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High concentrations of D-amino acids in human gastric juice

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Summary. The concentrations of free D- and L-amino acids were determined in the gastric juice from four groups: patients suffering from early gastric carcinoma with or without *Helicobacter pylori* infection, and patients without carcinoma but with peptic ulcers, duodenal ulcers or chronic gastritis with or without *H. pylori* infection. *H. pylori* is a bacterium associated with gastric inflammation and peptic ulcers and is a risk factor for stomach cancer. The highest D-amino acid ratios (free D-amino acid concentration to the total corresponding free D- and L-amino acid concentration) were 29%, 26%, 18%, 4% and 1% for proline, alanine, serine, aspartate and glutamate, respectively. The gastric juice levels of L-alanine, L-serine, L-proline, L-glutamate and D-alanine in the samples obtained from subjects bearing early gastric carcinoma and *H. pylori* were significantly higher than in the samples from the other three groups. Except for D-alanine, there was no correlation between the D-amino acid concentrations and presence of carcinoma or *H. pylori.*

Keywords: D-Amino acids – Gastric juice – Gastric carcinoma – *H. pylori*

Introduction

The ratio of free form D-alanine, D-serine and D-proline to the total corresponding free amino acid concentration (D- and L-amino acids) has been observed to be as high as 20% in the blood plasma of patients with renal diseases, whereas the ratio is less than 2% in healthy subjects (Nagata et al., 1992a). These D-amino acids were also found at high concentrations in the sera (Nagata et al., 1992b) and urine (Konno et al., 1989) of mutant mice lacking D-amino acid oxidase (EC 1.4.3.3). This enzyme catalyzes the oxidative deamination of free neutral D-amino acids (Krebs, 1935) and is localized in nephrons in the kidney at the highest concentration in the body (Chan et al., 1979). This enzyme maintains D-amino acid levels at low concentrations in normal mammalian plasma (Nagata et al., 1989).

Although the majority of serum D-alanine seems to be derived from intestinal microbes (Konno et al., 1990), some of the free D-amino acids have been suggested to be de novo synthesized in the metabolic pathways. A previous study demonstrated that the serum D-amino acid level in germ-free mice was not lower than that in specific pathogen-free (SPF) mice (Nagata and Akino, 1990). Serine racemase purified from rat brain (Wolosker et al., 1999) was the first racemase found in vertebrates. These findings suggest that the D-serine detected in the sera of humans, mice and rats did not originate from intestinal bacteria, but instead, was synthesized in the body. D-Aspartate synthesis in rat adrenal pheochromocytoma PC-12 cells is also strongly suggested (Long et al., 1989). At this time, the de novo synthesis of D-amino acids as a part of vertebrate metabolisms has been limited to only these two amino acids.

In search of sources of D-amino acids other than the brain and adrenal glands, five non-essential amino acids, serine, alanine, proline, aspartate and glutamate, were chosen for the present study. We analyzed gastric juice because it may not contain D-amino acid oxidase. Especially, we examined the gastric juice from cancer patients because a positive relationship was reported between tumor cells and D-amino acids (Kögl et al., 1939; Fisher et al., 1995).

Materials and methods

Patients

Nine patients carried early gastric carcinoma and were infected with *H. pylori* (3 males and 6 females; age, 66 to 75 years), 9 carried the carcinoma but were not carriers of *H. pylori* (5 males and 4 females; age, 59 to 75 years). Out of 18 patients with peptic ulcers, duodenal ulcers or chronic gastritis, 9 were carriers of *H. pylori* (6 males and 3 females; age, 53 to 82 years) and 9 were not carriers (4 males and 5 females; age, 28 to 69 years).

Collection of gastric juice and culture of H. pylori *cells*

After overnight fasting, gastric juice was collected from the subjects through the aspiration channel of an endoscope. The exclusion criteria of the sample included the following: (1) history of previous gastric surgery, (2) use of antimicrobial agents or proton pump inhibitor within 1 month preceding the gastric juice collection and (3) patients with bleeding gastric carcinoma. Informed consent was obtained from each patient prior to collection of gastric juice.

Type strain of *H. pylori* NCTC11637 was cultured on Brucella agar plates (Becton & Dickinson) containing 5% horse serum in an atmosphere containing 10% CO_2 in an incubator at 37 °C for 2 days.

