Table 1. Body weight and food consumption in rats dosed with SWCNT by gavage for 28 days

Sex	Male				Female			
Dose (mg/kg bw/day)	0	0.125	1.25	12.5	0	0.125	1.25	12.5
Body weight (g)								
Day 1	216 ± 7	212 ± 6	214 ± 8	214 ± 8	162 ± 5	165 ± 6	160 ± 7	161 ± 7
Day 4	243 ± 8	238 ± 8	241 ± 11	241 ± 10	174 ± 8	175 ± 5	172 ± 8	172 ± 7
Day 7	274 ± 12	267 ± 9	273 ± 16	270 ± 14	187 ± 9	186 ± 6	181 ± 9	184 ± 8
Day 14	335 ± 18	322 ± 16	337 ± 29	331 ± 20	211 ± 14	210 ± 10	198 ± 12	209 ± 10
Day 21	390 ± 26	368 ± 27	388 ± 34	378 ± 24	231 ± 18	234 ± 11	213 ± 17	227 ± 15
Day 28	426 ± 31	399 ± 34	426 ± 39	413 ± 30	250 ± 22	246 ± 15	227 ± 18	245 ± 17
Food consumption (g/rat/day)								
Day 0 - 1	25 ± 2	24 ± 1	25 ± 2	25 ± 1	18 ± 2	20 ± 1	18 ± 2	19 ± 2
Day 1 - 7	27 ± 2	26 ± 1	26 ± 2	26 ± 1	19 ± 2	19 ± 2	17 ± 1	19 ± 1
Day 7 - 14	29 ± 3	28 ± 2	29 ± 3	29 ± 2	19 ± 2	19 ± 2	17 ± 1	19 ± 1
Day 14 - 21	29 ± 3	28 ± 3	29 ± 3	29 ± 2	20 ± 2	20 ± 2	18 ± 1	19 ± 2
Day 21 - 28	29 ± 4	27 ± 3	29 ± 2	28 ± 2	20 ± 3	20 ± 2	18 ± 1	20 ± 2

Table 2. Histopathological findings of rats dosed with SWCNT by gavage for 28 days

Sex		Male				Female			
Dose (mg/kg bw/day)		0	0.125	1.25	12.5	0	0.125	1.25	12.5
	Grade								
Number of animals examined [a]		5	0	0	5	5	5	5	5
Kidney									
Tubular regeneration	+	2	-	-	1	0	1	0	0
Liver									
Vacuolation of midzonal hepatocyte	+	0	-	-	1	0	-	-	0
Vacuolation of periportal hepatocyte	+	0	-	-	0	2	-	-	3
	++	0	-	-	0	1	-	-	0
Focal necrosis	+	1	-	-	0	0	-	-	1
Microgranuloma	+	5	-	-	3	5	-	-	5
Lung (bronchus)									
Focal hemorrhage	+	1	-	-	1	0	-	-	0
Cell infiltration	+	0	-	-	1	0	-	-	0
Accumulation of foamy cell	+	0	-	-	1	0	-	-	0
Spleen									
Lymphoid hyperplasia	++	1	-	-	0	0	-	-	0

Grade; +: slight change; ++: mild change, -: Not applicable

[a] :The histopathological examination was carried out in 5 animals at 0 and 12.5 mg/kg bw/day and the kidney was examined in 5 females at 0.125 and 12.5 mg/kg bw/day.

Table 3. Water intake and urinalysis in rats dosed with SWCNT by gavage for 28 days

Sex	Male				Female			
Dose (mg/kg bw/day)	0	0.125	1.25	12.5	0	0.125	1.25	12.5
Water intake (ml/24 hr)	35 ± 6	30 ± 5	36 ± 4	35 ± 6	30 ± 8	31 ± 13	25 ± 5	26 ± 4
Urine volume (ml/24 hr)	17.7 ± 4.7	14.2 ± 2.7	16.7 ± 5	15.3 ± 3.6	12.8 ± 4.5	8.1 ± 1.5	5.5 ± 2.3**	8.1 ± 3.1*
Osmolality (mOsm/kg)	1696 ± 407	1710 ± 455	1459 ± 208	1758 ± 352	1874 ± 507	2046 ± 239	2296 ± 463	2104 ± 394

*: Significantly different from the control group ($p < 0.05$).

