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Antitumour activity and adverse reactions of combined treatment with chitosan and doxorubicin in tumour-bearing mice

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Abstract

We examined the antitumour activity and adverse reactions, such as myelotoxicity, gastrointestinal toxicity and body-weight loss, of the cancer chemotherapy drug doxorubicin when given with chitosan in sarcoma 180-bearing mice. Intraperitoneally administered doxorubicin (5 mg kg^{-1}) given on days 1 and 8 with or without orally administered chitosan (200, 400 and 800 mg kg^{-1} twice daily) inhibited tumour growth. The orally administered chitosan (400 and 800 mg kg^{-1} twice daily) prevented doxorubicin-induced body-weight loss and small-intestinal mucosal injury. Similarly, the reduction of leucocyte number induced by the intraperitoneally administered doxorubicin was restored to normal by the oral administration of chitosan (400 and 800 mg kg^{-1} twice daily). It seems likely that the mechanisms by which the orally administered chitosan protects against doxorubicin-induced gastrointestinal toxicity may be due to the formation of doxorubicin–chitosan complex in the small-intestinal mucosa through the diffusion of chitosan into the small-intestinal villi. In conclusion, our data suggest that the oral administration of chitosan prevents the gastrointestinal mucositis associated with doxorubicin therapy.

Introduction

Chitin and chitosan are polymers with molecular weights of about 1000 kDa, and contain more than 5000 acetylglucosamine and glucosamine units, respectively. Chitin is widely distributed in natural products such as the protective cuticles of crustaceans and insects, as well as being found in the cell walls of some fungi and microorganisms, and is usually prepared from the shells of crabs and shrimps. Chitin is converted to chitosan by deacetylation with 45% NaOH at 100°C for 2 h. It has been reported that chitosan augments the natural killer activity of mouse lymphocytes (Zhou et al 1994). Recently, we have also reported that chitosan prevents the adverse effects (myelotoxicity, gastrointestinal toxicity, immunocompetent organic toxicity and reduction of body weight) induced by the oral administration of cancer chemotherapy drugs, 5-fluorouracil and cisplatin, without interfering with their antitumour activity (Kimura & Okuda 1999; Kimura et al 2000). Although anthracycline derivatives have been used extensively in the treatment of certain types of cancer (Quantin et al 2000; Verweiji et al 2000), they induce vomiting (Luftner et al 1999), nephrotoxicity (Venkatesan et al 2000), myelotoxicity (Strurgill et al 2000), gastrointestinal toxicity (Morelli et al 1996) and cardiotoxicity (Wahab et al 2000). In this study, we examined the antitumour and possible adverse effects of doxorubicin administered intraperitoneally, together with chitosan, in solid-type sarcoma 180-bearing mice.

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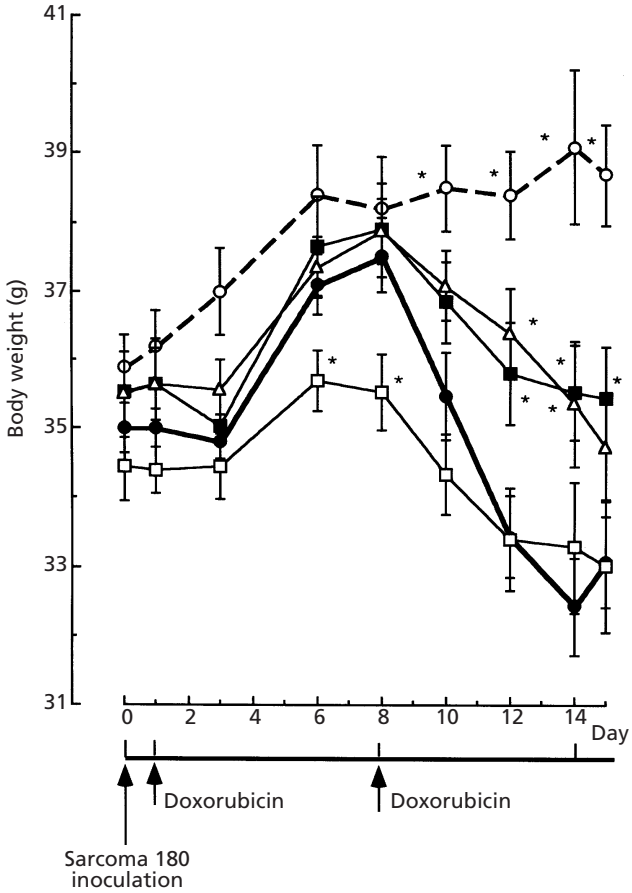