Determination of amino acids

The cultured *H. pylori* cells were harvested from the agar plates and collected by centrifugation at $10,000 \times g$ for 15 min at 4 °C. They were suspended in H_2O after washing with 50 mM Na-phosphate buffer (pH 7.4), and disrupted by sonication followed by a French press ($200,000\,lb/in^2$). The cell-free extract of *H. pylori* was obtained as the supernatant of centrifugation ($13,000 \times g$ for 20 min at 4 °C) of the homogenate and was used to measure the concentrations of free amino acids. Both the gastric juice and the *H. pylori* cell-free extract were treated with 5% trichloroacetic acid to precipitate and remove proteins followed by centrifugation. The resultant solution was passed through a Dowex $50\,W \times 8$ (H^+-form) column, and eluted with 2 M NH_4OH after washing the column with H_2O to obtain purified free amino acids. The eluate was evaporated to dryness *in vacuo* in a centrifugal evaporator (Taitec, Saitama, Japan) below 40 °C. The determination of amino acids for each isomer was performed as described previously (Nagata et al., 1992c). In brief, the free amino acids were derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) (Sigma, MO, USA) (Marfey, 1984). Diastereomers of amino acids were formed and were separated on a Silica Gel 60 plate (Merck, Darmstadt, Federal Republic of Germany) by two-dimensional thin-layer chromatography. FDAA-serine, alanine, proline, aspartate and glutamate recovered from the plate were analyzed by HPLC for the resolution of D- and L-isomers, using a Nova-Pak C_{18} reversed-phase column (150 by 3.9 mm i.d.; Waters, MA, USA) and a Hitachi (Tokyo, Japan) or a Jasco (Tokyo) gradient HPLC system. The eluate from the column was monitored at 340 nm with a D-2500 Chromato-Integrator (Hitachi). As internal controls, known amounts of each authentic D- and L-amino acid were added to the samples before the trichloroacetic acid treatment and derivatized with FDAA as described above. On the basis of the peak areas of these authentic FDAA-amino acids, amounts of D- and L-amino acids in the samples were calculated.

Results

The concentrations of the five D-amino acids and their ratios to the total of each L- and D-amino acid in human gastric juice are shown in Table 1. High concentrations of D-alanine (206 nmol/ml), D-proline (80 nmol/ml) and D-serine (24 nmol/ml) were observed. The D-alanine level was significantly higher in the cancer group of *H. pylori* carriers than in the other three groups (Table 1). The D-amino acid ratios were high for D-proline and

Table 1. Free D-amino acid in human gastric juice

Group	Gastric lesion	*H. pylori* infection	Concentration (nmol/ml)[a] of D-amino acid (% of total L- and D-amino acid)				
			Serine	Alanine	Proline	Aspartate	Glutamate
A (n = 9)	cancer	+	8.5 ± 6.5 (3.5)	205.8 ± 79.5 (25.9)	80.3 ± 34.2 (13.7)	2.2 ± 1.0 (1.7)	1.2 ± 0.5 (0.5)
B (n = 9)	cancer	−	24.2 ± 10.2 (18.4)	26.3 ± 6.0 (14.6)[b]	4.0 ± 2.1 (8.4)	0.6 ± 0.6 (1.3)	0.7 ± 0.3 (0.7)
C (n = 9)	noncancer[c]	+	3.3 ± 2.4 (5.3)	15.9 ± 8.5 (17.2)[b]	17.9 ± 11.9 (29.2)	1.2 ± 0.6 (3.8)	0.4 ± 0.4 (0.7)
D (n = 9)	noncancer[c]	−	2.3 ± 2.0 (8.0)	6.6 ± 1.2 (12.1)[b]	12.8 ± 5.5 (28.7)	0.4 ± 0.3 (3.4)	0.2 ± 0.1 (0.8)

[a] Values are means with standard errors
[b] Value for ratio significantly different from that obtained for group A by MANOVA at $P < 0.05$
[c] Subjects had peptic ulcers, duodenal ulcers or chronic gastritis

