Table 7 Correlation of the expression of A antigen in the tumor, dysplasia and normal urothelium specimens with the genetic and epigenetic changes in the primary tumor

Case	*Genotype*	*Tumor*			*Dysplasia expression*	*Urothelium expression*
		Expression	*LOH*	*Methylation status*		
A-22	A/O1	Positive	O1	M	Positive	Positive
A-1	A/O2	Positive	O2	UM	Positive	Positive
A-10	A/O2	Positive	O2	UM	Positive	Positive
A-3	A/O2	Positive	O2	UM	Positive	Positive
A-46	A/O2	Positive	O2	UM	Positive	Positive
A-5	A/O1	Positive	Ret	UM	Positive	Positive
A-43	A/O1	Positive	Ret	UM	Positive	Positive
AB-5	A/B	Positive	Ret	UM	Positive	Positive
A-5	A/O1	Positive	Ret	UM	Positive	Positive
A-18	A/O1	Positive	Ret	NA	Positive	Positive
A-29	A/A	Positive	NI	UM	Positive	Positive
A-47	A/A	Positive	NI	UM	Positive	Positive
A-14	A/A	Positive	NI	NA	Positive	Positive
A-15	A/O2	Negative	A	M	Positive	Positive
A-16	A/O2	Negative	A	M	Positive	Positive
A-48	A/O2	Negative	A	M	Positive	Positive
A-2	A/O2	Negative	A	UM	Positive	Positive
A-31	A/O1	Negative	A	UM	Positive	Positive
A-6	A/O1	Negative	A	NA	Positive	Positive
A-13	A/O1	Negative	O1	M	Positive	Positive
A-9	A/O1	Negative	O1	UM	Hetero	Positive
A-33	A/O2	Negative	O2	NA	Positive	Positive
AB-2	A/B	Negative	B	M	Positive	Positive

UM and M indicate whether the *RE 7* sequences were unmethylated and methylated, respectively;
Hetero: heterogenous expression; NA: not applicable.

tion, suggesting potential difficulty in assessing the allelic status of small lesions from archival materials. The aim of the present study was to elucidate the underlying mechanisms of reduced expression of the histo-blood group A antigen in bladder cancer, and to determine if IHC of the A antigen expression could be available as a hallmark to determine the allelic loss and/or epigenetic alterations of the *ABO* gene on a cell-to-cell basis.

In cases with radical cystectomy, allelic status was examined using DNAs extracted from histological slides and directly comparable with the A antigen expression in the same specimen and expression of the A antigen was negative in all cases showing A allelic loss. In cases that underwent TUR, three cases showed heterogenous expression of the A antigen, regardless of A allelic loss in the sample. In TUR cases, DNAs were extracted from fresh frozen samples obtained by cold-cup biopsies, while the expression of the A antigen was examined in formalin-fixed paraffin-embedded sections of the resected tumors. Discrepancies between A allelic loss and A antigen expression in TUR cases may be explained by the difference of materials subjected to analysis. As we indicated in Figure 4, some tumors show polyclonal development as to the allelic loss of chromosome 9 and direct comparison between biopsies and resected specimen may be difficult in such cases. In BiPS analysis, full methylation was observed in four cases and they all showed negative expression of the A antigen (Table 5). CpG islands were densely methylated in full methylation and they were closely correlated with the transcriptional silencing of the *ABO* gene. In cases with partial methylation, A antigen expression was also negative in 66.7% (6/9) of cases. Although partial methylation may play some role in transcriptional silencing, we used full methylation as an indicator of methylation in this study. As methylation extended to the most downstream of the *ABO* gene promoter region (*re 6*) in full methylation, we designed a primer set for MSP spanning region 7, which overlapped the downstream of region 6. The size of the amplified DNA fragment in MSP was as short as 96 bp and we used it as an indicator of full methylation in analysis of DNAs extracted from formalin-fixed paraffin-embedded sections. As MSP amplifies methylated DNA sequences selectively, its sensitivity is much higher than that of BiPS analysis and may have a risk of overestimation. In fact, MSP showed methylation in three cases that showed no methylation in BiPS analysis and the expression of the A antigen in these three cases were heterogenous. This may indicate the heterogeneity of the methylation status, suggesting only small number of cells harbored methylation (Table 3). In cases that underwent TUR, negative A antigen expression was signifi-

cantly correlated with full methylation ($P=0.035$), but not with A allelic loss ($P=0.26$) (Table 5). In cases that underwent radical cystectomy, both methylation and A allelic loss were significantly correlated with the expression of the A antigen ($P=0.003$ for A allelic loss, $P=0.03$ for MSP, respectively). Using these two indices in combination, 29.5% (13/44) of the cases that underwent TUR and 29.7% (11/37) of cases that underwent radical cystectomy showed loss of the A allele and/or hypermethylation of the *ABO* gene. They were significantly correlated with the expression of the A antigen ($P=0.02$ for TUR cases, $P=0.0005$ for radical cystectomy cases) (Tables 5 and 6). Negative A antigen expression was observed in 50.0% (22/44) in TUR cases and 48.6% (18/37) in cases that underwent radical cystectomy, which was attributable to genomic deletion and/or hypermethylation of the *ABO* gene in at least 45% (10/22) of cases that underwent TUR and 66.7% (10/15) of cases that underwent radical cystectomy. It is apparent that A allelic loss and/or hypermethylation of the *ABO* gene could not be the sole cause for negative A antigen expression. As the antigenic determinant of the A antigen is the terminal structure of the carbohydrate chains, incomplete synthesis of carbohydrate chains associated with oncogenesis may also be concerned with the reduced expression of the A antigen. Methylation seems to be more predominant than loss of the A allele in cases that underwent TUR. This might be explained by the observation that superficial papillary tumors such as pTa or pT1 stages comprised most of the TUR cases, while more than 70% of them were invasive cancers above Stage pT2 in cases that underwent radical cystectomy. In our previous study, frequencies of LOH on chromosome 9 were 67% in pTa, 71% in pT1 and 80% in tumors ≧pT2 stages.[4] As for the putative tumor suppressors found on chromosome 9, p16 and $p14^{ARF}$ are located on 9p21.[32,33] And an area on 9q31–34 is most prone to be deleted in TCC of the bladder,[34,35] which is also a candidate locus for a putative tumor suppressor gene. Reportedly, deletion of chromosome 9 is an early genetic event in the development of bladder cancers.[1] However, there is not enough evidence to support this hypothesis regarding the occurrence of chromosome 9 deletion in preneoplastic lesions. In a few studies using microsatellite markers from microdissected specimens, allelic loss on chromosome 9 was observed in bladder dysplasia.[3,7] We studied the expression of the A antigen on the dysplasia specimens by IHC, aiming at screening genetic alterations in precancerous lesions of the bladder. Expression of the A antigen was examined in 23 cases of bladder cancer comprising dysplasia, among which the numbers of tumors showing positive or negative expression were 13 and 10, respectively. All of the cases showing positive expression retained the A allele in the tumor and only one case showed hypermethylation, while the expression of the A antigen was preserved in dysplasia and normal urothelium in all cases. In the analysis of 10 cases showing negative A antigen expression in the tumor, loss of the A allele and/or the hypermethylation was observed in eight cases. Expression of the A antigen was preserved in normal urothelium and dysplasia in all but one case showing heterogenous expression in the dysplasia. This case did not exhibit LOH or hypermethylation in the tumor. These results suggested that LOH and/or hypermethylation of the *ABO* gene were infrequent genetic and epigenetic alterations in dysplasia and normal urothelium of the bladder bearing TCC. Furthermore, one case showed chimeric expression of the A antigen in the tumor, among which the expression of the A antigen coincided with loss or retention of the A allele (Figure 4). Analysis of two polymorphic markers in the vicinity of *ABO* gene locus also showed LOHs and the parental origin of the lost allele in these two loci was opposite as was shown in analysis of the *ABO* gene locus. Previously, we reported loss of chromosome 9 was observed in 71% of TCCs of the bladder and nearly 50% of them involved both 9p and 9q, suggesting monosomy or uniparental aneusomy of chromosome 9.[4] Thus, the deletion was considered to involve large chromosomal regions at least between 9q21.3 and 9q34.1 and possibly on the same allele. This finding may suggest the idea that the tumor showed polyclonal development as to the deletion of the 9q allele and that the loss of chromosome 9 might not be an early genetic event associated with tumorigenesis.

In conclusion, reduced expression of the A antigen in bladder cancer reflects allelic loss of the *ABO* gene assigned to 9q34.1 and/or hypermethylation of its promoter region, which is a specific marker for genetic and epigenetic alterations in bladder cancer but not in dysplasia.

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Randomized trial of dietary fiber and *Lactobacillus casei* administration for prevention of colorectal tumors

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The epidemiologic evidence that dietary fiber protects against colorectal cancer is equivocal. No large-scale clinical study of the administration of *Lactobacillus casei* has been reported. We examined whether dietary fiber and *L. casei* prevented the occurrence of colorectal tumors. Subjects were 398 men and women presently free from tumor who had had at least 2 colorectal tumors removed. Subjects were randomly assigned to 4 groups administered wheat bran, *L. casei,* both or neither. The primary end point was the presence or absence of new colorectal tumor(s) diagnosed by colonoscopy after 2 and 4 years. Among 380 subjects who completed the study, 95, 96, 96 and 93 were assigned to the wheat bran, *L. casei,* both and no treatment groups, respectively. Multivariate adjusted ORs for occurrence of tumors were 1.31 (95% CI 0.87–1.98) in the wheat bran group and 0.76 (0.50–1.15) in the *L. casei* group compared to the control group. There was a significantly higher number of large tumors after 4 years in the wheat bran group. The occurrence rate of tumors with a grade of moderate atypia or higher was significantly lower in the group administered *L. casei.* No significant difference in the development of new colorectal tumors was observed with administration of either wheat bran or *L. casei.* However, our results suggest that *L. casei* prevented atypia of colorectal tumors.
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Key words: colorectal cancer; *Lactobacillus casei*; dietary fiber; probiotic

The incidence of colon cancer is rapidly increasing in Japan.[1] It has been suggested that this trend is caused by the high-fat, low-dietary fiber diet resulting from Westernization of the lifestyle among Japanese. Indeed, intake of dietary fiber by the Japanese has decreased significantly over the past 10 years.[2]

Since Burkitt[3] proposed that a diet high in dietary fiber prevented colorectal cancer, basic studies have suggested the possibility of prevention of colorectal cancer by dietary fiber, through actions including absorption of carcinogens by insoluble dietary fiber[4] and dilution of bile acids and decrease of mutagenicity due to the increase in stool volume.[5,6]

A large number of case-control studies have suggested that dietary fiber may prevent the development of colorectal cancers.[7] However, reports of large-scale cohort studies have failed to show a preventive effect of dietary fiber against colorectal cancer, causing controversy.[8–11]

Randomized clinical trials have been conducted in Western countries[12–16] to evaluate the effectiveness of dietary fiber, using the development of colorectal adenoma as an end point. Many of these studies failed to prove that dietary fiber prevented the development of colorectal adenoma. No intervention study on dietary fiber has been reported in Asians.

It has been shown that *Lactobacillus casei* strain Shirota reduces DNA damage induced by chemical carcinogens in laboratory studies[17] and prevents carcinogenesis in animal experiments.[18,19] In addition, it has been reported, in humans, that lactobacilli reduce the level of mutagens in stool.[20] Furthermore, oral administration of *L. casei* strain Shirota preparation decreased the recurrence of superficial bladder cancer after transurethral resection,[21,22] and habitual intake of a fermented product with *L. casei* strain Shirota reduced the risk of bladder cancer in an epidemiologic study.[23] Thus, we decided to use a *L. casei* strain Shirota preparation in the present study. It has been suggested that high intake of yogurt and fermented milk is responsible for the low incidence of colon cancer in Finland, where consumption of fat is higher than in other countries.[24] Two case-control studies have shown that yogurt[25] and fermented milk[26] prevent colon cancer. In the Netherlands Cohort Study, it was reported that fermented milk intake showed an inverse relationship with the development of colon cancer, although there was no statistical significance.[27]

In 1993, we initiated a randomized clinical trial to determine whether dietary fiber from wheat bran and *L. casei* prevented the occurrence of colorectal tumors.