Figure 1 The combined effects of doxorubicin and chitosan on body weight in sarcoma 180-bearing mice. Results are expressed as mean ± s.e.m. of results from 10–20 mice. ○, Sarcoma 180-bearing mice (control, n = 20); ●, doxorubicin (5 mg kg^{-1} on days 1 and 8, i.p., n = 11); □, doxorubicin + chitosan (200 mg kg^{-1} twice daily, p.o., n = 10); ■, doxorubicin + chitosan (400 mg kg^{-1} twice daily, p.o., n = 10); △, doxorubicin + chitosan (800 mg kg^{-1} twice daily, p.o., n = 10). $^*P < 0.05$, vs intraperitoneal administration of doxorubicin alone.

Materials and Methods

Materials

Chitosan was supplied by Fuji Bio Co. (Shizuoka, Japan) and was converted to the chloride salt. Its intrinsic viscosity was about 113 cP. The average molecular weight was determined to be 500–700 kDa based on the viscosity, and the degree of acetylation was 14%. Chitosan was suspended in distilled water or 0.9% NaCl solution. Doxorubicin (Adriacin Injection) was supplied by Koywa Hakko Co. Ltd (Tokyo, Japan) and dissolved in 0.9% NaCl solution. Other chemicals were of reagent grade. Sarcoma 180 cells were maintained in the laboratory of the Second Department of Medical Biochemistry, School of Medicine, Ehime University, Japan.

Animals

Male ICR strain mice (6 weeks old) were obtained from Clea Japan (Osaka). ICR mice were housed for 1 week in a room maintained at 25 ± 1°C with 60% relative humidity and provided with free access to food and water. The room was lit for 12 h per day starting at 0700 h. Mice were treated according to the ethical guidelines of the Animal Center, School of Medicine, Ehime University. The experimental protocol was approved by the Animal Studies Committee of Ehime University.

Measurement of antitumour activity and adverse effects induced by doxorubicin in sarcoma 180-bearing mice

Solid-type sarcoma 180 was prepared by subcutaneous transplantation of 3×10^6 cells into the right abdomen of mice on day 0. Doxorubicin (5 mg kg^{-1}) was administered intraperitoneally on days 1 and 8 (at 0700 h), and chitosan (200, 400 or 800 mg kg^{-1}) was administered orally twice daily (at 0730 and 1930 h) for 14 consecutive days, starting 12 h after the implantation of tumour cells. Control mice were also given 0.9% NaCl solution or water on the same schedule. The tumour volume was determined by direct measurement with callipers and calculated by the formula length × $width^2/2$, every 2 or 3 days. Also, the food intake was measured every 2 or 3 days. On day 15, blood was obtained by venous puncture under anaesthesia with diethyl ether. Subsequently, the tumour, small intestine, liver, epididymal adipose tissue, kidney, heart, spleen and thymus were removed, and weighed for evaluation of antitumour activity and adverse effects. The blood samples were chilled in test-tubes containing heparin, and the number of leucocytes were measured using a Coulter Counter (Japan Scientific Instruments Co. Ltd, Tokyo, Japan). Blood urea nitrogen (BUN) and activities of transaminases (glutamic pyruvic transaminase (GPT) and glutamic oxaloacetic transaminase (GOT)) and creatine phosphokinase (CPK) were measured using Wako Bun-B-Test, Wako-S.T.A-Test and Wako CPK-test kits, respectively.

