New aspects in pathogenesis of konzo: neural cell damage directly caused by linamarin contained in cassava
(*Manihot esculenta Crantz*)

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Epidemic spastic paraparesis (konzo) found in tropical and subtropical countries is known to be caused by long-term intake of cassava (*Manihot esculenta Crantz*), which contains a cyanoglucoside linamarin (α-hydroxysobutyronitrile-β-D-glucopyranoside). It has been reported that linamarin is enzymatically converted to cyanide by bacteria in the intestine, and this is absorbed into the blood and then damages neural cells. However, unmetabolized linamarin was found in the urine after oral administration of cassava; thus, we hypothesized that konzo could be caused by direct toxicity of the unmetabolized linamarin that was transferred to the brain and could be transported into neural cells via a glucose transporter. In the present study it was confirmed that linamarin directly damaged neural culture pheochromocytoma cell (PC) 12 cells; 0·10mm-linamarin caused cell death at 13·31 (SD 2·07) %, which was significantly different from that of control group (3·18 (SD 0·92) %, P<0·0004). Additional 10 μM-cytochalasin B, an inhibitor of a glucose transporter, prevented cell death: the percentage of dead cells significantly decreased to 6·06 (SD 1·98), P=0·0088. Furthermore, glucose also prevented cell death. These present results strongly suggest that linamarin competes with cytochalasin B and glucose for binding to a glucose transporter and enters into cells via glucose transporter.

**Cassava: Linamarin: Konzo: Glucose transporter**

A root of cassava (*Manihot esculenta Crantz*) shrub is an important staple food to millions of people in tropical and subtropical countries. Lancaster et al. (1982) were the first to report that prolonged consumption of cassava caused a metabolic neurological disease, konzo. In the last decade, several epidemics of konzo have been reported among the rural populations in Africa, including Mozambique, Tanzania, Zaire and the Central African Republic (Howlett, 1990). The epidemiological studies suggest that the eating of cassava combined with malnutrition caused permanent damage to the nervous system (Howlett et al. 1990). The toxicity of cassava was caused by cyanogenic glucoside, linamarin (α-hydroxysobutyronitrile-β-D-glucopyranoside) (Sunderesan et al. 1987), which is contained mainly in the root. When processing of cassava is not sufficient, linamarin is not completely removed and a portion of it remains.

The ingested linamarin is though to be hydrolysed to glucose and cyanohydrins in the intestinal tract; hydrogen cyanide is produced by a catalytic reaction of bacterial β-glucosidase in the intestine (Winkler, 1958). The cyanide is rapidly absorbed from the intestine into the blood. In clinical cases, concentrations of cyanide in the blood of patients suffering from konzo were between 4 and 70 μmol/l (Tylleskar et al. 1992), but the greater part of cyanide can be metabolized to much less toxic thiocyanate, since there are defending enzymes against cyanide, such as rhodanese or mercaptopyruvate sulfurtransferase, in the tissues of the whole body (Sylverster & Sander, 1990; Nagahara et al. 1998) and the intravenous cyanide is eliminated at the rate of 17 μg/kg body weight per min (Macnamara, unpublished results).

It was also reported that after cassava porridge containing linamarin was eaten, about 25% linamarin was excreted in the urine within 24 h in unmetabolized form, and about 50% as the less-toxic thiocyanate (Barrett et al. 1977; Brimer & Rosling, 1993; Carlson et al. 1995, 1999; Hernandez et al. 1995); cyanide produced by hydrolysis of linamarin in the gut was absorbed into the blood and was systemically metabolized to thiocyanate (Maduagwu, 1989).

As linamarin contains a glucose moiety, linamarin could bind to a glucose transporter (GLUT), be carried through the blood–brain barrier and then transported into cells via GLUT; furthermore, neural cells take up and consume glucose much more than the other cells. Thus, we hypothesized that after linamarin was absorbed from the intestine into the blood and transferred to the brain, linamarin could...
be transported to the cytoplasm of neural cells via GLUT and then accumulated linamarin could degenerate the cells.