Material and methods

Study design and subjects

Part of the study design and methods have been previously described in detail.[28] Subjects were recruited at the Osaka Medical Center for Cancer and Cardiovascular Diseases between June 1993 and September 1997. The study protocol was approved by the Ethics Committee of the Osaka Medical Center for Cancer and Cardiovascular Diseases. Written informed consent was obtained from all subjects.

Inclusion criteria were men and women aged 40–65 years who had had at least 2 colorectal tumors (adenomas and/or early cancers) removed endoscopically within 3 months before recruitment. Endoscopic examination had been conducted twice, to detect and resect polyps, respectively. It must have been performed on the entire large intestine, and the subjects must have had an adequate nutritional status. Excluded were subjects with other malignant tumors, a history of intestinal or gastric resection (except appendectomy), familial adenomatous polyposis and severe illness.

Four regimens were incorporated for prevention of colorectal cancer: A, dietary instruction and regular intake of wheat bran biscuits; B, dietary instruction and regular intake of *L. casei* preparation; C, dietary instruction and regular intake of wheat bran biscuits and *L. casei* preparation; and D, dietary instruction alone.

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One of the 4 regimens was assigned randomly in advance each week. Physicians recruited subjects according to the regimen decided at the beginning of the week. Patients received consultation, including histologic diagnosis of the resected polyp, from group physicians as soon as possible from 1 week following endoscopic treatment. Since the regimen assignment could not be changed by the physicians or participants or arbitrarily manipulated by the authors, it was regarded as random. Trial physicians recruited all outpatients who met the inclusion criteria.

The amount of wheat bran biscuits and *L. casei* preparation to be consumed in 1 month was given to the participant at the start of the trial, and thereafter the amount for 3 months was given. The trial was started after confirming that the subject understood the procedure of the regimen. During the trial, consultation was performed every 3 months to check the participant's physical condition and to confirm the intake of wheat bran biscuits and the *L. casei* preparation. The regimen was continued for 4 years. Participants' compliance with taking wheat bran biscuits and/or the *L. casei* preparation was assessed at the consultations performed every 3 months. At each consultation, the numbers of unconsumed biscuit packages and silver packages of *L. casei* preparation were estimated by verbal inquiry of the patients, and the amounts of wheat bran biscuits and *L. casei* preparation taken in the past 3 months were calculated as the percentage of the target intake. Consultations were performed 16 times, every 3 months for 4 years, and the average at those 16 consultations was taken as the compliance over 4 years.

The target number of subjects was 100 in each group, for a total of 400 subjects. As the incidence of tumors in the control group can be estimated to be about 60%, a significant difference would be obtained if the occurrence rate of tumors could be reduced to 50% (suppression rate 17%) by administration of wheat bran or *L. casei* preparation. No midpoint analysis was performed.

Tumors discovered by colonoscopy performed at the end of the 2nd and 4th years were included in the analysis. The analysis included subjects with poor compliance with the regimen for intake of wheat bran biscuits and *L. casei* preparation on an intention-to-treat basis. For patients with early colorectal cancers resected during colonoscopy before entry in the trial, colonoscopy was performed 6 months after entry (35, 29, 25 and 24 subjects of groups A, B, C and D, respectively). Tumors discovered by colonoscopy performed at 6 months were excluded from analysis. Subjects who refused participation and dropouts were excluded from analysis.

Dietary instruction

The core purpose of the dietary instruction was to restrict fat intake so that the energy from fat constituted 18–22% of total energy intake. Subjects were asked to record, on a diet record form, the contents of their meals for the 3 days before consultation; and nutritionists calculated, from these records, the total energy intake and intake of fat and oil. Compliance with the dietary instructions on the restriction of fat intake was evaluated at dietary checkups 3 months and 4 years after beginning the regimen, and, when necessary, instruction was given again.

Wheat bran biscuits

Biscuits containing wheat bran at 30% of dry weight were prepared.[29] Patients were instructed to eat 25 g/day wheat bran biscuits (7.5 g as wheat bran) before each meal. Biscuits were developed by Ezaki Glico (Osaka, Japan) and Horii Pharmaceutical Industry (Osaka, Japan). The components and contents of the wheat bran biscuits were as follows: energy, 454 kcal/day; protein, 2.9 g/day; lipid, 3.3 g/day; and nonfibrous carbohydrate, 17.5 g/day.

L. casei *preparation*

The *L. casei* strain Shirota preparation was a powder containing approximately 10^{10} viable cells/g. It was stored in a refrigerator, and 1 g was taken after every meal. The *L. casei* preparation was provided by Yakult Honsha (Tokyo, Japan). The viable cell count of *L. casei* and absence of bacterial contamination were confirmed for all lots every 6 months during the 2-year storage period. To confirm the viable cell count of *L. casei*, MRS agar medium for detection of *L. casei* was used. It has been confirmed in previous studies that the number of bacteria per 1 g of *L. casei* preparation remained in the range of 1.5×10^9 to 2.1×10^{10} during 24 months when stored in a cool place (15°C). In addition, the average number of bacteria is 8.0×10^9 after 24 months.

Colonoscopy

The main end point of the trial was the presence or absence of new colorectal tumor(s). Colonoscopy was performed 2 and 4 years after the start of the regimen. The entire large intestine, from the anus to the cecum, was examined. Examinations for detection of new lesions were performed by 2 physicians. All lesions, except hyperplastic polyps clearly evaluated by colonoscopy, were examined histologically on the basis of the guidelines of the Japanese Society for Cancer of the Colon and Rectum.[30] All histologic diagnoses (inflammatory polyp; hyperplastic polyp; adenoma with mild atypia, with moderate atypia, with severe atypia; early cancer) were performed blindly without identification of the participant's dietary regimen.

In patients with early colorectal cancer, which was diagnosed from tumor tissue resected by colonoscopy before entry in the trial, colonoscopy was performed to detect local recurrence after 6 months of participation. All colorectal tumors discovered with this procedure were resected.

Statistical analysis

All colorectal tumors discovered at the end of the 2nd and 4th years were defined as "new". Analyses at years 2 and 4 were performed separately, and 2×2 contingency table analysis was performed. Comparison of baseline characteristics of subjects with or without wheat bran biscuits or *L. casei* intake was performed by appropriate tests such as *t*-test and the χ^2 test. Logistic regression models were used to estimate the odds ratio (OR) adjusted for covariates such as age and sex. Confidence intervals (CIs) based on Wald statistics were used to assess significance.

Results

Enrollment and randomization

The number of patients who met the inclusion criteria during the screening period was 470 (Fig. 1). All were invited to participate in the trial, but 60 patients (13%) declined. Of 410 patients who agreed to participate, 12 were excluded because of incompatibility with the protocol, including detection of cholangiocarcinoma and gastric cancer in 4, history of gastrectomy in 3, colectomy in one, familial adenomatous polyposis in one, advanced age in one, young age in one and more than 3 months after endoscopic treatment in one. Thus, 398 patients were assigned to the 4 groups.

Baseline characteristics of subjects

Table I shows the baseline characteristics of the 398 patients randomly assigned and the number of dropouts. There was no difference in baseline characteristics of subjects such as dietary content among the 4 groups. A total of 18 patients (4.5%) did not complete endoscopic examinations. The reasons for not receiving endoscopic examinations were death in 2 patients (from lung cancer and cerebral hemorrhage), serious illness in 5 patients and trial discontinuation in 11 patients. There was no difference in the rate of dropouts among all groups. Excluding 18 dropouts, 380 patients were included in the analysis.

Colonoscopy

Colonoscopic examination was possible throughout the length of the large intestine, up to the cecum, in all cases. There was no difference in the intervention period among groups (Table II).

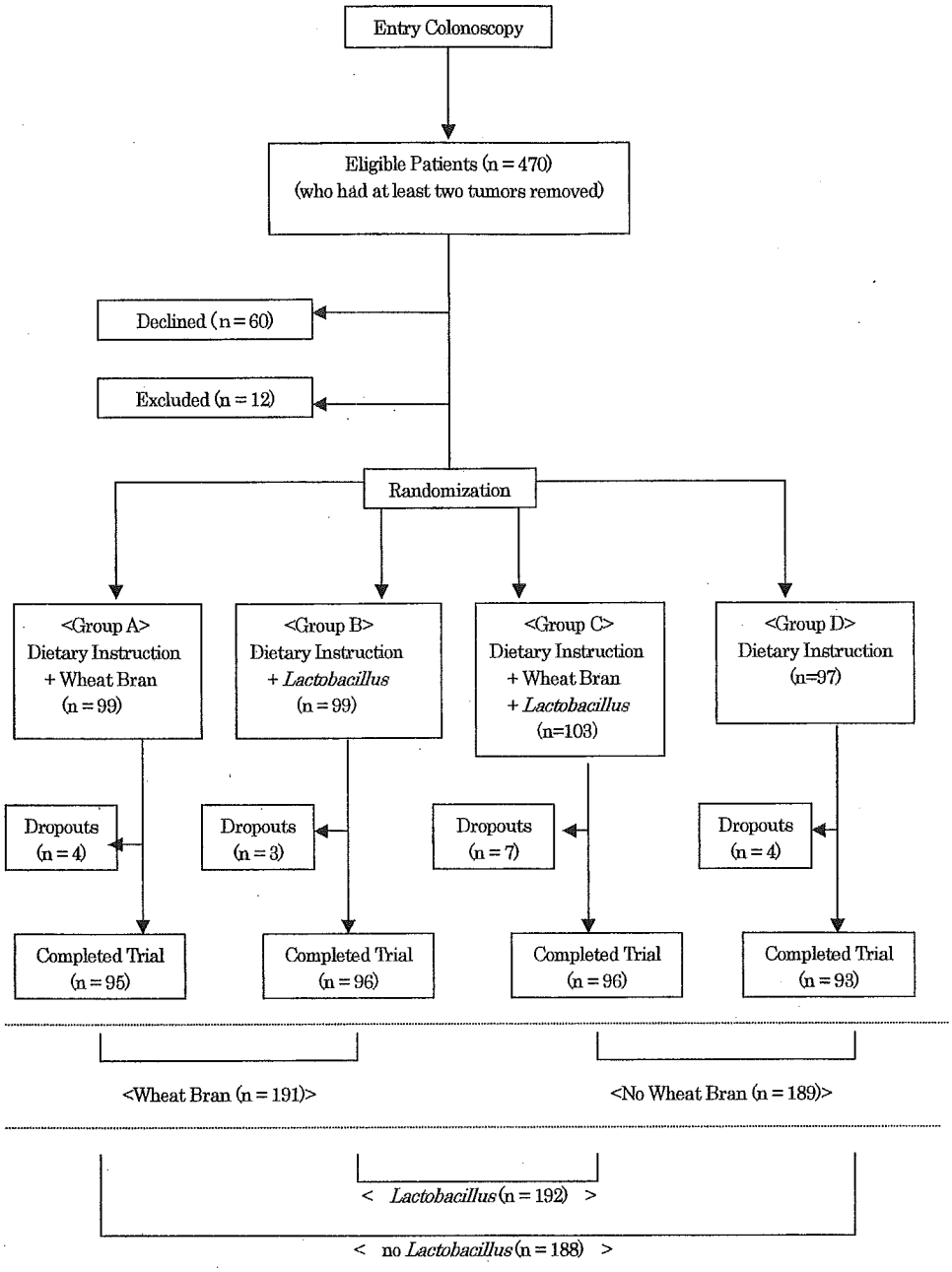

FIGURE 1 – Study participant data.

There was no difference in the time required for insertion into and removal from the cecum in all groups. No difference was found in the proportion of nonneoplastic lesions (inflammatory polyps and hyperplastic polyps).