Measurement of sucrase activity in small-intestinal mucosal membrane in sarcoma 180-bearing mice

The small intestine was washed with cold 0.9% NaCl, and the mucosa scraped off with a glass slide. It was then

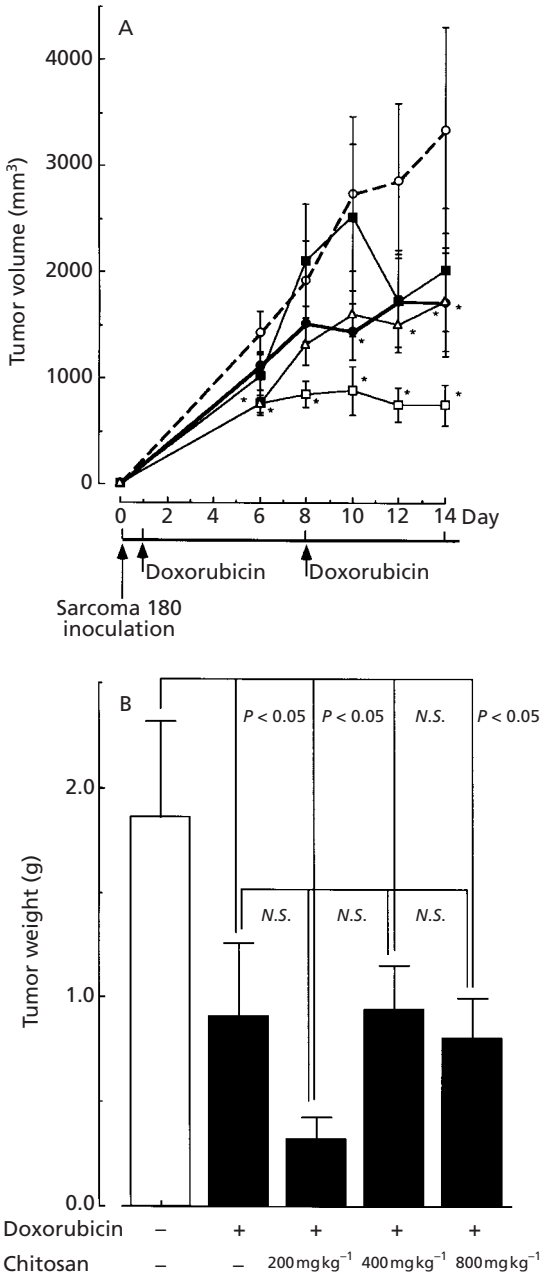

Figure 2 The combined antitumour activity of doxorubicin and chitosan in sarcoma 180-bearing mice. A. The combined effects of doxorubicin and chitosan on tumour volume in sarcoma 180-bearing mice. Results are expressed as mean ± s.e.m. of results from 10–20 mice. ○, Sarcoma 180-bearing mice (control, n = 20); ●, doxorubicin (5 mg kg^{-1} on days 1 and 8, i.p., n = 11); □, doxorubicin + chitosan (200 mg kg^{-1} twice daily, p.o., n = 10); ■, doxorubicin + chitosan (400 mg kg^{-1} twice daily, p.o., n = 10); △, doxorubicin + chitosan (800 mg kg^{-1} twice daily, p.o., n = 10). *$P < 0.05$, vs sarcoma 180-bearing mice (control). B. The combined effects of doxorubicin and chitosan on tumour weight in sarcoma 180-bearing mice. Results are expressed as mean ± s.e.m. of results from 10–20 mice. N.S., not significant.

homogenized with 80 mM sodium phosphate buffer (pH 7.0) in a final volume of 2 mL using a polytron homogenizer (Kinematica, Switzerland). Sucrase activity was determined by the methods of Kimura & Okuda (1999). Briefly, the assay was performed in a reaction mixture containing the homogenate (50 μL) in 5 mM sucrose and 80 mM sodium phosphate buffer (pH 7.0), in a total volume of 0.5 mL at 37°C for 30 min. The glucose liberated was determined using glucose oxidase reagents.