The purpose of the present study was to test this hypothesis. First, comparative studies on toxicity of linamarin and cyanide to hepatoma (Hepa) 1 cells and neural pheochromocytoma (PC) 12 cells were done; second, the effect of cytochalasin B, an endofacial inhibitor of GLUT, and/or glucose on toxicity of linamarin to PC12 cells was investigated. The results of the present study strongly suggest that linamarin binds to GLUT and can be transported into cytoplasm via GLUT.

Materials and methods
Roswell Park Memorial Institute medium 1640 (Nissui Pharmaceuticals, Tokyo, Japan), Dulbecco’s modified Eagle’s medium and fetal bovine serum (GIBCO BRL; Rockville, MD, USA), glucose (Wako Pure Chemicals Ltd, Osaka, Japan), linamarin (Toronto Research Chemicals Inc., North York, Canada) and cytochalasin B (ICN Pharmaceuticals Inc., Costa Mesa, CA, USA) were purchased. Rat PC12 cell line was the kind gift of Dr K. Watabe, Department of Molecular Neuropathology, Tokyo Pharmaceuticals Institute for Neuroscience, Japan. Mouse Hepa 1 cell line was kindly given by Professor Y. Fujii, University of Tsukuba, Centre for Tsukuba Advanced Research Alliance, Japan.

Cell culture
PC12 cells were cultured in Roswell Park Memorial Institute medium containing fetal bovine serum (100 ml/l), glutamine (0.3 mg/ml) and gentamycin (20 mg/ml) in 50 ml tissue-culture flasks coated with collagen (100 g/l) in methanol (300 ml/l)). Hepa1 cells were cultured in Dulbecco’s modified Eagle’s medium containing fetal bovine serum (100 ml/l) and kanamycin (60 mg/ml). Cultures were maintained at 37°C in humidified 5% CO2.

Toxicity of linamarin and potassium cyanide for neural (pheochromocytoma 12) and hepatic (hepatoma 1) cells
PC12 and Hepa1 cells (2 × 10^5 and 3 × 10^4 cells/ml respectively) were incubated with different concentrations of linamarin (about 0.01–5.00 mM) or potassium cyanide (about 0.001–0.250 mM) in twenty-four well plates for 48 h at 37°C in 5% CO2. Control experiment was also done without addition of linamarin and potassium cyanide. After incubation the cells were harvested and cell viability was determined by staining with Trypan Blue. Cell death (%) was calculated as dead cells (n) per cells counted (N).

Effects of concentration of cytochalasin B on toxicity of linamarin to pheochromocytoma 12 cells
PC12 cells (2 × 10^5 cells/ml) were incubated with different concentrations of linamarin (about 0.01–5.00 mM) and 10 μM-cytochalasin B, a glucose transport inhibitor, in twenty-four well plates for 48 h at 37°C in 5% CO2 (cytochalasin B did not show toxic effect on PC12 cells at 10 μM). In this experiment, 11 mM-glucose was contained in the medium (commercial concentration of Roswell Park Memorial Institute medium). For a control group, PC12 cells were incubated in medium containing no linamarin and cytochalasin B. As an experimental group, PC12 cells (2 × 10^5 cells/ml) were incubated with 0.001, 0.010, 0.250 and 0.500 mM-linamarin.

After incubation the cells were harvested and cell viability was determined by staining with Trypan Blue. Cell death (%) was calculated as dead cells (n) per cell counted (N).

Effects of concentration of glucose with or without cytochalasin B on toxicity of linamarin to pheochromocytoma 12 cells
To investigate a competitively protective effect of glucose on transport of linamarin into cells, PC12 cells (2 × 10^5 cells/ml) were incubated for 48 h at 37°C in 5% CO2 in medium containing 11 (commercial concentration), 15, 21, 26 or 43 mM-glucose for a control group. 11, 15, 21, 26 or 43 mM-glucose with 0.10 mM-linamarin for an experimental group, and 11, 15, 21, 26 or 43 mM-glucose with 0.10 mM-linamarin and 10 μM-cytochalasin B for another experimental group.

After incubation the cells were harvested and cell viability was determined by staining with Trypan Blue. Cell death (%) was calculated as dead cells (n) per cells counted (N).