Compliance

Compliance with intake of wheat bran biscuits was over 90% in 77 persons (40%) and over 70% in 135 persons (71%) for the entire 4 years. Compliance with intake of the *L. casei* preparation was over 90% in 130 persons (68%) and over 70% in 168 persons (88%).

Occurrence of tumors

The results after intake of wheat bran biscuits are shown in Table III. The wheat bran biscuit administration group included 191 persons, while the nonadministration group included 189 persons. The adjusted OR of developing at least one tumor was 1.31 (95% CI 0.87–1.98) after 2 years in the administration group compared to the nonadministration group. It was 1.31 (95% CI 0.87–1.97) after 4 years, showing some increase in adjusted OR, although not statistically significant. The adjusted OR for developing tumors larger than 3 mm was 1.14 (95% CI 0.76–1.72) in the administration group compared to the nonadministration group after 2 years and 1.57 (95% CI 1.04–2.37) after 4 years, showing a significant increase. In particular, the occurrence of tumors larger than 10 mm after 2 years showed no difference between the wheat bran administration and nonadministration groups. However, after 4 years, these tumors did not occur in the nonadministration group while they occurred in 7 patients (3.7%) in the administration group, showing a significant increase. There was no difference in the occurrence of more than one or more than 3 tumors with moderate or severe atypia.

Table IV shows the results after *L. casei* administration. The *L. casei* administration group included 192 persons and the nonad-

TABLE I – BASELINE CHARACTERISTICS OF SUBJECTS[1]

	Group A: wheat bran (n = 99)	Group B: *Lactobacillus* (n = 99)	Group C: wheat bran + *Lactobacillus* (n = 103)	Group D: no treatment (n = 97)
Age (years)	54.7±6.1	54.8±6.5	54.9±6.2	55.5±6.2
Male sex, number (%)	83 (83.8)	79 (79.8)	80 (77.7)	83 (85.6)
Height (cm)	164.6±8.4	164.6±7.3	163.0±7.1	164.1±7.6
Weight (kg)	66.1±10.5	64.6±10.5	62.7±8.7	63.2±9.4
Dietary intake				
Energy (kcal/day)	2,075±368	2,066±411	2,058±402	2,163±404
Total fat (g/day)	54.6±15.2	53.0±13.5	52.8±16.7	56.6±14.9
Dietary fiber (g/day)	15.1±3.9	14.5±3.9	15.4±4.7	15.5±4.0
Calcium (mg/day)	635.4±237.1	638.7±218.4	636.6±246.7	661.4±247.7
Alcohol drinking every day, number (%)	50 (50.5)	37 (37.4)	49 (47.6)	48 (49.5)
Current smoker, number (%)	47 (47.5)	41 (41.4)	43 (41.7)	44 (45.4)
Tumors before recruitment				
Total tumors	5.9±4.3	5.8±5.6	5.2±3.6	5.0±3.4
Adenomas with mild atypia	2.8±2.9	2.7±3.9	2.5±2.6	2.0±2.7
Adenomas with moderate atypia	2.1±2.2	2.4±2.1	1.9±1.8	2.0±1.9
Adenomas with severe atypia	0.6±0.8	0.4±0.7	0.5±1.1	2.0±1.9
Early cancers, number (%)	37 (37.4)	31 (31.3)	29 (28.2)	28 (28.9)
History of colorectal cancer in one parent or sibling, number (%)	15 (15.2)	8 (8.1)	15 (14.6)	11 (11.3)
Dropped out, number (%)	4 (4.0)	3 (3.0)	7 (6.8)	4 (4.1)

[1]Values are means ± SD.

TABLE II – INTERVENTION PERIOD OF COLONOSCOPY

Intervention period	Group A: wheat bran (n = 95)	Group B: *Lactobacillus* (n = 96)	Group C: wheat bran + *Lactobacillus* (n = 96)	Group D: no treatment (n = 93)
Period of 2nd year from entry (days)				
Mean ± SD	679.4±60.8	674.2±31.0	672.1±27.6	680.3±56.9
Maximum	1,009	827	778	925
Minimum	568	617	600	617
Period of 4th year from entry (days)				
Mean ± SD	1,339.6±46.9	1,339.7±51.1	1,338.1±40.5	1,367.4±120.4
Maximum	1,611	1,660	1,617	2,129
Minimum	1,275	1,275	1,233	1,201

ministration group, 188 persons. The adjusted OR of developing at least one tumor was 0.76 (95% CI 0.50–1.15) in the administration group compared to the nonadministration group after 2 years. After 4 years, it was 0.85 (95% CI 0.56–1.27), showing a decrease after both 2 and 4 years, although not statistically significant. For the occurrence of tumors with moderate or severe atypia, the adjusted OR was 0.80 (95% CI 0.52–1.22) in the administration group compared to the nonadministration group after 2 years and 0.65 (95% CI 0.43–0.98) after 4 years, showing a significant decrease after 4 years. There was no difference in the size and number of new tumors that developed.

When the results were examined separately for the different levels of compliance, they were similar to those described above.

Synergistic effects

Tumor occurrence in the group administered both wheat bran and *L. casei* was higher than that in the groups administered wheat bran or *L. casei* and lower than that in the nonadministered group (data not shown). No notable synergistic effects between the treatments were observed.

Adverse events

During the study period, colorectal cancers were discovered in 4 persons by endoscopy, including one person each in groups B, C and D after 2 years and one person in group B after 4 years. There was no bias in their occurrence among the groups. All were cancer invasion of mucosa and were completely resected endoscopically, not requiring colectomy. During the study period, 2 patients died, one of lung cancer in group A and one of cerebral hemorrhage in group C. One person each in groups A and C underwent surgery for peritonitis resulting from acute appendicitis. There was no other serious adverse event.

Discussion

It was found that *L. casei* intake appeared to suppress the development of colorectal tumors; in particular, it prevented, with statistical significance, the development of tumors with moderate and severe atypia. This large-scale randomized clinical study shows that an *L. casei* preparation prevented the development of colorectal tumors.

Since our study was performed at one hospital, the evaluations of endoscopic findings were thought to be consistent. All patients who satisfied the conditions for participation were asked to participate, and the rate of consent to participation was extremely high at 88%, supporting the high validity of the results. The reasons for the high rate of consent could be that a special organization was instituted in this hospital for this trial and that all participants were offered dietary instruction. In addition, the dropout rate was low at 4.5% and compliance was high, indicating that the results were highly reliable. Endoscopic examination was conducted twice before entry so that we could minimize oversights.

In our previous prevention studies, subjects were patients with at least one tumor, whereas the present study included patients with at least 2 tumors. It is known that patients with at least 2 tumors in the large intestine have a higher risk of colon cancer than those with only one tumor. It is difficult to apply the results of our clinical study to the general population. Many of the patients participating in this trial, different from other reports in the past, had a larger number of colorectal tumors together with a history of cancer. This difference appears to have resulted from the background of the population, who had a higher risk of colorectal cancer than those participating in previous clinical trials. Accordingly, our results should be discussed not on the basis of the general population but on the basis of a population with a high risk of colorectal cancer. Nonetheless, our study included patients

TABLE III – RISK OF TUMOR OCCURRENCE WITH WHEAT BRAN BISCUITS

	Year	Wheat bran (groups A + C) (n = 191)	No treatment (groups B + D) (n = 189)	Crude		Adjusted	
				relative risk	(95% CI)	OR	(95% CI)[1]
Number of tumors							
At least one	2	119 (62.3%)	106 (56.1%)	1.11	(0.94–1.31)	1.31	(0.87–1.98)
	4	106 (55.5%)	93 (49.2%)	1.13	(0.93–1.37)	1.31	(0.87–1.97)
≥ 2	2	57 (29.8%)	60 (31.7%)	0.94	(0.70–1.27)	0.92	(0.60–1.43)
	4	51 (26.7%)	53 (28.0%)	0.95	(0.69–1.32)	0.95	(0.60–1.50)
≥ 4	2	11 (5.8%)	14 (7.4%)	0.78	(0.36–1.67)	0.78	(0.34–1.76)
	4	11 (5.8%)	12 (6.3%)	0.91	(0.41–2.00)	0.91	(0.39–2.13)
Size of largest tumor (mm)							
≥ 3	2	95 (49.7%)	88 (46.6%)	1.07	(0.87–1.32)	1.14	(0.76–1.72)
	4	97 (50.8%)	76 (40.2%)	1.26	(1.01–1.58)	1.57	(1.04–2.37)
≥ 4	2	51 (26.7%)	52 (27.5%)	0.97	(0.70–1.35)	0.97	(0.61–1.54)
	4	52 (27.2%)	51 (27.0%)	1.01	(0.73–1.40)	1.02	(0.65–1.60)
≥ 10	2	4 (2.1%)	4 (2.1%)	0.99	(0.25–3.90)	1.00	(0.25–4.06)
	4	7 (3.7%)	0 (0.0%)	—	$p < 0.01$[2]		
Atypia of tumors							
≥ With moderate	2	64 (33.5%)	66 (34.9%)	0.96	(0.73–1.27)	0.94	(0.61–1.44)
	4	77 (40.3%)	74 (39.2%)	1.03	(0.80–1.32)	1.06	(0.70–1.60)

[1]OR of recurrent tumors in the wheat bran biscuits group compared to the no treatment group, adjusted for age, sex and *Lactobacillus* group.–[2]χ^2 test.

TABLE IV – RISK OF TUMOR OCCURRENCE WITH *LACTOBACILLUS* PREPARATION

	Year	*Lactobacillus* (groups B + C) (n = 192)	No treatment (groups A + D) (n = 188)	Crude		Adjusted[1]	
				relative risk	(95% CI)	OR	(95% CI)
Number of tumors							
At least one	2	107 (55.7%)	118 (62.8%)	0.89	(0.75–1.05)	0.76	(0.50–1.15)
	4	96 (50.0%)	103 (54.8%)	0.91	(0.75–1.11)	0.85	(0.56–1.27)
≥ 2	2	56 (29.2%)	61 (32.4%)	0.90	(0.66–1.22)	0.88	(0.57–1.36)
	4	53 (27.6%)	51 (27.1%)	1.02	(0.73–1.41)	1.08	(0.68–1.71)
≥ 4	2	10 (5.2%)	15 (8.0%)	0.65	(0.30–1.42)	0.67	(0.29–1.53)
	4	15 (7.8%)	8 (4.3%)	1.84	(0.79–4.23)	1.98	(0.81–4.83)
Size of largest tumor (mm)							
≥ 3	2	86 (44.8%)	97 (51.6%)	0.87	(0.70–1.07)	0.77	(0.51–1.15)
	4	83 (43.2%)	90 (47.9%)	0.90	(0.72–1.13)	0.85	(0.56–1.28)
≥ 4	2	41 (21.4%)	62 (33.0%)	0.65	(0.46–0.91)	0.56	(0.35–0.89)
	4	58 (30.2%)	45 (23.9%)	1.26	(0.90–1.76)	1.38	(0.87–2.19)
≥ 10	2	4 (2.1%)	4 (2.1%)	0.98	(0.45–3.86)	1.01	(0.25–4.12)
	4	4 (2.1%)	3 (1.6%)	1.31	(0.30–5.75)	1.29	(0.28–6.00)
Atypia of tumors							
≥ With moderate	2	61 (31.8%)	69 (36.7%)	0.87	(0.65–1.14)	0.80	(0.52–1.22)
	4	66 (34.4%)	85 (45.2%)	0.76	(0.59–0.98)	0.65	(0.43–0.98)

[1]OR of recurrent tumors in the *Lactobacillus* group compared to the no treatment group, adjusted for age, sex and wheat bran biscuit group.

with at least 2 tumors for the following reasons: *(i)* it is more efficient for the analysis of preventive methods against colon cancer to use subjects in higher-risk groups and *(ii)* since the occurrence rates of colon tumors after 2 and 4 years were higher in patients in the high-risk group, a preventive effect would be more prominent in this group.