Table 2. Free L-amino acids in human gastric juice

Group	Gastric lesion	*H. pylori* infection	Concentration (nmol/ml)[a] of L-amino acid				
			Serine	Alanine	Proline	Aspartate	Glutamate
A (n = 9)	cancer	+	237.7 ± 49.0	587.8 ± 128.0	503.8 ± 179.1	128.8 ± 40.0	236.0 ± 51.6
B (n = 9)	cancer	−	107.0 ± 13.8[b]	154.3 ± 12.3[c]	43.7 ± 4.5[b]	45.0 ± 7.4	95.7 ± 9.5[b]
C (n = 9)	noncancer[d]	+	59.5 ± 9.9[c]	76.3 ± 15.3[c]	43.5 ± 15.0[b]	30.3 ± 11.9[b]	60.0 ± 23.9[c]
D (n = 9)	noncancer[d]	−	26.5 ± 5.9[c]	48.0 ± 6.1[c]	31.8 ± 9.4[b]	11.5 ± 2.9[b]	25.0 ± 6.9[c]

[a] Values are means with standard errors. The data were obtained in the same experiments as in Table 1
[b] Value significantly different from that obtained for group A by MANOVA of $P < 0.05$
[c] Value significantly different from that obtained for group A by MANOVA at $P < 0.01$
[d] Subjects had peptic ulcers, duodenal ulcers or chronic gastritis

Table 3. L- and D-amino acids in *H. pylori*

Concentration (nmol/g wet wt)[a] of total L- and D-amino acid (% free D-amino acid)				
Serine	Alanine	Proline	Aspartate	Glutamate
95 ± 8.5 (2.4)	258 ± 20 (38.3)	31 ± 3.7 (0)	28 ± 4.6 (3.5)	108 ± 0.9 (1.4)

[a] Values are means with standard errors

D-alanine, up to 29% and 26%, respectively. For unknown reason, the D-serine level and the ratio were considerably higher in the cancer group not carrying *H. pylori* than in the other three groups. Except for D-alanine, there was no statistically significant difference between any two of the groups regarding the D-amino acid ratios.

On the other hand, the gastric juice levels of L-alanine, L-serine, L-proline and L-glutamate of the cancer-bearing group of *H. pylori* carriers were significantly higher than the levels of the other three groups (Table 2). The L-aspartate level of the cancer-bearing group was significantly higher than the levels of the noncancer groups (Table 2). The concentrations of free amino acids (total of D- and L-amino acids) in *H. pylori* cells are shown in Table 3. In the organism, the D-amino acid ratio was low, except for D-alanine (38%).

Discussion

High concentrations of D- and L-amino acids were detected in the gastric juice from subjects carrying both cancer and *H. pylori*. The amino acids might be supplied by the gastric mucosal cells, stimulated by the stomach cancer and *H. pylori* infection. Degraded neutrophils and monocytes may also be present in sites of mucosal inflammation caused by chronic infection with *H. pylori* (Zarilli et al., 1999), resulting in high L-amino acid levels in the gastric juice. The gastric-juice amino acid levels are equal to or slightly lower in the healthy subjects than in patients with peptic ulcers or chronic gastritis (Abasov, 1967; Segawa et al., 1983).

High concentrations of D-alanine, D-proline and D-serine have been detected in human gastric juice for the first time, possibly because gastric juice may not contain D-amino acid oxidase that metabolizes D-amino acids. The gastric juice is produced in many gastric glands and secreted into the stomach. In addition, saliva must be present in the gastric juice. The D- and L-amino acid contents in saliva (Nagata et al., 2005) are similar to those in gastric juice, except for serine. D- and L-serine concentrations are considerably low in saliva. Degraded *H. pylori* cells do not seem to affect the gastric-juice amino acid level because the D-amino acid concentrations of the bacterium are not high (Table 3). Even if bacterial cells were to be in the gastric juice samples, the cells had been removed by centrifugation before the Dowex 50 W-column chromatography. No correlation has been shown between the D-amino acid ratio of gastric juice and the presence of cancer. Although it was reported that tumor proteins contained D-amino acids such as D-glutamate (Kögl et al., 1939; Fisher et al., 1995), no significant difference was observed in the D-amino acid ratios between the cancer groups (A and B) and the noncancer groups (C and D) (Table 1). The D-glutamate concentration was low in all the samples, including those from patients with stomach cancer. The source of D-amino acids in the gastric juice has not been clearly shown except for saliva. Any dependency of the D-amino acid ratio on the sex or age of the subjects was not observed (data not shown).

It is interesting that the D-alanine concentration was significantly higher in the gastric juice from the patients carrying both carcinoma and *H. pylori* than in patients from the other categories. However, it is a matter for further study to elucidate the reason.

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