Statistical analysis

Data are expressed as mean ± s.e.m. Statistical analysis was performed with Fisher's Protected LSD test to determine significance ($P < 0.05$) using Super analysis of variance software (Abacus Concepts Inc., Berkeley, CA).

Results

Antitumour activity of intraperitoneal doxorubicin alone or plus oral chitosan in sarcoma 180-bearing mice

We examined the antitumour action and possible adverse effects of intraperitoneal doxorubicin administration (5 mg kg^{-1} on days 1 and 8 after inoculation of tumour cells) alone or plus the oral administration of chitosan (200, 400 or 800 mg kg^{-1} twice daily for 14 days). A remarkable reduction in body weight of mice after 8 days was observed in the mice receiving intraperitoneal doxorubicin compared with control mice (Figure 1). Oral administration of chitosan, at doses of 400 and 800 mg kg^{-1} twice daily, prevented the doxorubicin-induced reduction in body weight after 12 days (Figure 1).

Intraperitoneal doxorubicin significantly inhibited tumour volume and tumour weight, compared with control mice (Figure 2). Similarly, doxorubicin plus chitosan (200 and 800 mg kg^{-1} twice daily) also inhibited the tumour growth, compared with control mice (Figures 2A and 2B). However, there was no significant difference in tumour weight between the group receiving doxorubicin and those receiving doxorubicin plus oral chitosan (200, 400 and 800 mg kg^{-1} twice daily). The oral administration of chitosan, at a dose of 200 mg kg^{-1} twice daily, increased the antitumour activity of doxorubicin (Figure 2). These results indicate that the orally administered chitosan did not decrease the antitumour activity of intraperitoneal doxorubicin.

Effects of orally administered chitosan on adverse effects induced by intraperitoneal doxorubicin in sarcoma 180-bearing mice

The intraperitoneal administration of doxorubicin (5 mg kg^{-1} on days 1 and 8) resulted in a reduction in weight of the heart, liver, kidney and adipose tissue, compared with control mice (Table 1). Chitosan, at a dose of 400 mg kg^{-1} twice daily, prevented the reduction in liver and kidney weights induced by the intraperitoneal administration of doxorubicin but chitosan at the doses of 200 and 800 mg kg^{-1} twice daily did not. The reduction in weight of heart and adipose tissue induced by doxorubicin failed to be recovered by the oral administration of chitosan. Moreover, intraperitoneally administered doxorubicin reduced the weight of the thymus, but it did not affect the weight of the spleen. Chitosan had no effect on the reduction of thymus weight induced by doxorubicin. However, doxorubicin (5 mg kg^{-1} on days 1 and 8, i.p.) plus chitosan (400 and 800 mg kg^{-1} twice daily, p.o.) significantly increased the spleen weight, compared with control mice and mice treated with doxorubicin alone. The number of leucocytes was significantly decreased by the intraperitoneal administration of doxorubicin alone (Table 1). However, there was no significant difference in the leucocyte number between the control mice and mice treated with doxorubicin plus chitosan (400 and 800 mg kg^{-1} twice daily, p.o.). These results indicate that the reduction in leucocyte number induced by intraperitoneal doxorubicin was prevented by the oral administration of chitosan (400 and 800 mg kg^{-1} twice daily).

The intraperitoneal administration of doxorubicin to mice reduced the weight of small intestine and the sucrase activity of its mucosal membrane (Figure 3). The protein content of the small-intestinal mucosa was also reduced by intraperitoneally administered doxorubicin (data not shown). The oral administration of chitosan (400 and 800 mg kg^{-1} twice daily) prevented the doxorubicin-induced reduction in protein content of the small-intestinal mucosa (data not shown). In addition, doxorubicin had no effect on sucrase activity in in-vitro experiments (data not shown). Chitosan (400 or 800 mg kg^{-1} twice daily, p.o.) inhibited the doxorubicin-induced reduction in small-intestine weight and sucrase activity (Figures 3A and 3B). Therefore, it seems likely that chitosan prevents the intestinal mucosa injury induced by doxorubicin.