The weak point of this trial is that it was not a double-blind study. Therefore, there could be bias from the fact that the participants and medical professionals did know the group to which each participant belonged. However, since it is widely believed in Japan that dietary fiber prevents colorectal cancer and nobody would think that dietary fiber would cause tumors to enlarge, it is highly unlikely that the unexpected results obtained in this study were biased. Histologic evaluations were performed blindly, without group identification, by pathologists. Therefore, there is unlikely to be a bias resulting from this not being a double-blind study in the result that administration of *L. casei* prevented the development of tumors with moderate or severe atypia.

The occurrence of tumors larger than 4 mm was significantly suppressed by *L. casei* administration after 2 years but not after 4 years. This might have resulted from a suppressive effect of *L. casei* administration against enlargement of colon tumors lasting for only a limited period. At the present time, it is not clear how *L. casei* influences the early stages of tumor development. We are planning to examine the effect of *L. casei* administration on cellular proliferation histopathologically, to find the best administration method that will clearly show a suppressive effect on tumor development.

Although clinical studies on the administration of *L. casei* for the prevention of colorectal tumors have not been reported, there are a few reports of clinical studies aimed at changing the intestinal flora. Roncucci *et al.*[31] reported that lactulose appeared to slightly suppress the development of colorectal tumors, although without statistical significance.

The *L. casei* preparation used in our study was a quality-controlled homogeneous live preparation. *L. casei* survives well in gastric acid[32] and is used as an intestinal conditioning agent in Japan. It is known to augment immunity[33] and inhibit enzyme activity involved in carcinogenesis.[17] It has been reported to suppress the development of colorectal tumors in rats.[34]

The mechanism of the suppression by *L. casei* of the development of colorectal tumors with moderate or severe atypia is not clear. Further analyses are in progress examining stools, colonic mucous membrane and serum collected from patients who participated in this study.

Several similar studies from Western countries have reported that dietary fiber supplementation did not prevent or promote the

development of colorectal tumors. In the clinical study by Bonithon-Kopp *et al.*,[16] dietary fiber-rich psyllium significantly increased the development of adenomas after 3 years as analyzed by endoscopy (OR = 1.67), consistent with our results. Alberts *et al.*[15] reported, from a clinical study with large and small quantities of wheat bran cereal, that there was no difference in the development of adenomas but that the number of patients who developed at least 3 adenomas was significantly higher in the high-dietary fiber group. Since it was found in a previous study[29] that the diets of participants were changed by administration of a large quantity of dietary fiber, the quantity of dietary fiber was lower in the present study than that used in other studies. To target the high-risk group for colorectal cancer, patients with multiple colorectal tumors were included as subjects. In spite of these differences from previous studies, the development of colorectal tumors was not prevented by dietary fiber also in this study.

Thus, there has been no consensus on the efficacy of dietary fiber against colorectal cancer. From the results of our study as well as the previous results of supplementation studies, it is not recommended to take supplements containing a high concentration of dietary fiber for the prevention of colorectal cancer.

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Cisplatin-incorporating polymeric micelles (NC-6004) can reduce nephrotoxicity and neurotoxicity of cisplatin in rats

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In spite of the clinical usefulness of cisplatin (CDDP), there are many occasions in which it is difficult to continue the administration of CDDP due to its nephrotoxicity and neurotoxicity. We examined the incorporation of CDDP into polymeric micelles to see if this allowed the resolution of these disadvantages. Cisplatin was incorporated into polymeric micelles through the polymer–metal complex formation between polyethylene glycol poly(glutamic acid) block copolymers and CDDP (NC-6004). The pharmacokinetics, pharmacodynamics, and toxicity studies of CDDP and NC-6004 were conducted in rats or mice. The particle size of NC-6004 was approximately 30 nm, with a narrow size distribution. In rats, the area under the curve and total body clearance values for NC-6004 were 65-fold and one-nineteenth the values for CDDP ($P<0.001$ and 0.01, respectively). In MKN-45-implanted mice, NC-6004 tended to show antitumour activity, which was comparable to or greater than that of CDDP. Histopathological and biochemical studies revealed that NC-6004 significantly inhibited the nephrotoxicity of CDDP. On the other hand, blood biochemistry revealed transient hepatotoxicity on day 7 after the administration of NC-6004. Furthermore, rats given CDDP showed a significant delay ($P<0.05$) in sensory nerve conduction velocity in their hind paws as compared with rats given NC-6004. Electron microscopy in rats given CDDP indicated the degeneration of the sciatic nerve, but these findings were not seen in rats given NC-6004. These results were presumably attributable to the significantly reduced accumulation of platinum in nerve tissue when NC-6004 was administered ($P<0.05$). NC-6004 preserved the antitumour activity of CDDP and reduced its nephrotoxicity and neurotoxicity, which would therefore seem to suggest that NC-6004 could allow the long-term administration of CDDP where caution against hepatic dysfunction must be exercised.

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Translational Therapeutics

Cisplatin (*cis*-dichlorodiammineplatinum (II): CDDP) is a key drug in the chemotherapy for cancers, including lung, gastrointestinal, and genitourinary cancer (Roth, 1996; Boulikas and Vougiouka, 2004). However, we often find that it is necessary to discontinue treatment with CDDP due to its adverse reactions, for example, nephrotoxicity and neurotoxicity, despite its persisting effects (Pinzani *et al*, 1994). Platinum (Pt) analogues, for example, carboplatin and oxaliplatin (Cleare *et al*, 1978), have been developed to date to overcome these CDDP-related disadvantages. Consequently, these analogues are becoming the standard drugs for ovarian cancer (du Bois *et al*, 2003) and colon cancer (Cassidy *et al*, 2004). However, those regimens including CDDP are considered to constitute the standard treatment for lung cancer, stomach cancer, testicular cancer (Horwich *et al*, 1997), and urothelial cancer (Bellmunt *et al*, 1997). Therefore, the development of a drug delivery system (DDS) technology is anticipated, which would offer the better selective accumulation of CDDP into solid tumours while lessening its distribution into normal tissue.

Drug delivery system targeting involves two concepts: active targeting and passive targeting. Active targeting aims drug targeting through antigen–antibody reactions and specific bindings between molecules, for example, receptor and ligand. On the other hand, passive targeting is an approach in which the drug accumulates in tumour tissue using the pathophysiological characteristics of solid tumours such as the hyperplasia of tumour vasculature which generally occurs in solid tumours, but which is not seen in a comparable way in lymph nodes. Marked vascular hyperpermeability is also found in the tumour vasculature, and the combination of hyperplasia and hyperpermeability facilitate the extravasation of high-molecular-weight polymers or nanoparticles, which are less prone to leak from intact vasculature, and which can be retained in solid tumour tissue for a longer time (enhanced permeability and retention effect (EPR) effect) (Matsumura and Maeda, 1986; Maeda and Matsumura, 1989; Maeda, 2000, 2001). This effect allows passive targeting of macromolecules with a high blood retention profile into the site of tumour.

Simple polymerisation only is not sufficient to bring about the EPR effect, and strategies are also required to suppress trapping by

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the reticuloendothelial system (RES) and to enhance the blood retention profile (Klibanov *et al*, 1990, 1991; Allen, 1994; Gabizon *etal*, 1996; Lasic, 1996). Polyethylene glycol-tagged liposomal adriamycin (Doxil®) has recently been reported as a clinical success (Orditura *etal*, 2004). We have recently been conducting research dedicated to the development of polymeric micelles capable of incorporating anticancer drugs (Yokoyama *et al*, 1990, 1991, 1999). The Phase I clinical trial of adriamycin-incorporating polymeric micelles has been completed (Matsumura *et al*, 2004). Furthermore, in an animal model, the plasma and tumour area under the curve (AUC) values for taxol-incorporating polymeric micelle (NK105) showed 85- and 25-fold increases, respectively, as compared with those for taxol. Therefore, NK105 showed significant enhancement ($P<0.001$) of the antitumour activity of free taxol and a significant reduction ($P<0.05$) in its neurotoxicity (Hamaguchi *et al*, 2005). Based on these results, the Phase I clinical trial of NK105 is currently being conducted at the National Cancer Center Hospital, Tokyo. We have also been conducting research dedicated to the development of CDDP-incorporating polymeric micelles and have made a number of improvements, in the *in vivo* antitumour activity, reduction of nephrotoxicity, particle size, and particle size distribution as variables (Nishiyama and Kataoka, 2001; Nishiyama *et al*, 2001). Consequently, we discovered that block copolymers, which react with CDDP, acquire a long blood retention profile with the use of polyethylene glycol poly(glutamic acid) block copolymers (PEG-P(Glu)) (Nishiyama *et al*, 2003). In the present study, we used the final development of the technology to prepare CDDP-incorporating polymeric micelles (NC-6004) in an attempt to investigate the following objectives: (1) calculation of pharmacokinetic (PK) parameters in a detailed PK study of CDDP and NC-6004 in rats; (2) a comparison between CDDP and NC-6004 with respect to their antitumour activity in a human cancer cell line; and (3) a detailed comparison between CDDP and NC-6004 with respect to nephrotoxicity and neurotoxicity, which constitute the dose-limiting factors of CDDP.

MATERIALS AND METHODS

Materials

Cisplatin was purchased from WC Heraeus GmbH & Co., KG (Hanau, Germany). γ-Benzyl-L-glutamate *N*-carboxy anhydride was purchased from a supplier. *N,N*-dimethylformamide and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were purchased from Wako Pure Chemical Co., Inc. (Osaka, Japan). α-Methoxy-ω-aminopropyl polyethylene glycol ($CH_3O-PEG-CH_2CH_2CH_2-NH_2$; MW = 12 000) was purchased from NOF Corporation (Tokyo, Japan).

Following cell lines, MKN-45, MKN-28, EJ-1, J82, MBT-2, colo201, colo320, HT-29, A549, EBC-1, PC-14, and MCF-7 cells were purchased from the American Type Culture Collection.

Female BALB/*c nu/nu* mice were purchased from SLC (Shizuoka, Japan). Female Sprague-Dawley rats were purchased from Charles River Japan (Kanagawa, Japan). All animal procedures were performed in compliance with the guidelines for the care and use of experimental animals, which had been drawn up by the Committee for Animal Experimentation at the National Cancer Center; these guidelines meet the ethical standards required by law and also comply with the guidelines for the use of experimental animals in Japan and the UKCCCR guidelines (UKCCCR, 1998).

Preparation of PEG-P(Glu) and preparation of CDDP-incorporating polymeric micelles (NC-6004)

Polyethylene glycol-P(Glu) block copolymers were synthesised according to the slightly modified procedure of the previously reported synthetic method of PEG-P(Asp) (Nishiyama and Kataoka, 2001). γ-Benzyl L-glutamate *N*-carboxy anhydride was polymerised in *N,N*-dimethylformamide, initiated with the NH_2 amino group of $CH_3O-PEG-CH_2CH_2CH_2NH_2$, to obtain PEG-poly(γ-benzyl L-glutamate) block copolymers (PEG-PBLG). The polymerisation degree of PBLG was determined to be 40 by comparing proton ratios between PEG ($-OCH_2CH_2-$: $\delta = 3.7$ p.p.m.) and phenyl groups of PBLG ($-CH_2C_6H_5$: $\delta = 7.3$ p.p.m.) in ^{1}H NMR measurement (Mercury plus 300 (Varian Technologies); solvent: DMSO-d_6; and temperature: 25°C). The benzyl group was deprotected by mixing with 0.5 N NaOH at ambient temperature to obtain PEG-P(Glu) as a sodium salt.