Study on metabolism of linamarin in the cell
To test whether linamarin serves as a substrate or an inhibitor of yeast hexokinase (Roche Diagnostics, Basel, Switzerland), a key enzyme of glycolysis, or whether linamarin is catalysed by rat recombinant rhodanese (Nagahara et al. 1995) or rat recombinant mercaptopyruvate sulfurtransferase (Nagahara & Nishino, 1996) (cyanide metabolizing enzyme; Nagahara et al. 1999), linamarin was added (0.05–2.00 mM) in each assay system for hexokinase (Harrison & Gray, 1972), rhodanese (Sorbo, 1953) and mercaptopyruvate sulfurtransferase (Nagahara & Nishino, 1996) and kinetic studies were carried out.

Statistical analysis
All values were expressed as mean values and standard deviations. The significance of difference between values was estimated by Student’s t test and two-way ANOVA.

Results
Comparison of toxicity of linamarin and cyanide for pheochromocytoma 12 and hepatoma 1 cells
As shown in Fig. 1(a), 32.27 (SD 5.54) % (n=3) PC12 cells were dead at 2.0 mM-linamarin; on the other hand, 35.58 (SD 3.54) % (n=3) Hepa1 cells were dead at 5.0 mM-linamarin (Fig. 1(b)). Two-way ANOVA to compare values in two groups exposed to linamarin at 0.01, 0.10, 1.00 and 2.00 (for PC12) or 2.50 mM (for Hepa1) represented significant difference (P=0.0000018). These results...
suggest that linamarin damages both PC12 and Hepa1 cells and PC12 cells are more sensitive to linamarin than Hepa1 cells.

As for a toxicity of cyanide, at 0.01 mM-potassium cyanide 25.40 (SD 1.44) % (n 3) PC12 cells were dead (Fig. 1(c); on the other hand, at 0.25 mM 24.84 (SD 8.93) % (n 3) of Hepa1 cells were dead (Fig. 1(d)). Two-way ANOVA to compare values in two groups exposed to cyanide at 0.001, 0.005, 0.010, 0.050 and 0.250 mM-potassium cyanide; (d), cyanide toxicity to Hepa1 cells: Hepa1 cells at 3 x 10^5 cells/ml were incubated with 0.050, 0.100 and 0.250 mM-potassium cyanide. For details of procedures, see p. 468. Values are means with standard deviations shown by vertical bars (n 3).

In comparing the toxic effect of linamarin with that of cyanide, at 0.01 mM-linamarin 25.40 (SD 1.44) % (n 3) PC12 cells were dead (Fig. 1(a)), whereas at 0.25 mM-cyanide 46.86 (SD 1.79) % (n 3) PC12 cells died (Fig. 1(c)). At 0.10 mM-linamarin, 8.89 (SD 2.31) % (n 3) Hepa1 cells were dead (Fig. 1(d)), whereas at 0.100 mM-cyanide 20.67 (SD 6.23) % (n 3) Hepa1 cells were dead (Fig. 1(d)). These findings suggest that cyanide is much more toxic to PC12 and Hepa1 cells than linamarin.

Possible mechanism of toxicity of linamarin in pheochromocytoma 12 cells

When cells were incubated with linamarin combined with 10 μM-cytochalasin B, dead cells (%) were significantly decreased (Fig. 2); 0.001, 0.010, 0.100, 0.250 and 0.500 mM-linamarin caused cell death of 4.22 (SD 2.43), 8.69 (SD 2.31), 15.67 (SD 2.75), 22.54 (SD 4.33) and 29.49 (SD 5.06) %, respectively (n 5), whereas cytochalasin B protected for the cell death caused by linamarin, and dead cell (%) significantly decreased to 2.14 (SD 1.97), 4.22 (SD 2.07), 8.34 (SD 3.27), 13.41 (SD 3.87) and 17.47 (SD 5.46) (n 5). These values in two groups except values at 1.00 mM-linamarin were significantly different (P=0.05, 0.032, 0.04 and 0.039, respectively). These findings indicate that the toxicity of linamarin to PC12 cells is partially protected by cytochalasin B.