The intraperitoneal administration of doxorubicin did not cause elevation of plasma GOT, GPT, CPK and BUN. These results indicate that administration of doxorubicin (5 mg kg^{-1}, i.p.) to mice on days 1 and 8, does not cause liver injury, cardiotoxicity or nephrotoxicity. The intraperitoneal administration of doxorubicin plus the oral administration of chitosan (200, 400 and 800 mg kg^{-1} twice daily) had no effect on the plasma levels of GOT, GPT, CPK and BUN (data not shown).

Discussion

This study indicated that doxorubicin, administered intraperitoneally (5 mg kg^{-1}) on days 1 and 8, had potent

Table 1 Effects of intraperitoneally administered doxorubicin alone or doxorubicin plus orally administered chitosan on the weights of various tissues and the numbers of leucocytes in sarcoma 180-bearing mice.

	Liver (g)	Kidney (mg)	Heart (mg)	Adipose tissue (mg)	Thymus (mg)	Spleen (mg)	Leucocytes (10^3 μL^{-1})
Sarcoma 180-bearing mice (control, n = 20)	2.79 ± 0.061*	801.8 ± 14.8*	159.1 ± 5.13*	603.6 ± 25.6*	48.2 ± 6.72*	150.0 ± 14.2	6.55 ± 0.98*
Doxorubicin (n = 11)	1.79 ± 0.080	557.5 ± 21.0	128.5 ± 3.86	162.9 ± 36.4	14.1 ± 1.98	164.8 ± 11.6	4.87 ± 0.327
Doxorubicin + chitosan (200 mg kg^{-1}; n = 10)	1.83 ± 0.082	583.0 ± 33.8	135.0 ± 5.00	275.0 ± 47.7	14.5 ± 1.58	146.6 ± 5.50	4.49 ± 0.387
Doxorubicin + chitosan (400 mg kg^{-1}; n = 10)	2.14 ± 0.063*	635.0 ± 16.8*	132.0 ± 2.91	330.0 ± 29.2	15.5 ± 3.22	250.1 ± 29.0*	5.02 ± 0.294#
Doxorubicin + chitosan (800 mg kg^{-1}; n = 10)	1.92 ± 0.111	604.0 ± 31.6	135.0 ± 6.19	285.0 ± 47.4	17.2 ± 3.39	218.7 ± 23.6*	5.37 ± 0.258#

Results are expressed as mean ± s.e.m. of 10–20 mice. Doxorubicin (5 mg kg^{-1}) was administered intraperitoneally to mice on days 1 and 8 after implantation of tumour cells; chitosan (200, 400 or 800 mg kg^{-1}) was administered orally twice daily for 14 days starting 12 h after implantation of tumour cells. *$P < 0.05$ vs intraperitoneally administered doxorubicin alone. #Not significantly different from control group.