Cisplatin-incorporating polymeric micelles (NC-6004) were prepared according to the slightly modified procedure of the previously reported synthetic method of CDDP-incorporating polymeric micelles (Nishiyama *et al*, 2003). Briefly, the sodium salt of PEG-P(Glu) and CDDP were dissolved in distilled water ([Glu] = 4.7 mmol l^{-1}; [CDDP]/[Glu] = 1.0) and were allowed to react for 72 h. NC-6004 thus prepared was purified with ultrafiltration (molecular weight cutoff size: 100 000). The size distribution of NC-6004 was evaluated by dynamic light scattering (DLS) at 23°C using the NICOMP 380 ZLS particle sizer (Particle Sizing Systems, Santa Barbara, CA).

Release of CDDP from NC-6004 dissolved in saline

NC-6004 was dissolved in saline and was then incubated at 37°C. In all, 80 μl of the solution was then harvested at 3, 6, 24, and 96 h after the onset of incubation. The release of CDDP from NC-6004 in the solution harvested at 37°C was quantified by gel permeation chromatography (column: Waters Ultrahydrogel 500 (ϕ7.8 × 300 mm); Waters GPC system equipped with a UV detector (310 nm); and eluent: 10 mmol l^{-1} phosphate-buffered 50 mmol l^{-1} NaCl solution).

In vitro cytoxicity

Various human cancer cell lines were evaluated in the present study. The cell lines were maintained in monolayer cultures in Dulbecco's modified Eagle's medium containing 10% (v v^{-1}) focal calf serum and 600 mg l^{-1} glutamine. WST-8 Cell Counting kit-8 (Dojindo, Kumamoto, Japan) was used for cell proliferation assay. In all, 2000 cells of each cell line in 90 μl of culture medium were plated in 96-well plates and were then incubated for 24 h at 37°C. Serial dilutions of CDDP and NC-6004 in a volume of 10 μl were added, and the cells incubated for 48 or 72 h. All dates were expressed as mean ± s.e. of triplicate of the date triplicate cultures. The data were then plotted as a percentage of the data from the control cultures, which were treated identically to the experimental cultures, except that no drug was added.

Pharmacokinetics and pharmacodynamics of CDDP and NC-6004

Under isoflurane anaesthesia, a polyethylene catheter was inserted into the right internal jugular vein of female Sprague-Dawley female rats. Rats ($n = 3$) were given a single intravenous (i.v.) injection of CDDP (5 mg kg^{-1}) or NC-6004 (an equivalent dose of 5 mg kg^{-1} CDDP) via the tail vein. At 5, 15, and 30 min, as well as at 1, 4, 12, 24, and 48 h after injection of each drug, blood (0.2 ml) was collected into a heparinised microtube via the polyethylene catheter. The blood samples were centrifuged (1000 g) for 10 min at room temperature to obtain the plasma. The plasma samples were stored below −80°C until the analysis. In a tissue distribution study, rats were injected i.v. with CDDP (5 mg kg^{-1}) or NC-6004 (an equivalent dose of 5 mg kg^{-1} CDDP) via the tail vein, and were then killed in groups of three animals at 10 min, at 1, 6, 24, and 48 h, and on day 7 day after injection of each drug under intraperitoneal pentobarbital anaesthesia (50 mg kg^{-1}). Various organs (kidney, liver, spleen, heart, lung, small intestine, colon,

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and stomach) were dissected. The organ samples were stored below −80°C until the analysis. Female BALB/*c* mice were inoculated subcutaneously on the back with 10^6 MKN-45 cells (UKCCCR, 1998). After 10 days, when the tumour size had reached approximately 50 mm^2, mice were injected i.v. with CDDP (5 $mg\,kg^{-1}$) or NC-6004 (an equivalent dose of 5 $mg\,kg^{-1}$ CDDP) via the tail vein and were then killed in groups of three animals at 10 min, at 1, 6, 24, and 48 h, and on day 7 after injection of each drug. The tumours were dissected and stored below −80°C until the analysis. The plasma samples were diluted with 0.1 N HCl, vortexed, and analysed for elemental Pt by frameless atomic absorption spectrophotometry (FAAS). The tissue samples were decomposed by heating in concentrated nitric acid, evaporated to dryness, and redissolved in 0.1 N HCl. Elemental Pt was measured by FAAS.

The PK parameters were calculated using noncompartmental analysis (WinNonlin standard software, version 3.1; Pharsight Corporation, Palo Alto, CA, USA). The following PK parameters were obtained: AUC, maximum Pt concentration (C_{max}), time to obtain C_{max} (T_{max}), total body clearance (CL_{tot}), terminal half-life of Pt ($t_{1/2}z$), and steady-state volume of distribution (V_{ss}). The area under the tumour concentration–time curve (tumour AUC) was calculated based on the trapezoidal rule up to 48 h. The parameters were calculated using the following equations:

$$AUC_{0-t}$$

was calculated by the trapezoidal rule to the last measurable data point:

$$AUC_{0-inf.} = \int_0^\infty C(t)dt$$

$$t_{1/2}z(\text{terminal half-life}) = 0.693/\lambda z$$

λz: first-order rate constant associated with terminal portion of the curve)

$$CL_{tot} = \text{Dose}/AUC_{0-inf.}$$

$$V_{ss} = MRT \times CL_{tot}(MRT : \text{mean residence time})$$

In vivo antitumour activity

Antitumour activity was evaluated using nude mice implanted with a human gastric cancer cell line MKN-4. BALB/*c nu/nu* female mice (aged 6 weeks) were inoculated subcutaneously with 10^6 MKN-45 cells on the right dorsal skin. After 3 days, when tumour diameter had reached approximately 3 mm, tumour-bearing mice were allocated randomly to drug administration groups of six animals each. The drugs were administered as follows: animals in the CDDP group were given doses of 0.5, 2.5, 5 $mg\,kg^{-1}$; animals in the NC-6004 group were given doses of 0.5, 2.5, and 5 $mg\,kg^{-1}$; and animals in the control group were given the 5% glucose solution. Cisplatin or NC-6004 was administered to mice at any of the above dose levels per dose every 3 days. Antitumour activity was evaluated in terms of tumour size by measuring two orthogonal diameters ($a \times b$: a, long diameter; b, short diameter) at various time points. Animals were killed by cervical dislocation when the tumour size reached approximately 15 mm (UKCCCR, 1998). Changes in body weight were also monitored for the mice which were used in the present study.

Nephrotoxicity and hepatotoxicity of CDDP and NC-6004

Under isoflurane anaesthesia, five groups of Sprague–Dawley female rats (aged 6 weeks; 185–215 g initial body weight) were given a single i.v. injection of 5% glucose ($n=8$), CDDP at a dose of 10 $mg\,kg^{-1}$ ($n=12$), NC-6004 at a dose of 10 $mg\,kg^{-1}$ on a CDDP basis ($n=13$), or NC-6004 at a dose of 15 $mg\,kg^{-1}$ on a CDDP basis ($n=8$). Samples of blood and major organs were taken on day 7 after administration (UKCCCR, 1998). In the case of administering NC-6004 at a dose of 10 $mg\,kg^{-1}$ on a CDDP basis, five samples of blood and major organs were taken on day 14 after administration. The organs were immersed in 10% formalin solution. In each blood sample, plasma concentrations of blood urea nitrogen (BUN), creatinine, glutamic oxaloacetic transaminase (GOT), and glutamic pyruvic transaminase (GPT) were measured by SRL Laboratories (Tokyo, Japan). In addition, WBC and platelet were counted for blood samples 7 and 14 days after each drug administration in SRL Laboratories (Tokyo, Japan).

Evaluation of neurotoxicity

The severity of neurotoxicity was assessed by electrophysiological and histopathological procedures. Under isoflurane anaesthesia, rats ($n=5$) were given CDDP (2 $mg\,kg^{-1}$), NC-6004 (an equivalent dose of 2 $mg\,kg^{-1}$ CDDP), or 5% glucose, all i.v., twice a week, to a total of 11 administrations. Electrophysiological measurements were conducted at week 6 after the first administration, using the method described previously (McKeage *et al*, 1994; Screnci *et al*, 2000). Under light anaesthesia with phenobarbital, responses were evoked by stimulating the sciatic nerve at its notch and the tibial nerve at the ankle of the right hind paw, using a percutaneous needle electrode. The plantar muscle H- and M-waves were recorded using a pair of superficial silver–silver chloride electrodes applied to the sole and dorsum of the hind paw. H-response-related sensory nerve conduction velocity (SNCV) was calculated by dividing the distance between the stimulation sites at the sciatic notch and ankle by the difference in H-response latency after stimulation at the ankle and sciatic notch. M-response-related motor nerve conduction velocity (MNCV) was calculated by dividing the distance between the stimulation sites at the sciatic notch and ankle by the difference in M-response latency after stimulation at the sciatic notch and ankle. At week 7 after the initial administration, rats under deep anaesthesia with phenobarbital were subjected to intracardiac catheterisation and were rinsed with saline, followed by perfusion with 4% glutaraldehyde in 0.12 M PBS. Subsequently, a segment of the sciatic nerve was carefully removed. One part of the sciatic nerve was post-fixed with 4% glutaraldehyde in 0.12 M PBS for 24 h and was then embedded in epoxy resin as described previously (Cavaletti *et al*, 1992). The remaining parts of the sciatic nerve were immersed in a 10% formalin solution. Semi-thin (1 μm thick) and thin sections were prepared from the resin-embedded sciatic nerve for light microscopic observation and electron microscopic observation, respectively.

To determine the Pt concentration in the sciatic nerve, rats were given CDDP (5 $mg\,kg^{-1}$, $n=5$), NC-6004 (an equivalent dose of 5 $mg\,kg^{-1}$ CDDP, $n=5$), or 5% glucose ($n=2$), all i.v. twice a week, to a total of four administrations. On day 3 after the final administration, a segment of the sciatic nerve was removed. The removed sciatic nerve was prepared for ICP-MS analysis as described previously (Screnci *et al*, 2000). Briefly, the nerve was immersed in 1 ml of 70% nitric acid overnight. On the next day, the nerve was digested for 2 h at 90°C and Milli-Q was then added to a final volume of 5 ml. Finally, the Pt concentration in the sample solution was analysed with an ICP-MS spectrometer (SPQ 9000; Seiko Instruments Inc., Tokyo, Japan).

Statistical analysis

Data on therapeutic efficacy and body weight change were expressed as the mean ± s.e. The other data were expressed as the mean ± s.d. The statistical significance of differences in therapeutic efficacy and body weight change between two administration groups was calculated by repeated-measured

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analysis of variance (ANOVA). The statistical significance of differences in other data between two administration groups was calculated with the Student's *t*-test. All data were calculated with StatView® Software, version 5 (ABACUS Concepts, Berkeley, CA). A value of $P<0.05$ was considered statistically significant.

