D-glucuronidase activity by GAMG was lower than GL β -D-glucuronidase activity by GL during culture of 24 h. It was found that GAMG β -D-glucuronidase has the ability to hydrolyze well at a lower activity.

The amount of metabolite GA produced from 1.0 mM GL and 1.0 mM GAMG by *Ruminococcus sp. PO1-3* coincided in the pattern of each bacterial growth and was about 50% in

Fig. 2. Metabolism of GL and GAMG by *Eubacterium* sp. GLH, *Ruminococcus* sp. PO1-3 and a Mixture of Both Bacteria

Each bacteria was cultured in GAM medium with 1.0 mM GL or 1.0 mM GAMG for 3 or 4 d at 37°C under anaerobic conditions. Samples were the same bottle as those in Fig. 1. The quantities of GL (—○—), GAMG (—△—), GA (—●—) and 3-oxo-GA (—▲—) were measured as described in Materials and Methods.

culture of 3 d and about 90% in culture of 24 h, respectively. The metabolism of GL started from a lower level for slowly in bacterial growth and low GL β -D-glucuronidase activity incident to it. GAMG was metabolized to a remarkable large amount of GA at an overwhelming fast for the increase of GAMG β -D-glucuronidase rapidly till culture 12 h and by the ability to hydrolyze well at low GAMG β -D-glucuronidase

activity as shown in *Eubacterium* sp. GLH of Figs. 1A and 2A (Fig. 2B).

GL (1.0 mM) and 1.0 mM GAMG by these mixed bacteria were metabolized almost completely to GA at culture of 48 and 24 h, respectively. GL β -D-glucuronidase activity increased gradually by the exposure of GL during culture of 36 h led to the increase of metabolite GA gradually during culture 48 h. GAMG β -D-glucuronidase activity increased more quickly by the exposure of GAMG at culture of 12 h contributed to the increase of metabolite GA linearly at culture of 24 h (Fig. 2C). These differences appeared in each influence of GL and GAMG added to each characteristic of bacteria of two species.

Effect of GL with GAMG on the Growth and a Maximal Stage of Mixed *Eubacterium* sp. GLH and *Ruminococcus* sp. PO1-3 and Their Metabolism GL (1.67 mM) with 1.67 mM GAMG added to mixed *Eubacterium* sp. GLH and *Ruminococcus* sp. PO1-3 after culture of 24 h enhanced gradually the growth till culture of 36 h, and after it, the bacterial growth was suppressed. GL and GAMG β -D-glucuronidases and 3 β -HSD activity showed a similar pattern at a low level as compared to control (Fig. 1C). GAMG β -D-glucuronidase activity was the highest among these enzyme activities (Fig. 3A). Mixing these components showed an enhanced action on further growth of bacteria. This may be the effect of GAMG (Fig. 1).

The growth of these mixed bacteria cultured with mixed GL (1.0 mM) and 1.0 mM GAMG was more slowly at a lower level till culture of 6 h and enhanced rapidly at culture of 4 d. This late stage of bacterial growth showed the suppression of GL and GAMG β -D-glucuronidase activities. It is considered that these enzyme activities and bacterial growth appeared in mixed effects of GL and GAMG of Figs. 1A and 1B (Fig. 3B) and showed a similar effect of GAMG with *Eubacterium* sp. GLH in the early stage of culture.

The competition in the metabolism of GL with GAMG was examined by these mixed components added to these mixed bacteria cultured for 0 and 1 d (Figs. 3C and 3D). Mixed GL (1.67 mM) and 1.67 mM GAMG with these mixed bacteria possessing high enzyme activities at culture 24 h was converted to about 20% of GA by the consumption of GAMG more quickly, indicating the ability to hydrolyze well at low GAMG β -D-glucuronidase activity. GL was not consumed for 12 h, and after it, the consumption of GL started slowly. The amount of metabolite GA showed little increase at culture of 2 d after addition of these components. It is considered that intestinal flora reaching a maximal stage can be metabolized more quickly against that one among various components.