On the other hand, as shown in Fig. 3, glucose also affected the mortality of PC12 cells, depending on the
concentration, probably due to increase in osmolarity by glucose. When glucose concentration was 11 mM (the concentration of glucose in a commercial medium) without addition of linamarin and cytochalasin B, 2.58 ± SD 0.44 % (n 5) cells were dead; 0.10 mM-linamarin (with 11 mM-glucose) significantly increased cell death to 15.67 ± SD 2.76 % (n 5) (P = 0.00039), whereas 10 μM-cytochalasin B (with 11 mM-glucose) protected the cell death and cell death (%) significantly decreased to 8.34 ± SD 3.27 % (n 5) (P = 0.032), which was significantly different from that of a control group (P = 0.025).

When glucose concentration was 15 mM without addition of linamarin and cytochalasin B, 3.18 ± SD 0.92 % (n 5) of cells were dead; 0.10 mM-linamarin (with 15 mM-glucose) significantly increased cell death to 13.31 ± SD 3.06 % (n 5) (P = 0.0004), whereas 10 μM-cytochalasin B (with 15 mM-glucose) protected from cell death and dead cells significantly decreased to 6.06 ± SD 1.98 % (n 5) (P = 0.0011). When glucose concentration was 21 mM, 5.17 ± SD 1.25 % (n 5) (P = 0.068).

In comparing linamarin toxicity among groups in the medium containing different concentrations of glucose, there was no significant difference between groups in the medium containing 11 mM- and 15 mM-glucose. On the other hand, 21 mM-glucose was more toxic to PC12 cells than 15 mM-glucose, although the difference was not significant (P = 0.11); however, linamarin toxicity was significantly reduced compared with the group treated with 0.1 mM-linamarin in the medium containing 15 mM-glucose (P = 0.01), suggest that glucose competes linamarin for GLUT. These findings conclude that cell damage by linamarin is protected not only by cytochalasin B, but also by glucose.

**Effect of transported linamarin on metabolism in the cell**

Linamarin (about 0.50–2.00 mM) did not inhibit hexokinase, a key enzyme in the first step of glycolysis, and hexokinase did not catalyse linamarin, suggesting that transported linamarin does not serve as a sugar source for glycolysis and reduce ATP production. Furthermore, rhodanese and mercaptopyruvate sulfurtransferase, cyanide detoxification enzymes, were not inhibited by linamarin, suggesting that transported linamarin did not inhibit detoxification of cyanide via transulfuration.

**Discussion**

The results of the present study strongly suggest that linamarin could bind to GLUT and enter cells. Transportedlinamarin directly damages both neural PC12 and hepatic Hepa1 cells, which is consistent with the fact that neural cells consume glucose more than hepatic cells and hepatic cells have more defence mechanisms, i.e. against cyanide than neural cells (Nagahara et al. 1999).

In the present study, we regarded cell death (%) as dysfunction of the cells exposed to toxic reagents, as there is no suitable marker for cell dysfunction by different toxic reagents, linamarin and cyanide. It is proper that dose of the reagent needed to impair the cell function is much less than that to cause cell death.

Previous reports show that factors other than cyanide were responsible for histopathological changes observed in the liver of fed gari (a cassava product) (Kamalu, 1991). On the other hand, in chronic cyanide poisoning, enlargement of the thyroid gland a distinctive finding, but this is not observed in konzo. It is possible that there is a change in normal flora in the intestine, resulting in reduction of the hydrolysis of linamarin contained in cassava for a long time (Kamalu, 1993), indicating that enzymatic conversion of linamarin to cyanide is reduced in gastrointestinal tract. However when cassava was orally administrated, unmetabolized linamarin was found in the urine (Barrett et al. 1977; Brimer & Rosling, 1993; Carlsson et al. 1995, 1999; Hernandez et al. 1995); therefore, linamarin can be directly absorbed in the intestine.

There are no reports of the concentration of linamarin in the serum of patients suffering from konzo, but thiocyanate concentration has been reported: the mean value for three patients was about 300 μM (Carlsson et al. 1999). Based on these results, in spite of the difference in metabolism and renal clearance between thiocyanate and linamarin, it is estimated that concentration of linamarin in the patient’s serum was > 150 μM. Linamarin at this concentration in a medium caused 15 % cell death in PC12. We consider that chronic exposure of linamarin at