RESULTS

Preparation and characterisation of CDDP-incorporating polymeric micelles (NC-6004)

Cisplatin-incorporating polymeric micelles (NC-6004) consist of CDDP and PEG–P(Glu) (Figure 1A). Furthermore, NC-6004 consists of PEG, a hydrophilic chain which constitutes the outer shell of the micelles, and the coordinate complex of P(Glu) and CDDP, a polymer–metal complex-forming chain which constitutes the inner core of the micelles. The molecular weight of PEG–P(Glu) as a sodium salt was approximately 18 000 (PEG: 12 000; P(Glu): 6000). The CDDP-incorporated polymeric micelles were clearly discriminated from typical micelles from amphiphilic block copolymers. The driving force of the formation of the CDDP-incorporated micelles is the ligand substitution of Pt(II) atom from chloride to carboxylate in the side chain of P(Glu). The molar ratio of CDDP to the carboxyl groups in the copolymers was 0.71 (Nishiyama *et al*, 2003). A narrowly distributed size of polymeric micelles (30 nm) was confirmed by the DLS measurement

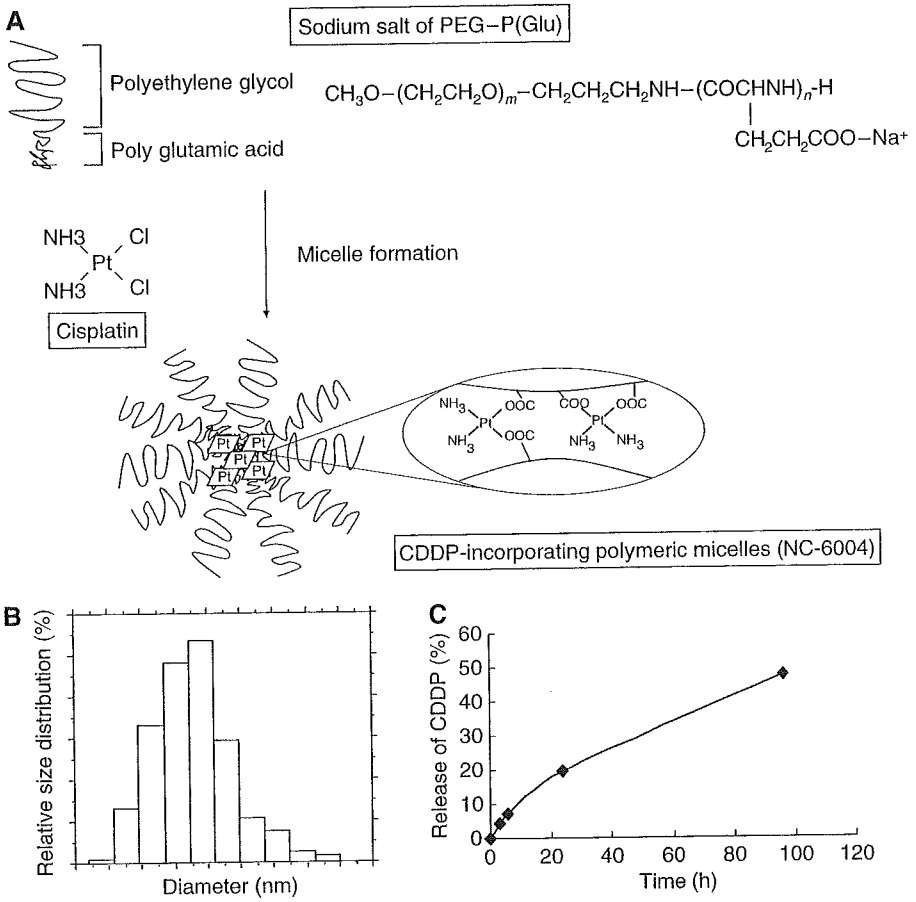

Figure 1 Preparation and characterisation of CDDP-incorporating polymeric micelles (NC-6004). (**A**) Chemical structures of CDDP and PEG–P(Glu) block copolymers, and the micellar structures of CDDP-incorporating polymeric micelles (NC-6004). (**B**) The particle size distribution of NC-6004 measured by the dynamic light-scattering method. The mean particle size of NC-6004 was approximately 30 nm. (**C**) Release of CDDP from NC-6004 in saline at 37°C.

Table 1 Pharmacokinetic parameter estimates for CDDP and NC-6004 in rats (see text for definitions of parameters)

Compound	Rat	T_{max}[a] (h)	C_{max}[a] ($\mu g\,ml^{-1}$)	$t_{1/2z}$ (h)	AUC_{0-t} ($\mu g\,h\,ml^{-1}$)	$AUC_{0-inf.}$ ($\mu g\,h\,ml^{-1}$)	CL_{tot} ($ml\,h^{-1}\,kg^{-1}$)	$MRT_{0-inf.}$ (h)	V_{ss} ($l\,kg^{-1}$)
CDDP	Mean	0.083	11.67	34.50	20.47	75.73	70.67	46.57	3.00
	s.d.		0.57	16.14	2.25	26.13	20.34	22.38	0.61
NC-6004	Mean	0.50	89.90	6.43	1325.90	1335.47	3.77	10.67	0.04
	s.d.		4.29	0.55	77.85	75.99	0.21	0.15	0.0023

The pharmacokinetic parameters were calculated after fitting to a noncompartment model using WinNonlin program. [a]For CDDP group, T_{max} represents time of maximum concentration.

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(Figure 1B). Also, the static light scattering (SLS) measurement revealed that the CDDP-loaded micelles showed no dissociation upon dilution and the CMC was less than 5×10^{-7}, suggesting remarkable stability compared with typical micelles from amphiphilic block copolymers (Nishiyama *et al.* 1999). It is assumed that the interpolymer crosslinking by Pt(II) atom might contribute to stabilisation of the micellar structure.

The release rates of CDDP from NC-6004 were 19.6 and 47.8% at 24 and 96 h, respectively (Figure 1C). Therefore, the release of CDDP was as slow as the previously reported release (Nishiyama *et al*, 2003). In distilled water, furthermore, NC-6004 was stable without releasing CDDP (data not shown).

Pharmacokinetics and pharmacodynamics

Frameless atomic absorption spectrophotometry could measure serum concentrations of Pt up to 48 h after i.v. injection of NC-6004, but could measure them only up to 4 h after i.v. injection of CDDP. NC-6004 showed a very long blood retention profile as compared with CDDP. The AUC_{0-t} and C_{max} values were significantly higher in animals given NC-6004 than in animals given CDDP, namely, 65- and 8-fold, respectively ($P<0.001$ and 0.001, respectively) (Table 1, Figure 2A). Furthermore, the CL_{tot} and V_{ss} values were significantly lower in animals given NC-6004 than in animals given CDDP, that is, one-nineteenth and one-seventy-fifth, respectively ($P<0.01$ and 0.01, respectively) (Table 1).

Regarding the concentration–time profile of Pt in various tissues after i.v. injection of CDDP or NC-6004, all organs measured exhibited the highest concentrations of Pt within 1 h after administration in all animals given CDDP (Figure 2B). Furthermore, animals given NC-6004 exhibited the highest tissue concentrations of Pt in the liver and spleen at late time points (24 and 48 h after administration, respectively). However, the concentrations decreased on day 7 after administration (Figure 2C). In addition, and in a similar manner to other drugs which are incorporated in polymeric carriers, NC-6004 demonstrated accumulation in organs of the reticuloendothelial system, for example, liver and spleen. At 48 h after administration, tissue concentrations of Pt in the liver and spleen were 4.6- and 24.4-fold higher for NC-6004 than for CDDP. On the other hand, a marked increase in tissue Pt concentration was observed immediately after administration in the kidneys of animals given CDDP. Renal Pt concentration at 10 min and 1 h after administration were 11.6- and 3.1-fold lower, respectively, in animals given NC-6004 than in animals given CDDP. Furthermore, the maximum concentration (C_{max}) in the kidney was 3.8-fold lower at the time of NC-6004 administration than at the time of CDDP administration.

Regarding the tumour accumulation of Pt, tumour concentrations of Pt peaked at 10 min after administration of CDDP. On the other hand, tumour concentrations of Pt peaked at 48 h after administration of NC-6004 (Figure 2D). The maximum concentration (C_{max}) in tumour was 2.5-fold higher for NC-6004 than for CDDP ($P<0.001$). Furthermore, the tumour AUC was 3.6-fold higher for NC-6004 than for CDDP (81.2 and 22.6 $\mu g\,ml\,h^{-1}$ in animals given NC-6004 and CDDP, respectively).

In vitro cytotoxicity

NC-6004 was tasted on 12 human tumour cell lines derived from bladder, colon, lung, gastric, and breast cancers. The IC_{50} values of NC-6004 were 6-to 15-fold higher than those of CDDP (Table 2).

In vivo antitumour activity

BALB/*c* nude mice implanted with a human gastric cancer cell line MKN-45 showed decreased tumour growth rates after i.v. injection of CDDP and NC-6004 (Figure 3A). In the administration of CDDP,

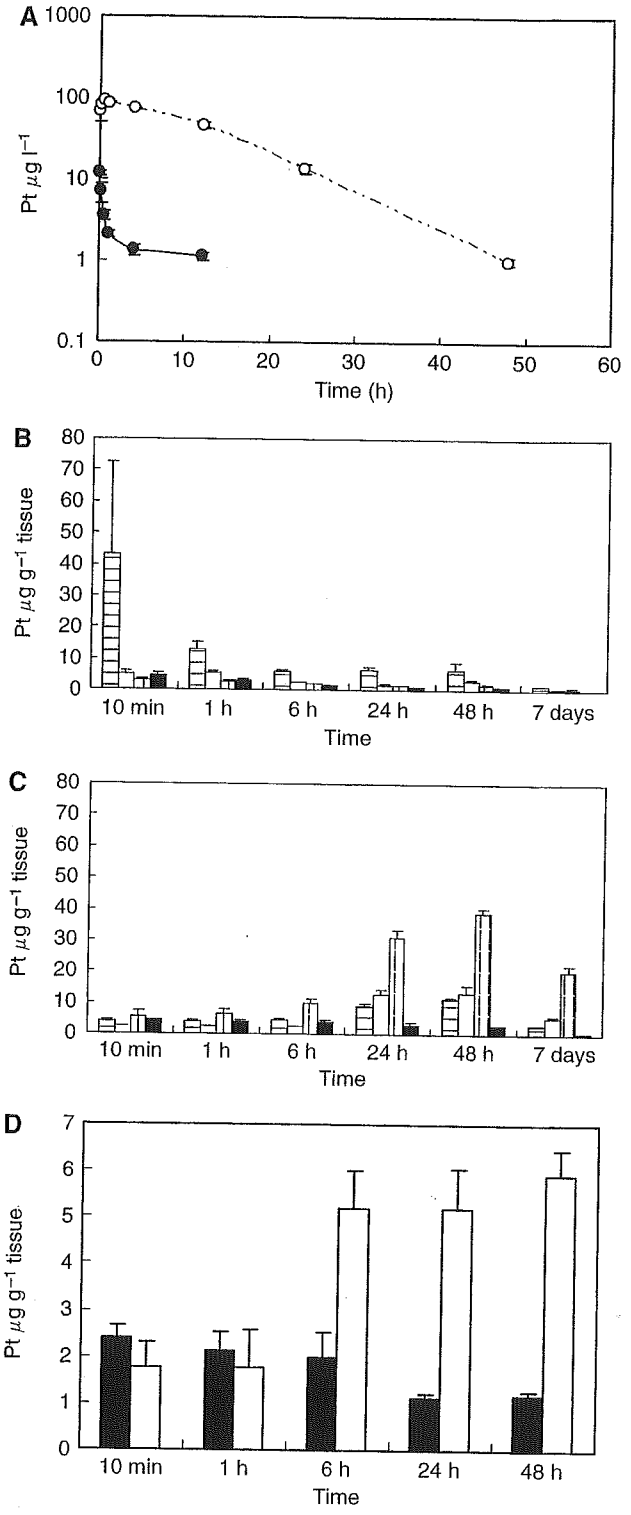

Figure 2 Time profiles of Pt concentration in the plasma and tissue distribution of Pt after a single i.v. injection of CDDP (5 mg kg^{-1}) or NC-6004 (an equivalent dose of 5 mg kg^{-1} CDDP). (**A**) Concentration–time profile of Pt in the plasma after a single i.v. injection of CDDP (●) and NC-6004 (○) in rats ($n=3$). Tissue distribution of Pt after a single i.v. injection of CDDP (**B**) and NC-6004 (**C**) in rats ($n=3$) (kidney (▤), liver (□), spleen (▥), and lung (■)). (**D**) Time profiles of Pt concentration in the MKN-45 solid tumour after a single i.v. injection of CDDP (■) and NC-6004 (□) in MKN-45 bearing BALB/*c* nude mice ($n=3$). Values are expressed as the mean ± s.d.