Mixed 1.0 mM GL and 1.0 mM GAMG cultured with mixed *Eubacterium* sp. GLH and *Ruminococcus* sp. PO1-3 showed the consumption of GAMG linearly during culture of 2 d, and after that, the consumption of GL started slowly (Fig. 3D). This showed that the metabolism of GL was started by the lack of GAMG. It is found that in the presence of GL with GAMG, GL was difficult for intestinal bacteria to metabolize. A mixture of various drugs such as the Kampo medicine may be different in the beginning time of metabolism of each drug by intestinal flora, and it may be due to the growth phase of intestinal flora to metabolize at the same time. Also, each drug may be metabolized in order during the exponen-

Fig. 3. Effect of GL with GAMG on the Growth and a Maximal Stage of Mixed *Eubacterium* sp. GLH and *Ruminococcus* sp. PO1-3 and Their Metabolism

These mixed bacteria were cultured in GAM medium by the addition of 1.0 mM GL with 1.0 mM GAMG and 1.67 mM GL and 1.67 mM GAMG after culture of 0 and 1 d, respectively. Samples were taken at the indicated times. Bacterial growth, GL and GAMG β -D-glucuronidase activities, 3 β -HSD activity and metabolite were measured as described in Materials and Methods. GL (—○—), GAMG (—△—), GA (—●—) and 3-oxo-GA (—▲—).

tial growth phase of intestinal flora.

DISCUSSION

Most Kampo medicines contain liquorice, being one of the herbal medicines and administered orally to human as a decoction. Its major component, GL is metabolized to a small amount or a large amount of GA in culture after 24 h by *Eubacterium* sp. GLH, *Ruminococcus* sp. PO1-3, both (Fig. 2) and intestinal flora,^{4,13} being present in the gastrointestinal tract. GL and GA are absorbed from the intestinal tract by the presence in the epithelial cells of rat intestinal tract¹⁴ and appear in rat plasma at 30 min.¹¹ The time for metabolizing by intestinal flora and its appearance in plasma were different in a longer time and a short time, respectively. However, the

bacterial mass as high enzymatic reaction by controlled condition metabolized GL to a large amount of GA at between 60 and 180 min.¹⁵ While, absorbed GL was metabolized to a large amount of GAMG and a negligible amount of GA at 60 min by rat intestinal tract, lung, spleen, liver and kidney.³ It is suggested that in fact it takes less time to metabolize at the internal organs. GL and GAMG were detected in rat liver by intravenous administration of GL,¹⁶ causing the same metabolism in oral, intravenous and intraperitoneal administration. The main component of the extract of liquorice was GL and GA, and it is used as healing drugs by their administration. The combined effects of paeoniflorin in peony root and liquorice component were synergistic in tests to evaluate their inhibitory effect on gastric and anti-inflammatory disease by intraperitoneal administration¹⁷ and the variation of pharmacokinetic behaviour of GL in acute hepatitis and liver cirrhosis receiving continuous intravenous administration was closely related to the extent of the liver function.¹ Finally, GL and GAMG from GA were excreted to bile-duct in about 80%¹² and a small amount,¹⁸ respectively, from the examination of intravenous administration using rats. The excretion of GAMG metabolized from GL has not been reported yet. It is considered that GL and GAMG were exposed simultaneously to intestinal flora in intestinal tract. GL and GAMG were each metabolized to a small amount and a remarkable large amount of GA at culture 24 h by *Eubacterium* sp. GLH, *Ruminococcus* sp. PO1-3, these mixed bacteria and intestinal flora (Figs. 2A and 2B).⁵ However, the fact is GL with GAMG and is not the metabolism of one component by single bacterium. The competition in the metabolism of GL with GAMG by a good substitute, mixed *Eubacterium* sp. GLH and *Ruminococcus* sp. PO1-3 for intestinal flora appeared in the consumption of GAMG, not in GL. GAMG consumed more quickly and 80% linearly at culture 2 d, indicating the conversion of GA of about 80% and 3-oxo-GA of a negligible amount. The consumption of GL was started from culture 2 d (Fig. 3D). It is the turn of GL and GAMG to metabolize and difficult in the metabolism of GL by intestinal flora. This examination caused the residue of GL. These results suggested that GL can be metabolized by intestinal flora at the passage of first time. It is a tremendous phenomenon get from examination. Also, GL was transferred to internal organs by the enterohepatic circulation and was metabolized to GAMG by their organs at all times. GA from GAMG was greater in the metabolism by intestinal flora than in that by internal organs (Fig. 2).³ The conversion of 3-oxo-GA from GA was enhanced by *Ruminococcus* sp. PO1-3 of intestinal bacteria in the presence of conjugated bile acids at glycine¹⁹ and the mass of *Eubacterium* sp. GLH and *Ruminococcus* sp., PO1-3 possessing high enzyme activities.²⁰ Moreover, rat liver possessed GA dehydrogenase, 3 α -hydroxyGA dehydrogenase and 22 α - and 24-hydroxylizing enzymes.^{3,9,21} It is considered that the hydroxylation of position 22 α and 24 of metabolite GAMG, GA, 3-oxo-GA and 3 α -hydroxyGA including GL occurs in rat liver. The active metabolites from among these components are under active investigation.

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