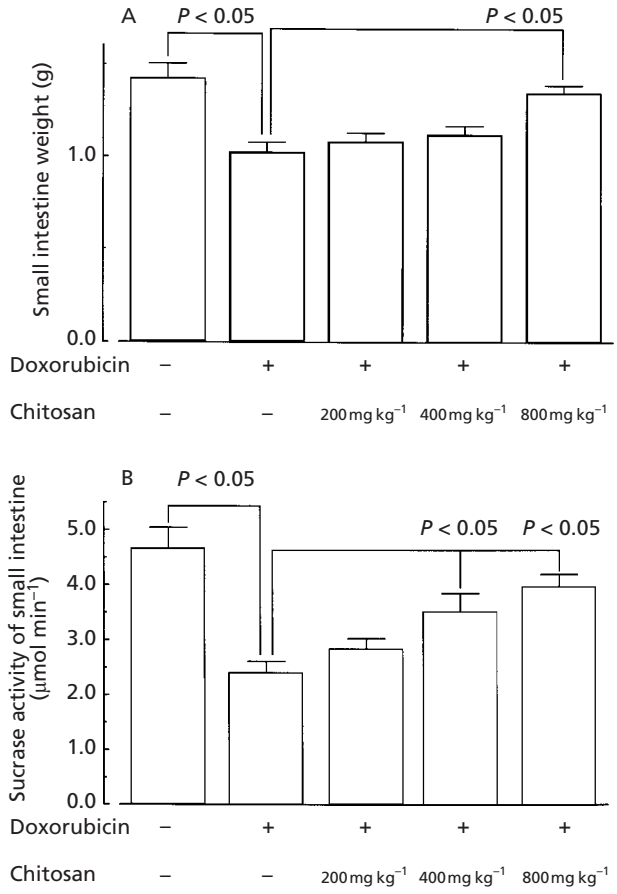

Figure 3 The preventive effects of chitosan on doxorubicin-induced gastrointestinal toxicity in sarcoma 180-bearing mice. A. The preventive effects of orally administered chitosan (200, 400 or 800 mg kg^{-1}, twice daily for 14 days) on the reduction in small-intestine weight induced by intraperitoneally administered doxorubicin (5 mg kg^{-1} on days 1 and 8). B. The preventive effects of orally administered chitosan on the reduction of sucrase activity in small-intestinal mucosa induced by intraperitoneally administered doxorubicin. Results are expressed as mean ± s.e.m. of 10–20 mice.

antitumour activity, while it caused adverse reactions such as myelotoxicity (reduction of leucocyte number), gastrointestinal toxicity (reduction of the weights of small intestine and sucrase activity in the small-intestinal mucosa) and body-weight loss. However, liver injury, nephrotoxicity and cardiotoxicity with elevated levels of plasma GOT, GPT, BUN and CPK were not markedly caused by doxorubicin (5 mg kg^{-1}, i.p., on days 1 and 8). Orally administered chitosan (400 mg and 800 mg kg^{-1} twice daily) prevented the body-weight loss and reduction of sucrase activity in small-intestinal mucosa induced by doxorubicin (5 mg kg^{-1}, i.p.) without interfering with the antitumour activity of doxorubicin alone.

Generally, the dose-limiting toxicity of most chemotherapeutic drugs are myelotoxicity and gastrointestinal toxicity. The discovery and development of colony-stimulating factors have reduced the severity and duration of haematopoietic toxicity (Bronchud et al 1989; Gianni et al 1990). Mucositis still represents an obstacle of paramount importance. In fact, severe mucositis (grade 3 and 4) that has not fully resolved at the time of retreatment usually suggests that not only should treatment be withheld until the mucosa has healed but also that the drug dose must be decreased, with consequently reduced therapeutic results. Approaches to prevent and treat gastrointestinal toxicity, which is induced by a wide range of therapeutic regimes, are under study (Blijham 1993). In this study, the oral administration of chitosan prevented the body-weight loss and damage to the mucosal membrane of the small intestine induced by the intraperitoneal administration of doxorubicin. It has been reported that doxorubicin induces cytotoxic effects on small-intestinal mucosal cells characterized by nuclear and cytoplasmic condensation and preservation of the organelles in the early stages (Anilkumar et al 1992; Thakkar & Potten 1992). Orally administered chitosan was retained for a long period in the small intestine, suggestive of possible diffusion of chitosan into the intracellular space of villi in the small intestine. Therefore, it seems likely that the protective mechanism for the doxorubicin-treated gastrointestinal toxicity by the orally administered chitosan may be due to the formation of doxorubicin–chitosan complex in the small intestinal mucosa through the diffusion of chitosan into the small intestinal villi without interfering with the antitumour activity of doxorubicin. Further work is needed to clarify the doxorubicin content in the small intestine following combined treatment with doxorubicin and chitosan at lower or higher doses. We found that oral administration of chitosan prevented the reduction in leucocyte number induced by intraperitoneally administered doxorubicin. Moreover, the orally administered chitosan plus intraperitoneally administered doxorubicin increased the spleen weight, as compared with the control and doxorubicin-treated mice. Further studies are needed to clarify these indications. We conclude that the combination of chitosan with doxorubicin might be useful for the prevention of gastrointestinal toxicity caused by doxorubicin. Experiments are now in progress to examine the clinical usefulness of the administration of chitosan together with doxorubicin in cancer chemotherapy.

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