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Table 2 IC_{50} values(μM) of CDDP and NC-6004 in various cancer cell lines

		Exposure time (h)			
		48		72	
Cancer	Cell line	CDDP	NC-6004	CDDP	NC-6004
Bladder cancer	EJ-1	2.46	25.45	1.86	18.44
	J82	2.78	42.89	2.42	20.27
	MBT-2	15.88	>100	5.67	71.67
Colon cancer	Colo201	34.77	>100	28.52	>100
	Colo320	16.32	>100	9.71	81.15
	HT-29	14.44	>100	8.83	>100
Lung cancer	A549	21.43	>100	20	>100
	EBC-1	>100	>100	9.36	84.78
	PC-14	16.81	>100	8.73	87.11
Gastric cancer	MKN-28	>100	>100	8.23	76.81
	MKN-45	7.12	68.36	6.94	43.81
Breast cancer	MCF-7	12.78	>100	5.71	54.71

the CDDP 5 mg kg^{-1} administration group showed a significant decrease ($P<0.01$) in tumour growth rate as compared with the control group. In the administration of NC-6004, NC-6004 2.5 mg kg^{-1} administration group ($P<0.05$) and 5 mg kg^{-1} administration group ($P<0.01$) showed significant decreases in tumour growth rate as compared with the control group. However, the NC-6004 administration groups at the same dose levels as CDDP showed no significant difference in tumour growth rate. The same animal model was used to repeat the study using the drugs at different dose levels, and similar tendencies were observed (data not shown). Regarding time-course changes in body weight change rate, the CDDP 5 mg kg^{-1} administration group showed a significant decrease ($P<0.001$) in body weight as compared with the control group. On the other hand, none of the NC-6004 administration groups showed a decrease in body weight as compared with the control group (Figure 3B).

Nephrotoxicity and hepatotoxicity of CDDP and NC-6004

In the CDDP 10 mg kg^{-1} administration group, four of 12 animals died from toxicity within 7 days after drug administration. No deaths occurred in the NC-6004 10 mg kg^{-1} administration group and the NC-6004 15 mg kg^{-1} administration group. Regarding renal function, the BUN concentrations on day 7 after the administration of 5% glucose, CDDP 10 mg kg^{-1}, NC-6004 10 mg kg^{-1}, and NC-6004 15 mg kg^{-1} were 20.8$\pm$3.0, 65.3$\pm$44.4, 20$\pm$4.5, and 24.6$\pm$18.2 mg dl^{-1}, respectively. The plasma concentrations of creatinine on day 7 after the administration of 5% glucose, CDDP 10 mg kg^{-1}, NC-6004 10 mg kg^{-1}, and NC-6004 15 mg kg^{-1} were 0.27$\pm$0.03, 0.68$\pm$0.23, 0.28$\pm$0.04, and 0.45$\pm$0.11 mg dl^{-1}, respectively. The CDDP 10 mg kg^{-1} administration group showed significantly higher plasma concentrations of BUN and creatinine as compared with the control group ($P<0.05$ and 0.001, respectively), with the NC-6004 10 mg kg^{-1} administration group ($P<0.05$ and 0.001, respectively), and also with the NC-6004 15 mg kg^{-1} administration group ($P<0.05$ and 0.05, respectively) (Figure 4A and B). Light microscopy indicated tubular dilation with flattening of the lining cells of the tubular epithelium in the kidney from all animals in the CDDP 10 mg kg^{-1} administration group. On the other hand, no histopathological change was observed in the kidneys from all animals in the NC-6004 10 mg kg^{-1} administration group (Figure 4C and D). Regarding hepatic function, the plasma concentrations of GOT on day 7 after the administration of 5% glucose, CDDP 10 mg kg^{-1}, NC-6004 10 mg kg^{-1}, and NC-6004 15 mg kg^{-1} were 68$\pm$6.8, 65.1$\pm$5.5, 106$\pm$13.1, and 97$\pm$16.2 IU l^{-1}, respectively. The plasma

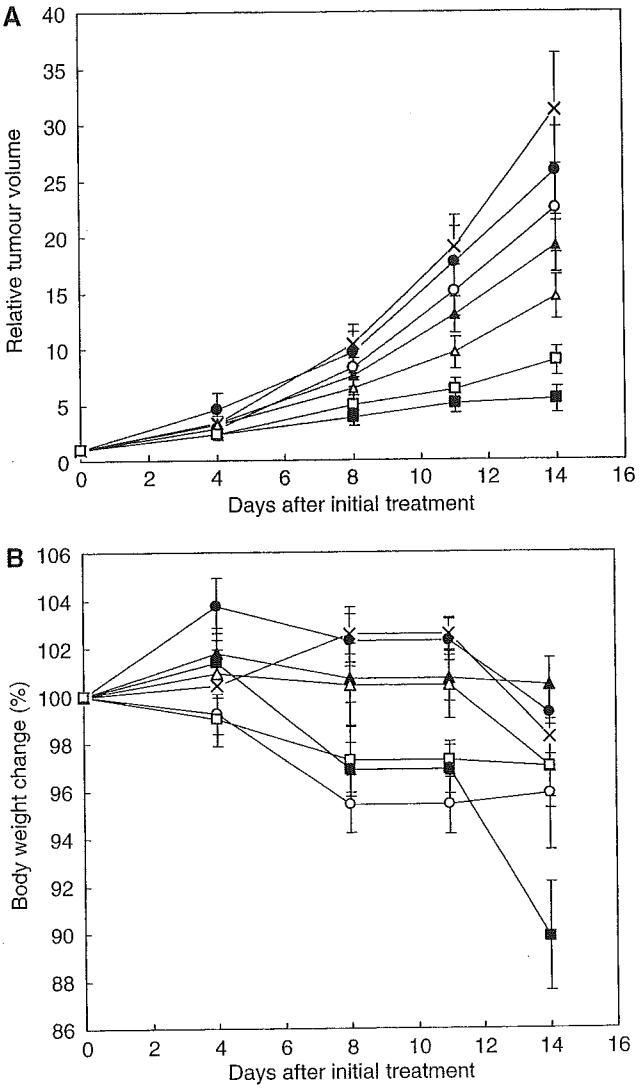

Figure 3 Relative changes in MKN-45 tumour growth rates in nude mice. (**A**) Cisplatin and NC-6004 were injected i.v. every 3 days, three administrations in total, at CDDP-equivalent doses of 0.5 mg kg^{-1} (●, ○), 2.5 mg kg^{-1} (▲, △), and 5 mg kg^{-1} (■, □), respectively. Glucose (5%) was injected in the control mice (×). (**B**) Changes in relative body weight. Data were derived from the same mice as those used in the present study. Values are expressed as the mean$\pm$s.e.

concentrations of GPT on day 7 after the administration of 5% glucose, CDDP 10 mg kg^{-1}, NC-6004 10 mg kg^{-1}, and NC-6004 15 mg kg^{-1} were 39.6$\pm$10, 32$\pm$6.4, 92$\pm$18.9, and 55$\pm$11.3 IU l^{-1}, respectively. The CDDP 10 mg kg^{-1} administration group showed plasma concentrations of GOT and GPT which were comparable to those in the control group. However, the NC-6004 10 mg kg^{-1} administration group, which presented the same dose level as the CDDP 10 mg kg^{-1} administration group, showed significantly higher plasma concentrations of GOT and GPT ($P<0.001$ and 0.01, respectively) as compared with the control group. Furthermore, the NC-6004 15 mg kg^{-1} administration group also showed significantly higher plasma concentrations of GOT ($P<0.001$) as compared with the control group. However, the plasma concentrations of GOT and GPT on day 14 after the administration of NC-6004 10 mg kg^{-1} were comparable to those in the control group (74$\pm$2.3 and 42.8$\pm$5.1 IU l^{-1}, respectively) (Figure 4E). These results lead to the conjecture that rats which were given NC-6004 10 mg kg^{-1}, i.v., showed transient and reversible hepatotoxicity.

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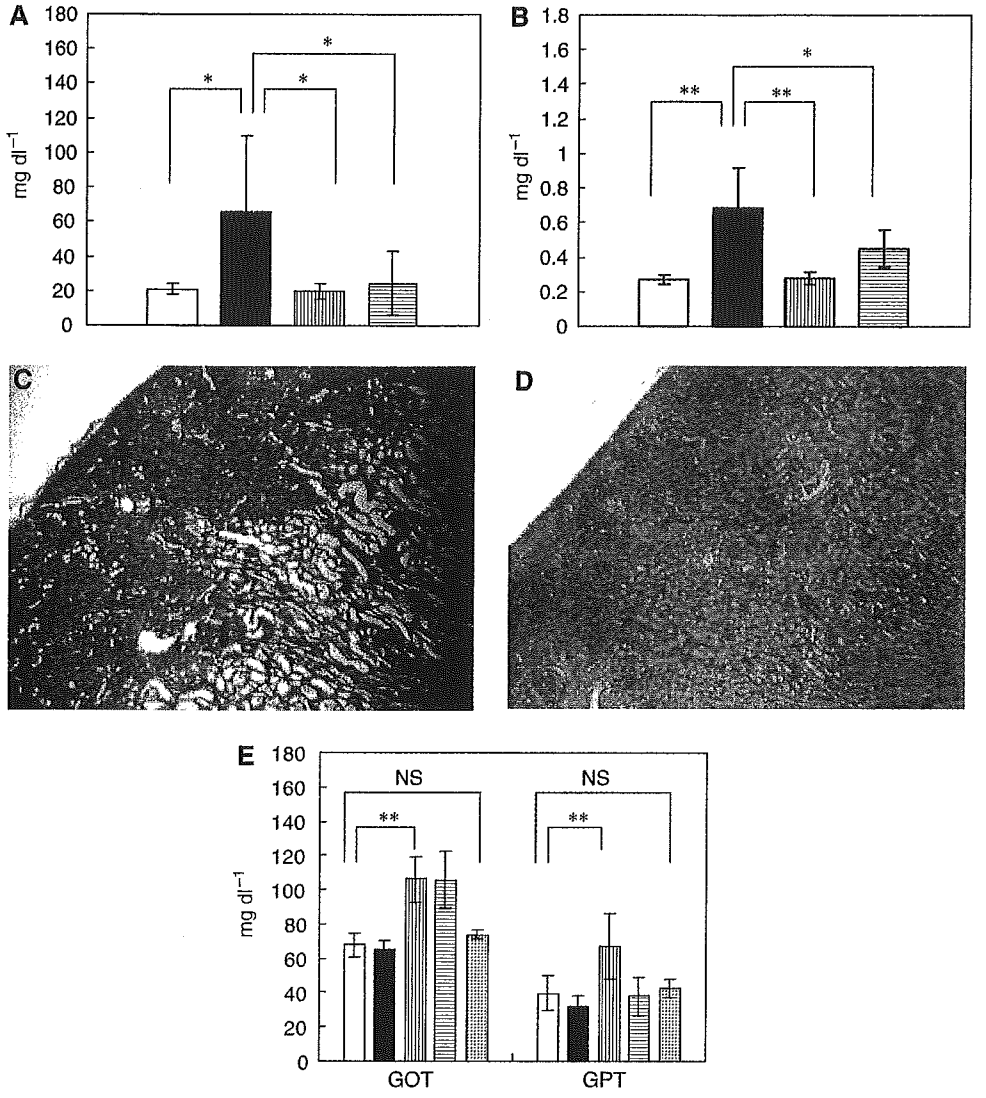

Figure 4 Nephrotoxicity and hepatotoxicity of CDDP and NC-6004. Plasma concentrations of BUN (**A**) and creatinine (**B**) were measured after a single i.v. injection of 5% glucose (□) ($n=8$), CDDP at a dose of 10 mg kg^{-1} (■) ($n=12$), NC-6004 at a dose of 10 mg kg^{-1} ($n=13$) on a CDDP basis (▥), and at a dose of 15 mg kg^{-1} on a CDDP basis (▤) ($n=8$) to rats. Histopathological changes in the kidney on day 7 after the i.v. injection of CDDP (**C**, ×4) and NC-6004 (**D**, ×4) in rats at an equivalent dose of 10 mg kg^{-1} CDDP. In rats given CDDP, widespread tubular degeneration as indicated by tubular dilation with flattening of the lining cells of tubular epithelium was seen. On the other hand, no histopathological change was observed in the kidney from all animals in the NC-6004 10 mg kg^{-1} administration group. For hepatotoxicity (**E**), the plasma concentrations of GOT and GPT were measured on day 7 after administration. When administering NC-6004 at a dose of 10 mg kg^{-1} on a CDDP basis, five of 13 blood samples were taken on day 14 after administration (▦). The other samples were taken on day 7 administration. In the group given CDDP at a dose of 10 mg kg^{-1}, four of 12 rats died within 7 days. Values are expressed as the mean ± s.d. *$P<0.05$, **$P<0.001$, NS: not significant.

