Protection from spontaneous hepatocellular damage by *N*-benzyl-D-glucamine dithiocarbamate in Long-Evans Cinnamon rats, an animal model of Wilson's disease

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Abstract

The Long-Evans Cinnamon (LEC) rat is a mutant strain that accumulates excessive tissue copper (Cu) and models the clinical symptoms and biological features of Wilson's disease in humans. We compared the effects of three metal chelating agents, *N*-benzyl-D-glucamine dithiocarbamate (BGD), D-penicillamine (D-PEN), and triethylenetetramine (TETA) on the biliary and urinary excretions of Cu using LEC rats. The animals were treated ip with each chelating agent (1 mmol/kg body weight) and then the bile and urine samples were collected for 3 h. Since the single treatment with BGD markedly stimulated biliary excretion of Cu, the protective effect of repeated BGD injection on spontaneous hepatocellular damage was further examined. Separate groups received two weekly injections of BGD starting at 11 weeks of age and were compared to saline injected controls. Serum alanine aminotransferase (ALT) activity and bilirubin level were significantly increased in control LEC rats by 19 weeks of age and histopathological analysis demonstrated extensive hepatic damage in these rats. However, repeated BGD injections prevented the increases in serum ALT and bilirubin and blocked the histopathological changes in the liver. Furthermore, although Cu rapidly accumulated in the liver, kidney, spleen and serum of control LEC rats during the test period, repeated BGD injection largely prevented these increases. These results indicate that BGD treatment is effective in blocking excessive Cu accumulation in LEC rats which, in turn, provides protection from spontaneous liver damage.

Introduction

Wilson's disease is an autosomal recessive disease caused by a functional disorder of copper (Cu) transport. This disease is characterized by failure to incorporate Cu into ceruloplasmin in the liver and to excrete the metal from the liver into bile. As a result, abnormal accumulation of Cu in various tissues, particularly the liver, occurs which ultimately leads to hepatitis and hepatocarcinoma (Nazer et al., 1986; Scheinberg and Sternlieb, 1996; Schaefer and Gitlin, 1999). Wilson's disease patients have a greatly reduced life-span.

Long-Evans Cinnamon (LEC) rat is a mutant strain that spontaneously develops hepatitis and hepatocarcinomas (Li et al., 1991), accompanied by abnormal accumulation of Cu in the liver (Sugawara et al., 1991a). The mutant rat shows many similar clinical symptoms and biological features with Wilson's disease (Li et al., 1991; Okayasu et al., 1992). The causative genes of this Cu disorder have been identified as encoding the same gene for P-type ATPase transporter involved in the incorporation of Cu into apo-ceruloplasmin (Bull et al., 1993; Wu et al., 1994). The defective expression of the gene results in the accumulation of Cu in the livers of Wilson's disease patients and LEC rats (Li et al., 1991; Sternlieb and Scheinberg, 1993). Thus, the LEC rats can be considered as an useful animal model for Wilson's disease. It has been reported that 90% of LEC rats at 14 weeks of age suffer severe jaundice, bleeding tendency, loss of body weight, and elevated serum transaminase activities caused by fulminant hepatitis and the 60% are led to death (Nagayama et al., 1991; Sawaki et al., 1994, 1998; Suzuki et al., 1995). Finally around 40% of LEC rats suffer hepatocarcinoma within 12 to 18 months (Takeichi, 1991). Both sexes of LEC rats are affected, but females are more vulnerable to fulminant hepatitis (Kasai et al., 1990).

Cu accumulated in the liver of LEC rats binds to metallothionein (MT) (Li et al., 1991; Sakurai et al., 1992a, 1992b; Sugawara et al., 1991a, 1991b), a low-molecular-weight, thiol-rich protein. Cu is thought to be non-toxic as long as it is bound to MT (Sakurai et al., 1992a; Sugawara et al., 1991b) because the protein plays an important role in cellular defense against many heavy metals such as cadmium (Waalkes and Goering, 1990). However, when Cu accumulation exceeds the capacity of the liver to synthesize MT, the excess unbound Cu is toxic (Takeichi, 1991; Suzuki, 1995). Acute hepatitis in LEC rat can occur when Cu accumulates beyond the capacity of MT synthesis (Takeichi, 1991; Suzuki, 1995; Suzuki et al., 1995).

