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Plasma citrulline is a sensitive safety biomarker for small intestinal injury in rats

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ABSTRACT

Plasma citrulline is decreased in cases of severe intestinal injury with apparent villus and cellular atrophy. However, the fluctuation of plasma citrulline in slight intestinal injury remains to be investigated. To clarify this, irinotecan at 30 mg/kg or 60 mg/kg was administered intravenously to rats. Irinotecan reduced plasma citrulline concentrations compared to those in the pair-fed control, being concurrent with slight single cell necrosis and mucosal epithelium regeneration in the small intestine without apparent villus and cellular atrophy. Gene expression of enzymes converting glutamine to citrulline was decreased in the small intestine of the injury model. Moreover, citrulline and arginine levels in the ileum were decreased without alterations to glutamine and glutamate levels, indicating that citrulline synthesis from glutamine was impaired. Metabolome analysis revealed that plasma citrulline and arginine levels were decreased, while there were no marked alterations in other amino acids, metabolites of glycolysis, ketone bodies, or fatty acids. These results suggested that a decreased plasma citrulline level was unlikely to result from amino acid catabolism in response to malnutrition. In conclusion, plasma citrulline concentration reflects slight intestinal injury without apparent villus and cellular atrophy, and thus, it would be a sensitive biomarker for the small intestinal injury.

1. Introduction

Intestinal injury is one of the dose-limiting toxicities induced by anticancer drugs or non-steroidal anti-inflammatory drugs in humans. In preclinical settings, intestinal injury is identified using histopathological examination. However, histopathology can profile intestinal toxicity only at the time of necropsy, and the procedure to make thin sections and to assess tissues under a microscope is low-throughput. To screen out toxic compounds to the intestine effectively, sensitive, non-invasive, and reliable biomarkers are required. Although several potential intestinal biomarkers in plasma or feces, such as diamine oxidase, calprotectin, and miRNAs, have been reported, no sensitive or translational biomarkers from preclinical or clinical studies on intestinal injury has been validated to date (John-Baptiste et al., 2012).

Citrulline, a non-protein amino acid, is exclusively synthesized from glutamine by enterocytes, since the enzyme activities for citrulline synthesis are predominant (*e.g.* pyrroline-5-carboxylate synthase: P5CS) and the enzyme activities for citrulline catabolism are low (*e.g.* argininosuccinate synthase; ASS, and argininosuccinate lyase; ASL) in the small intestine (Barzal et al., 2014; Crenn et al., 2008; van de Poll et al., 2007). Released citrulline from enterocytes into the blood circulation is primarily metabolized to arginine in the kidneys. Although the liver is a main site of the urea cycle, the release and uptake of citrulline by the liver are negligible (Windmueller and Spaeth, 1981). Thus, plasma citrulline derived from the small intestine is considered to be a biomarker of intestinal failure reflecting reduced intestinal enterocyte mass (Crenn et al., 2008).

Plasma citrulline has been reported to be decreased in short bowel syndrome and intestinal injury induced by anticancer drugs or radiation in clinical and preclinical settings; however, the decreases were

Abbreviations: P5CS, pyrroline-5-carboxylate synthase; ASS, argininosuccinate synthase; ASL, argininosuccinate lyase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GLS, glutaminase; OAT, ornithine aminotransferase; OCT, ornithine carbamoyltransferase; PRODH, proline dehydrogenase; ARG2, arginase 2

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accompanied by apparent villus atrophy or wide surgical resection (Boukhettala et al., 2009; Crenn et al., 2003; Gutierrez et al., 2014; Herbers et al., 2010; John-Baptiste et al., 2012; Lutgens et al., 2003). The fluctuations of plasma citrulline in slight intestinal injury without apparent villus and cellular atrophy remain to be clarified.

In the present study, we investigated the plasma citrulline concentration in a rat model of slight small intestinal injury without apparent villus and cellular atrophy, induced by administration of irinotecan, which is a camptothecin-derived anticancer drug that induces adverse effects on rapidly proliferating cells such as enterocytes (Takasuna et al., 1996). It is understood that plasma citrulline levels are decreased under fasted or restricted feeding conditions in rats (Bloxam, 1972; Kume et al., 2015). In addition, the small intestine is sensitive to food restriction, due to its high rate of protein turnover (Coeffier et al., 2003). Indeed, the mucosal mass of the small intestine is reduced under fasting conditions for 1 or 2 days in rats (Samuels et al., 1996). Therefore, we compared plasma citrulline concentrations between the irinotecan-treated group and the pair-fed feeding control group, in which food consumption was restricted to an equivalent amount to that of the irinotecan-treated group. Next, gene expression and endogenous metabolites in plasma and the small intestine (jejunum and ileum), where citrulline is predominantly synthesized, were analyzed to clarify the relationship between plasma citrulline concentrations and citrulline synthesis in the small intestine.

2. Materials and methods

2.1. Chemicals and reagents

An intravenous infusion of irinotecan (irinotecan hydrochloride 100 mg/5 mL, Daiichi Sankyo Co., Ltd., Tokyo, Japan) was used. A solution of 3 mg/mL or 6 mg/mL was made by dilution with physiological saline (Otsuka Pharmaceutical Factory, Inc. Tokushima, Japan). Citrulline and arginine were purchased from Wako Pure Chemicals Industries (Osaka, Japan). D_6-citrulline was purchased from Toronto Research Chemicals Inc. (Toronto, Canada). These amino acids were dissolved with Milli-Q water to prepare stock solutions of 1 mM, respectively. LC/MS grade acetonitrile and formic acid were purchased from Kanto Chemical (Tokyo, Japan) and Wako Pure Chemicals Industries, respectively. The stock solution of citrulline was further diluted with Milli-Q water to prepare calibration standard solutions at concentration from 1 to 200 μM.