Neurotoxicity of CDDP and NC-6004

Neurophysiological examination revealed that MNCVs in animals given 5% glucose, CDDP, and NC-6004 were 44.2±3.5, 40.94±5.08, and 40.62±0.63 m s^{-1}, respectively. No significant difference was found among the groups with respect to MNCV. Furthermore, SNCVs in animals given 5% glucose, CDDP, and NC-6004 were 42.86±8.07, 35.48±4.91, and 43.74±5.3 m s^{-1}, respectively. Animals given NC-6004 showed no delay in SNCV as compared with animals given 5% glucose. On the other hand, animals given CDDP showed a significant delay ($P<0.05$) in SNCV as compared with animals given NC-6004 (Figure 5A). In addition, histopathological examination with electron microscopy revealed degenerations, as manifested by electron photomicrographs indicating degenerative changes, for example, loss of microtubules, degeneration in the cytoplasm of Schwann cells, loss of filaments, and an irregular inner loop, in approximately 80% of myelinated segments of the sciatic nerve from animals given CDDP. On the other hand, animals given NC-6004 exhibited nearly normal electron photomicrographs of the sciatic nerve as the control animals did (Figure 5B and C). These results indicate that NC-6004 reduced peripheral neurotoxicity as compared with CDDP. Furthermore, regarding body weight change as an indication of general toxicity, furthermore, the NC-6004 administration groups showed significant inhibition of body weight decrease ($P<0.001$) as compared with the CDDP administration group ($P<0.001$) (Figure 5D).

The analysis by ICP-MS on sciatic nerve concentrations of Pt could not detect Pt in the sciatic nerve from animals given 5% glucose (data not shown). Sciatic nerve concentrations of Pt in

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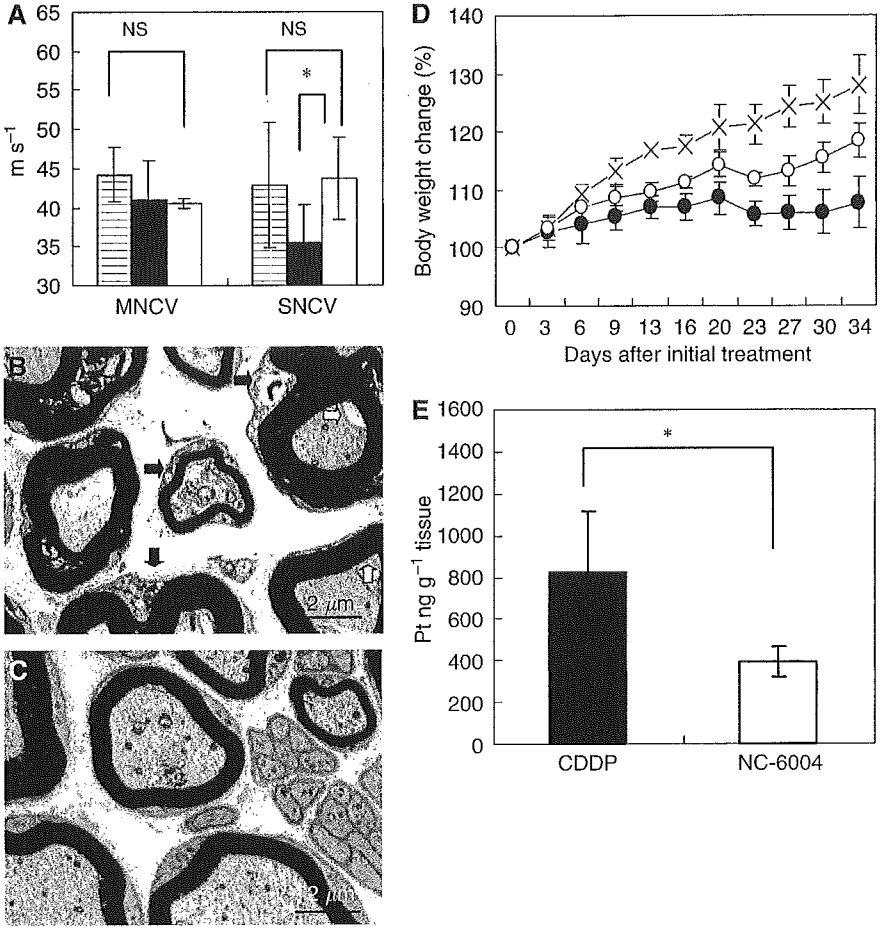

Figure 5 Neurotoxicity of CDDP and NC-6004 in rats. Rats ($n=5$) were given CDDP (2 mg kg^{-1}), NC-6004 (an equivalent dose of 2 mg kg^{-1} CDDP), or 5% glucose, all i.v. twice a week, 11 administrations in total. (**A**) Sensory nerve conduction velocity and MNCV of the sciatic nerve at week 6 after the initial administration (control (⊟), CDDP (●), and NC-6004 (□)). Histopathological changes of the sciatic nerve were examined by electron microscopy after the administration of CDDP (**B**) and NC-6004 (**C**). In rats given CDDP, widespread degenerations as indicated by loss of microtubules, loss of filaments, degeneration in the cytoplasm of Schwann cells (➡), and an irregular inner loop (⇨) were seen. On the other hand, animals given NC-6004 exhibited nearly normal electron micrographs of the sciatic nerve as the control animals. (**D**) Changes in relative body weight. Data were derived from the same rats as those used in the present study (control (×), CDDP (●), and NC-6004 (○)). (**E**) The Pt concentration in the sciatic nerve. Rats were given CDDP (■) (5 mg kg^{-1}, $n=5$), NC-6004 (□) (an equivalent dose of 5 mg kg^{-1} CDDP, $n=5$), or 5% glucose ($n=2$), all i.v. twice a week, four administrations in total. On day 3 after the final administration, a segment of the sciatic nerve was removed and the Pt concentration in the sciatic nerve was measured by ICP-MS. Body weight changes are expressed as the mean ± s.e. The other data are expressed as the mean ± s.d. $^*P<0.05$, $^{**}P<0.001$, NS: not significant.

animals given CDDP and NC-6004 were 827.2 ± 291.3 and 395.5 ± 73.1 ng g^{-1} tissue. Therefore, the concentrations were significantly ($P<0.05$) lower in animals given NC-6004 (Figure 5E). This finding is believed to be a factor which reduced neurotoxicity following NC-6004 administration as compared with the CDDP administration.

DISCUSSION

The present study indicated that CDDP-incorporating polymeric micelles (NC-6004) are stable nanoparticles with a long blood retention profile as compared with free CDDP. NC-6004 showed 6- to 15-fold less potent *in vitro* cytotoxic activity in several human cancer cell lines as compared with CDDP. These findings are considered attributable to the slow release of free CDDP in the presence of abundant chloride ions because NC-6004 contains coordination bonds between the atoms of Pt(II) of CDDP and the carboxylic group in the side chain of P(Glu). *In vivo*, however, in contrast to the *in vitro* findings, NC-6004 was found to markedly reduce nephrotoxicity and neurotoxicity – dose-limiting factors of CDDP, while preserving antitumour activity, which was equivalent to or better than that of free CDDP.

Nephrotoxicity of CDDP is considered to depend on the peak urinary CDDP concentration and on the maximum CDDP concentration in the uriniferous tubules (Levi *et al*, 1982). We consider that the reduced nephrotoxicity of NC-6004 may be explained by the following facts: (1) the tendency of micelles to be less prone to filtration by nephrons because of the NC-6004 particle size (approximately 30 nm), and (2) the much lower C_{max} value for CDDP at least in the uriniferous tubules than the value following CDDP administration. NC-6004 possibly facilitates treatment on an outpatient basis because it allows safer administration to patients with decreased renal function and requires no massive fluid replacement to protect renal tissue after the administration of CDDP.

The main neuropathy of CDDP is sensory peripheral neuropathy (van der Hoop *et al*, 1990; Gregg *et al*, 1992). A delay in SNCV due to the injury of dorsal root ganglia and peripheral nerve has previously been reported in rats given CDDP, although MNCV was

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preserved in the tail and hind paws of rats (McKeage *et al*, 1994; Tredici *et al*, 1998; Meijer *et al*, 1999; Tredici *et al*, 1999). Furthermore, histopathological examination revealed degenerative changes in the sciatic nerve in similar experimental animals (Cavaletti *et al*, 1992; Tredici *et al*, 1999). In the present study, animals given NC-6004 showed no delay in the SNCV, while animals given CDDP showed a significant delay in the SNCV as compared with animals given NC-6004. Neuropathologically, neuronal degeneration, which was observed following CDDP administration, was not observed with NC-6004 administration. This result is considered attributable principally to the fact that the peripheral nerve concentration of Pt decreased to half or less following NC-6004 administration than with CDDP administration. The nervous tissue concentration of Pt at the time of NC-6004 administration decreased significantly despite the fact that the plasma AUC at the time of NC-6004 administration was high, being 65-fold higher than the plasma AUC concentration with CDDP administration. We consider that this result is attributed to the marked inhibition of Pt distribution into nervous tissue in the NC-6004 administration groups as manifested by V_{ss} of 3.00 ± 0.61 and $0.04 \pm 0.0023\,l\,kg^{-1}$ in the CDDP and NC-6004 groups, respectively. In any event, we believe that the neurotoxicity of CDDP reduced by NC-6004 allows its long-term administration.

On the other hand, transient hepatic dysfunction was observed in rats. This observation indicates the proneness of Pt to accumulate in the RES of the liver because NC-6004 is, after all, said and done, a macromolecule, although preserving a stealth effect through its outer shell of PEG. We consider that caution should be exercised against hepatic dysfunction in conducting a clinical trial of NC-6004 in the future. However, the accumulation of Pt was lower following NC-6004 administration due to a decrease in V_{ss} in other organs including nerve. As shown by changes in body weight in multiple dose studies in rats, the NC-6004 administration groups have been demonstrated to show a smaller decrease in body weight as compared with the CDDP administration groups. In single-dose studies, furthermore, one dose of CDDP $10\,mg\,kg^{-1}$ was equivalent to the 50% of the lethal dose. In fact, four of 12 animals died within 7 days after administration. However, none of the eight animals in the NC-6004 group died after the administration of NC-6004 at a CDDP equivalent dose of $15\,mg\,kg^{-1}$. In terms of haematological toxicity, there was no significant difference between the CDDP and NC-6004 groups in rats (data not shown).

In murine tumour strains, CDDP-incorporating polymeric micelles showed significantly high antitumour activity (Nishiyama *et al*, 2003). In the human gastric cancer strain used in the present study, however, no significant difference was found between the NC-6004 and CDDP administration groups. A significant difference was found in antitumour activity between the NC-6004 low-dose group ($2.5\,mg\,kg^{-1}$ administration group) and the control group, while no significant difference was found between the CDDP low-dose group ($2.5\,mg\,kg^{-1}$ administration group) and the control group. Results available to date and the results from the present study lead to the consideration that the incorporation of CDDP into polymeric micelles does not reduce its antitumour activity.

Data from the present study warrant the clinical evaluation of NC-6004. We consider that the protocol for the Phase I clinical trial of NC-6004 should employ a regimen without massive i.v. drip infusion.

Translational Therapeutics

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TRANSRECTAL HIGH-INTENSITY FOCUSED ULTRASOUND IN THE TREATMENT OF LOCALIZED PROSTATE CANCER : A MULTICENTER STUDY

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