**Fig. 3.** Effect of glucose and/or cytochalasin B on toxicity of linamarin to pheochromocytoma (PC) 12 cells. PC12 cells at 2 x 10^5 cells/ml were incubated in the medium containing 11, 15, 21, 26 or 43 mM-glucose alone as a control group ([], 11, 15, 21, 26 or 43 mM-glucose with 0-10 mM-linamarin ([], and 11, 15, 21, 26 or 43 mM-glucose with 0-10 mM-linamarin and 10 μM-cytochalasin B ([]). For details of procedures, see p. 468. Values are means with standard deviations shown by vertical bars (n 5). Mean values were significantly different from those of the group treated with linamarin in the medium containing 11 mM-glucose: *P = 0.032,**P = 0.0039. Mean values were significantly different from those of the group treated with linamarin in the medium containing 15 mM-glucose: †P = 0.00043, ††P = 0.0088. Mean values were significantly different from those of the group treated with linamarin in the medium containing 15 mM-glucose: §P = 0.0011.
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this concentration would damage to neural cells in vivo. In cyanide poisoning, lethal concentration of cyanide in blood is >90 μM in man (Ellenhorn, 1997). Cyanide at this concentration does not cause histological change in the brain in vivo, but causes about 40% cell death in PC12. Thus, there is a discrepancy between in vivo and in vitro results.

When PC12 cells were incubated with cytochalasin B and linamarin, the proportion of dead cells (%) significantly decreased, suggesting that cytochalasin B prevented linamarin from entering the cells and consequently reduced the toxicity of linamarin. In fact, cytochalasin B binds specifically to a GLUT and inhibits uptake of 2-deoxyglucose, a glucose analogue (Bloch, 1973). Furthermore, it was found that glucose uptake was inhibited by low concentration of cytochalasin B (Stahl et al. 1989). In the brain, glucose is taken up from blood across the blood–brain barrier via GLUT1 (Gerhart et al. 1989; Maher et al. 1994; Vannucci et al. 1997b; Choi et al. 2001; McAllister et al. 2001; Simpson et al. 2001), and is transported into neural cells via GLUT3 (Nagamatsu et al. 1993; Zeller et al. 1995; Vannucci et al. 1997a). Since linamarin is an analogue of glucose, linamarin could also bind to GLUT, pass through the blood–brain barrier and enter neural cells via GLUT.

3-O-methylglucose, a glucose analogue, also shares GLUT1 across the blood–brain barrier (Dauterive et al. 1996) and is transported into neural cells via GLUT3 (Maher et al. 1996). These facts suggest that a compound carried via GLUT1 also can be transported via GLUT3 and support our hypothesis that linamarin, a glucose analogue, could be carried through blood–brain barrier and be transported into neural cells via GLUT. In the present study, however, when PC12 cells were incubated with glucose alone, cell viability decreased with increase in concentration of glucose (15–43 mM), probably due to increase in osmolarity by glucose added. When cytochalasin B, an inhibitor of GLUT, was added to the medium containing 15 mM-glucose, the toxicity by linamarin significantly reduced. When glucose was added to medium (final concentration was 21 mM), toxicity of linamarin to PC12 cells was significantly reduced. These findings strongly suggest that linamarin competes with glucose for GLUT and is transported into the cells via GLUT, whereas cyanide diffusely enters cells.

Transported linamarin does not serve as a substrate or an inhibitor of hexokinase, the key enzyme of glycolytic pathway nor does it serve as a substrate of rhodanese and mercaptopyrurate sulfurtransferase. A greater part of transported linamarin would bind to GLUT and be transported into lysosome via GLUT1 (Mancini et al. 1990) and could be hydrolysed by membrane-bound glucosylceramidease, which has β-glucosidase activity. Cyanohydrin produced would be non-enzymatically hydrolysed to cyanide, which diffuses in cytoplasm and is converted to less toxic thiocyanate by two sulfurtransferases.

Based on the results of the present and previous studies, we propose that pathogenesis of konzo could be complicated; chronic exposure of unmetabolized linamarin causes degenerative change in neural cells; first, chronic exposure of cyanide which is produced from linamarin in the intestine causes decrease in ATP production followed by cell dysfunction; second, chronic exposure of unknown metabolites that are metabolized from linamarin in unknown tissues also cause cell dysfunction (Carlsson et al. 1999). Impairment of detoxification enzyme, rhodanese or mercaptopyrurate sulfurtransferase, cannot be excluded as a possible additional aetiology of konzo.

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