2.2. Animals

Six-week-old male Sprague Dawley rats were purchased from Charles River Japan, Inc. (Kanagawa, Japan). Rats were housed individually in stainless steel bracket cages under a controlled environment (temperature 20–26 °C, humidity 30–70%, 12 h light/12 h dark cycle). Rats were acclimatized for a week before use. A commercial powder or pellet-type diet for rodents (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) was available *ad libitum* in irinotecan-treated and *ad libitum* control groups. During the dosing period, an equivalent amount of food to that of the irinotecan-treated group was given to the pair-fed control group for 6 days. Tap water containing chlorine at 1 to 5 ppm was available *ad libitum* in any group. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Daiichi Sankyo Co., Ltd. All animal procedures were performed in accordance with the guidelines of the Animal Care and Use Committee of Daiichi Sankyo Co., Ltd.

2.3. Experimental design

Irinotecan at doses of 0 mg/kg (saline), 30 mg/kg, and 60 mg/kg was intravenously administered to male rats (five or six rats/group) *via* the tail vein at a dosing rate of 1.2 mL/min for 2, 4, or 6 days. The animals were sacrificed 24 h after the final dose and the days of necropsy were shown as Days 3, 5, and 7. The dose level and sampling point of irinotecan were selected to induce slight small intestinal injury (Takasuna et al., 1996). Following necropsy, blood samples were obtained from the abdominal aorta under isoflurane anesthesia. Whole blood was placed in blood sampling tubes containing lithium heparin (Microtainer, Nippon Becton Dickinson Company, Ltd., Tokyo, Japan) for blood chemistry analysis, or containing EDTA (Vacutainer, Nippon Becton Dickinson Company, Ltd.) for measurement of plasma citrulline levels or metabolome analysis. The obtained blood was centrifuged at 3000 rpm for 15 min at 4 °C with a refrigerated centrifuge to obtain plasma. The plasma samples were kept at −80 °C until use. After blood collection, the animals were euthanized by exsanguination, and the stomach and intestine (duodenum, jejunum, ileum, cecum, colon, and rectum) were excised for histopathological examination. For gene expression and metabolome analysis, two samples of the jejunum and ileum (100 mg each) were removed on Day 7 of necropsy and rinsed with physiological saline (Otsuka Pharmaceutical Factory, Inc.). The tissue specimens were then snap-frozen in liquid nitrogen and stored at −80 °C until use.

2.4. Blood chemistry and histopathology

Since hepatic injury or renal injury have an effect on urea cycle-related metabolites in plasma, such as arginine and citrulline (Crenn et al., 2008; Saitoh et al., 2014), plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities, creatinine, and urea nitrogen levels were measured by an automated clinical analyzer (TBA-2000FR, Toshiba Medical Systems Co., Ltd., Tokyo, Japan). For microscopic examination, the forestomach, glandular stomach, duodenum, jejunum, ileum, Peyer's patch, cecum, colon, and rectum specimens were fixed in 10% buffered neutral formalin, embedded in paraffin, sectioned, and then stained with hematoxylin and eosin.

2.5. Quantitation of plasma citrulline concentrations

Plasma citrulline concentration was quantitated with liquid chromatography/tandem mass spectrometry using D_6-citrulline as the internal standard. Briefly, analytical samples were prepared as described below. For each 10 μL of plasma, 2 μL of internal standard solution containing 100 μM of D_6-citrulline, 178 μL of acetonitrile, and 10 μL of 2 N hydrochloride were added, and then centrifuged at 11,000 × *g* for 1 min. The supernatants were collected and 8 μL of each of them were injected. Authentic solutions of citrulline at concentrations from 1 to 200 μM were used instead of plasma to prepare calibration standard samples. Chromatographic separation was performed on the Acquity Ultra High Performance Liquid Chromatography (UPLC) system (Waters, Milford, MA, USA) using an Inertsil NH_2 column (2.1 × 150 mm, 3 μm, GL Sciences Inc., Tokyo, Japan), which was maintained at 40 °C. The mobile phases A and B, and weak wash solvent used in this study were 0.5% formic acid in water, 0.5% formic acid in acetonitrile, and 95% acetonitrile containing 0.5% formic acid, respectively. The separations were performed at a flow rate of 0.4 mL/min with the following gradient elution conditions: 5% of B from 0 min to 0.6 min, linear increase to 100% of B at 3.5 min, which was maintained at 100% B to 5.0 min. The composition was then returned to the initial conditions at 5.0 min and held up to 10.0 min. The UPLC instrument was coupled to a Xevo TQ MS mass spectrometer (Waters). Detection was performed with the selected reaction monitoring mode using the electro-spray ionization technique operated in positive mode with the following transitions: citrulline (m/z 176 > 70) and D_6-citrulline (m/z 182 > 76). Chromatographic separation of citrulline from M + 1 isotopologue of arginine, which was detected in m/z 176 > 70, was confirmed (Supplemental Fig. 1). The other state file parameters used were as follows: capillary voltage 2500 V; source temperature 150 °C; desolvation temperature 550 °C; cone gas flow

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