To date, metal chelating agents such as D-penicillamine (D-PEN) and triethylenetetramine (TETA) have been clinically used in therapy for Wilson's disease (Walshe, 1982a, 1982b; Scheinberg et al., 1987; Gibbs and Walshe, 1990). Although D-PEN is the first choice as a Cu-chelating agent, it often worsens the neurologic symptoms in Wilson's disease possibly due to the redistribution of Cu to the brain (Brewer et al., 1994). TETA is usually used when D-PEN has to be withdrawn, although the effectiveness of this drug is not satisfactory. We have previously reported that *N*-benzyl-D-glucamine dithiocarbamate (BGD), a metal chelating agent, was quite effective in decreasing the cadmium concentration in the liver and kidney without promoting concomitant redistribution of the metal to other tissues such as the brain (Kojima et al., 1986, 1987). Thus, the present study was to undertaken to evaluate the comparative effects of BGD, D-PEN, and TETA on the biliary and urinary excretions of Cu in LEC rats, and to determine whether BGD can protect from the spontaneous hepatocellular damage in the rats.

Materials and methods

Chemicals

BGD was synthesized according to the procedures of Kojima et al. (1986). D-PEN and TETA (as tetrahydrochloride) were purchased from Nacalai Tesque (Kyoto, Japan). All other reagents were of the highest purity commercially available.

Animals

Female LEC rats at 5 weeks of age were purchased from Charles River Japan (Kanagawa, Japan). Rats were maintained on a 12-h light/dark cycle and were given free access to diet (CE-2, Japan CREA, Osaka, Japan) and water ad libitum. All animal experiments were undertaken in compliance with the guideline principles and procedures of Kumamoto University for the care and use of laboratory animals.

Biliary and urinary excretions of Cu after a single injection of chelating agent

Rats at 7 weeks of age were anesthetized with urethane (1 g/kg, ip), and the bile duct was exposed through a midline abdominal incision and cannulated with polyethylene tubing. Then the rats were injected ip 1 mmol/kg with one of the chelating agents (D-PEN, TETA and BGD) in 0.5 ml saline. Bile and urine samples were collected for an experimental period of 3 h. The contents of the urinary bladder were collected by syringe at the end of the experiment and combined with the previously collected urine. A follow up experiment looked at the dose-response relationship between BGD treatment (1, 2 and 3 mmol/kg, ip) and Cu excretion as above

Repeated BGD Injection

Rats at 11 weeks of age were divided into 2 groups; one served as control (injected with saline, n = 17) and another group was injected ip with BGD (2 mmol/kg, n = 9) twice per week for 9 weeks (total 18 injections). The body weights of rats were measured during the experiment. The animals of each group were killed at 15, 18, and 19 weeks of age, then immediately processed as described below. In preliminary experimentaion, all the control rats died from spontaneous acute hepatitis by 20 weeks of age, so this experiment was terminated at 19 weeks of age.

Assay of alanine aminotransferase (ALT) activity and bilirubin level

To assess the protective effect of BGD on hepatic damage, serum indicators were measured. ALT activity and bilirubin level were assayed with commercially available kits (Wako Pure Chemicals, Osaka, Japan), and expressed as units per liter and milligrams per deciliter, respectively.

Tissue metal content

The contents of Cu, Zn and Fe in liver, kidney, spleen, brain and serum were measured using atomic absorption spectrophotometry with a Hitachi Model Z-8000 spectrophotometer after the tissues were digested with nitric acid. The data was expressed as micrograms metal per gram wet weight of tissue.

Histochemical analysis

For histopathological analysis of hepatic tissue damage, the liver was fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with

hematoxylin and eosin. Paraffin sections were also processed for rhodanine stain (Lindquist, 1969) for Cu. Briefly, paraffin sections were treated with the rhodanine stock solution consisting of *p*-dimethylaminobenzylidene rhodanine and ethanol for 18 h at 37°C. Lesions were assessed by light microscopy.

Statistical analysis

Statistical analysis was performed either by analysis of variance (ANOVA) followed by Duncan's new multiple range test for multiple comparisons or by Student's t-test for paired comparisons. Probability values of < 0.05 were considered statistically significant.

Results

Effects of a single treatment with various chelating agents on biliary and urinary excretion of Cu

Figure 1 shows the cumulative biliary and urinary excretions of Cu in LEC rats treated with the chelating agents at a dose of 1 mmol/kg. Of single treatments with the various chelating agents, only BGD treatment markedly enhanced the biliary excretion of Cu (the amount of Cu excreted, 22.1 ± 2.4 μg/3h), while the treatments with D-PEN and TETA enhanced the urinary excretion of the metal only to a small extent.

Dose dependency of a single treatment with BGD on biliary and urinary excretion of Cu

Since the single treatment with BGD showed a clear stimulatory effect on biliary excretion of Cu, dose dependency of single treatment with BGD for biliary and urinary excretions of Cu was further examined. As shown in Fig. 2, the biliary excretion of Cu increased in a dose-dependent manner, although the urinary excretion of the metal did not change even at the higher doses of 2 and 3 mmol/kg.

Effect of repeated BGD injection

1) Body weight

To test the effect of repeated BGD injection, animals at 11 weeks of age were treated ip with BGD (2 mmol/kg) twice per week for 9 weeks (total 18 injections). Figure 3 shows time-course profiles for body weight changes of LEC rats treated repeatedly with BGD. Up to 26 days after beginning of repeated BGD injection, no change in body weight was observed between control and BGD-treated rats. However, the body

weight gain in control rats showed a tendency to be disturbed on 31-58 days. In BGD-treated rats, stable body weight gain was observed during this experimental period.

2) Serum alanine aminotransferase and bilirubin

The effects of repeated BGD injections on serum ALT activity and bilirubin level, which are indicators of hepatic damage, were examined in LEC rats. In control rats, both serum ALT activity and bilirubin level were significantly increased at 19 weeks of age (Fig. 4). However, in BGD-treated rats, these serum indicators did not increase.

3) Histopathological effects

The liver from 19-week-old control LEC rat (Fig. 5A) showed marked changes including necrosis, and granular and vacuolar degeneration of hepatocytes. Proliferated bile ducts and signs of regeneration of hepatocytes were associated with these changes. Increased numbers of macrophages containing hemolytic erythrocytes were observed in sinusoid. Intense rhodanine positive findings, which are bright red and indicate presence of Cu deposits, were observed frequently in macrophages and occasionally in hepatocytes (Fig. 5C). However, the liver from 19-week-old BGD treated rat did not develop overt pathology and only a few hepatocytes showed signs of degeneration (Fig. 5B) or slight rhodanine positive staining (Fig. 5D).

4) Metal concentrations

Figure 6 shows the effect of repeated BGD injection on Cu contents in the liver, kidney, spleen, brain and serum of LEC rats. In 18- and 19-week-old control rats, Cu

contents in liver, kidney, spleen and serum were markedly increased, indicating abnormal Cu accumulation. However, BGD treatment markedly decreased the Cu contents. In addition, Cu content in the brain did not change after BGD treatment. In order to examine whether repeated BGD injection can affect accumulation of other essential metals in these tissues, Fe and Zn contents were also analyzed. Fe contents were increased in the liver, kidney and spleen of 19-week-old control LEC rats, possibly from hepatic pathology, but this increase was prevented by the treatment with BGD (Fig. 7). No pronounced alteration in Zn content was observed in control or BGD-treated rats (Fig. 8).

Discussion

A key strategy in the treatment of Wilson's disease is to develop new effective Cu-chelating agents. The present study demonstrated that single BGD treatment markedly increases the biliary excretion of Cu in LEC rats, a model animal for Wilson's disease. Furthermore, repeated BGD injections blocked the spontaneous hepatocellular damage seen in LEC rats while markedly reducing tissue Cu accumulation. It has been reported that in LEC rats, increases in serum enzyme indicators of hepatocellular damage are observed in accordance with the onset of jaundice at about 16 weeks of age, followed by the marked increases at 18 weeks of age (Suzuki et al., 1995). In the present study, significant increases in serum ALT activity and bilirubin level were observed at 19 weeks of age in control LEC rats. However, the repeated treatment with BGD decreased these serum markers of hepatic damage, indicating a protection from spontaneous hepatocellular damage in LEC rats. This was consistent with the results of histopathological analysis in which pathological changes in 19-week-old BGD treated LEC rats was negligible in contrast with the marked hepatopathology damage in 19-week-old control rats.

In the present study, the repeated treatment with BGD was effective for removal of Cu accumulated in the liver, kidney, spleen and serum of LEC rats. Most of Cu accumulated in the liver of LEC rats binds to MT (Li et al., 1991; Sakurai et al., 1992a, 1992b; Sugawara et al., 1991a, 1991b), but the capacity to synthesize MT is limited and the resulting unbound Cu is probably the toxic spars (Takeichi, 1991; Suzuki, 1995). Thus, when Cu is accumulated at more than the capacity of the hepatic MT synthesis, hepatitis likely occurs (Takeichi, 1991; Suzuki, 1995; Suzuki et al., 1995). Previously,

we reported that BGD is capable of removing Cd and Hg from MT (Kojima et al., 1986; Kiyozumi et al., 1988). On the other hand, prior work indicates D-PEN and TETA cannot remove Cu bound to MT (McQuaid and Mason, 1990). BGD, unlike D-PEN and TETA, may be effective at removing Cu even from tightly bound sites and have a distinct advantage in treatment of Wilson's disease. In fact, the removal of Cu from tissues of LEC rats by BGD was much greater than that resulting from D-PEN or TETA treatment. Tetrathiomolybdate is also known as a potent and selective Cu-chelating agent that has been shown to be effective in Wilson's disease patients (Brewer et al., 1991; Suzuki and Ogura, 2000). This chelating agent can remove Cu bound to MT (Suzuki and Ogura, 2000). Therefore, it is necessary to evaluate the real utility of BGD, by comparing with the ability of tetrathiomolybdate to block excessive Cu accumulation in the tissues of LEC rats.

Redistribution of metals removed from the target organs sometimes occurs in therapy using chelating agents and this can cause severe side effects. For instance, the treatment with 2, 3-dimercaptopropanol (BAL) to animals exposed to Cd or Hg causes the redistribution of the metal to other tissues such as the brain (Kiyozumi et al., 1988; Gale et al., 1983). Diethyldithiocarbamate (DDTC) also causes the redistribution of nickel and Cd to the brain (Oskarsson and Tjalve, 1980; Gale et al., 1983). Previously, we have reported that BGD has no effect on redistribution of heavy metals including Cd and Hg to other tissues such as the brain (Kojima et al., 1986, 1989; Kiyozumi et al., 1988). In the present study, redistribution of Cu to the brain was not observed in BGD-treated LEC rats. In addition, BGD is much less toxic (LD_{50} = 4320 mg/kg) (Kojima et al., 1987) than D-PEN (377 mg/kg) (Cantilena and Klaassen, 1981) or TETA (38.5 mg/kg) (Williams et al., 2001) in mice. The diminished toxicity of BGD

together with the lack of redistribution of Cu to critical target tissue (i.e. brain) would provide further advantages in treatment of Wilson's disease.

Several studies have shown that LEC rats also exhibit abnormal hepatic Fe overload (Kato et al., 1996). Fe is well known to efficiently produce reactive oxygen species (Stohs and Bagchi, 1995) which can damage proteins and lipids by reacting with them. In the present study, Fe was accumulated not only in the liver, but also in the kidney and spleen of LEC rats. The accumulated Fe may be responsible, at least in part, for the spontaneous hepatocellular damage in LEC rats. Repeated treatment with BGD blocked this Fe accumulation. In contrast, Zn was not accumulated markedly in the tissues of LEC rats, even though Zn content in the kidney was slightly increased at 18 and 19 weeks of age. In BGD-treated rats, Zn contents in the tissues were stable during the experimental period. At the present time, we can not explain the reason why BGD increases Zn contents in the spleen and serum. Further studies are needed to elucidate the effect of BGD treatment on the homeostasis of essential metals including Fe and Zn in LEC rats.

In conclusion, the results of the present study demonstrate that the repeated treatment with BGD in rats with a genetically-based Cu storage disease effectively removes Cu from the body through biliary excretion, and prevents resulting hepatopathology. BGD has several advantages over chelators presently used in Wilson's disease, including lower toxicity and the absence of Cu redistribution to the brain, which make it a good candidate for clinical trials.

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Figure legends

Fig. 1. Effects of a single treatment with various chelating agents on biliary and urinary excretions of Cu in LEC rats. The animals at 7 weeks of age were injected i.p. with each chelating agent (1 mmol/kg) and then bile and urine samples were collected for 3 h. Each bar represents the mean ± S.D. (n = 3). *Significantly different from control ($p < 0.05$).

Fig. 2. Dose dependency of a single treatment with BGD on biliary and urinary excretions of Cu in LEC rats. The animals at 7 weeks of age were injected i.p. with BGD (1, 2 or 3 mmol/kg) and then bile and urine samples were collected for 3 h. Each bar represents the mean ± S.D. (n = 3 to 5). *Significantly different from control ($p < 0.05$).

Fig. 3. Effect of repeated BGD injection on body weight of LEC rats. The animals at 11 weeks of age were divided into 2 groups; one served as control (●, n = 17) and another group was injected i.p. with BGD (○, 2 mmol/kg, n = 9) twice per week for 9 weeks (total 18 injections). The beginning of BGD treatment set as day zero. The body weights of rats were measured during the experiment. *Significantly different from control ($p < 0.05$).

Fig. 4. Effect of repeated BGD injection on serum ALT activity and bilirubin levels in LEC rats. For experimental details refer to the legend for Fig. 3. ●, control; ○, BGD. Activity and levels were determined at 11, 15, 18 and 19 weeks of age. Each

point represents the mean ± S.D. (n = 3). *Significantly different from control (p < 0.05).

Fig. 5. Effect of repeated BGD injection on hepatocellular damage and Cu distribution in LEC rats. For experimental details refer to the legend for Fig. 3. Livers were excised 11, 15, 18 and 19 weeks of age. Histopathology: (A) control rat (19 weeks); (B) BGD-treated rat (19 weeks), (HE, x400). Rhodanine histochemistry: (C) control rat (19 weeks); (D) BGD-treated rat (19 weeks). Note: the marked changes including necrosis, granular and vacuolar degeneration of hepatocytes (A) with intense rhodanine positive findings in macrophages and hepatocytes (C) in the control rat. Signs of mild degeneration (B) and slight rhodanin positive findings (D) of hepatocytes.

Fig. 6. Effect of repeated BGD injection on Cu contents in the liver, kidney, spleen, brain and serum of LEC rats. For experimental details refer to the legend for Fig. 3. The concentration was determined at 11, 15, 18 and 19 weeks of age. Each point represents the mean ±S.D. (n = 3 to 5). *Significantly different from control (p < 0.05).

Fig. 7. Effect of repeated BGD injection on Fe contents in the liver, kidney, spleen, brain and serum of LEC rats. For experimental details refer to the legend for Fig. 3. The concentration was determined at 11, 15, 18 and 19 weeks of age. Each point represents the mean ±S.D. (n = 3 to 5). *Significantly different from control (p < 0.05).

Fig. 8. Effect of repeated BGD injection on Zn contents in the liver, kidney, spleen, brain and serum of LEC rats. For experimental details refer to the legend for Fig. 3. The concentration was determined at 11, 15, 18 and 19 weeks of age. Each point represents the mean ± S.D. (n = 3 to 5). *Significantly different from control ($p < 0.05$).