**: Significantly different from the control group ($p < 0.01$).

Table 4. Hematology in rats dosed with SWCNT by gavage for 28 days

Sex	Male				Female			
Dose (mg/kg bw/day)	0	0.125	1.25	12.5	0	0.125	1.25	12.5
RBC ($\times 10^4/\mu l$)	841 ± 32	845 ± 30	819 ± 26	816 ± 83	810 ± 43	841 ± 13	858 ± 18	859 ± 34*
Hemoglobin (g/dl)	15.5 ± 0.6	15.6 ± 0.4	15.7 ± 0.6	15.2 ± 1.6	15.4 ± 0.7	15.9 ± 0.4	15.9 ± 0.4	16.2 ± 0.7
Hematocrit (%)	45.7 ± 1.4	45.5 ± 1.3	46 ± 1.8	44.8 ± 4.7	43.6 ± 2.3	45.5 ± 1	45.4 ± 1.1	46.3 ± 1.9
MCV (fl)	54.3 ± 1.3	53.8 ± 0.9	56.1 ± 1	54.9 ± 2	53.9 ± 2.5	54.1 ± 1.2	52.9 ± 0.9	53.9 ± 1.3
MCH (pg)	18.5 ± 0.5	18.5 ± 0.4	19.2 ± 0.3	18.6 ± 0.8	19 ± 0.8	19 ± 0.5	18.5 ± 0.4	18.8 ± 0.4
MCHC (g/dl)	34 ± 0.3	34.4 ± 0.1	34.2 ± 0.2	34 ± 0.2	35.3 ± 0.3	35 ± 0.3	35 ± 0.4	34.9 ± 0.1
Reticulocyte (%)	2.4 ± 0.4	1.9 ± 0.5	2.6 ± 0.3	2.8 ± 1.5	1.8 ± 0.3	1.5 ± 0.2	1.5 ± 0.1	1.7 ± 0.4
WBC ($\times 10^2/\mu l$)	89.1 ± 14.0	110.5 ± 24.2	105.5 ± 23.7	123.8 ± 34.8	70 ± 22.4	67.8 ± 11.4	81.0 ± 30.8	64.1 ± 10.9
Differential leukocyte counts ($\times 10^2/\mu l$)								
Lymphocyte	70.6 ± 9.7	86 ± 14.8	76.7 ± 13.6	99.9 ± 25.2*	52.4 ± 18	53.3 ± 13.7	64.1 ± 31.2	48.9 ± 11.3
Neutrophil	14.8 ± 3.2	20.1 ± 8.9	24.6 ± 16.3	19.2 ± 8.2	14.7 ± 3.4	11.8 ± 2.8	14.1 ± 5.4	12.7 ± 4.7
Eosinophil	1.0 ± 0.8	1.1 ± 0.7	0.9 ± 0.1	0.9 ± 0.4	1.0 ± 0.7	0.9 ± 0.2	0.9 ± 0.4	1.0 ± 0.3
Basophil	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.5 ± 0.3*	0.1 ± 0.1	0.1 ± 0.1	0.3 ± 0.3	0.1 ± 0.1
Monocyte	1.9 ± 0.8	2.3 ± 1.5	2.4 ± 1	2.6 ± 1.8	1.3 ± 0.7	1.3 ± 0.4	1.2 ± 0.5	1.2 ± 0.3

RBC: Red blood cell; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; WBC: White blood cell.
*: Significantly different from the control group ($p < 0.05$).

Table 5. Serum biochemistry in rats dosed with SWCNT by gavage for 28 days

Dose (mg/kg bw/day)	Male				Female			
	0	0.125	1.25	12.5	0	0.125	1.25	12.5
AST (IU/l)	68 ± 4	68 ± 5	62 ± 4	64 ± 6	60 ± 7	86 ± 23	79 ± 29	72 ± 11
ALT (IU/l)	28 ± 5	30 ± 4	25 ± 3	28 ± 7	19 ± 2	34 ± 10**	27 ± 10	22 ± 1
LDH (IU/l)	54 ± 8	53 ± 7	52 ± 5	54 ± 6	52 ± 3	63 ± 16	57 ± 12	56 ± 9
γ-GTP (IU/l)	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 1	1 ± 0	1 ± 0
ALP (IU/l)	647 ± 198	686 ± 82	655 ± 73	663 ± 84	415 ± 127	359 ± 102	378 ± 34	470 ± 102
T. bile acid (μmol/l)	15.1 ± 12.7	12.5 ± 4.9	10.5 ± 7.5	7.6 ± 2.9	12.2 ± 2.3	15.4 ± 4.9	11.3 ± 3.1	11.6 ± 6.3
T. cholesterol (mg/dl)	50 ± 5	48 ± 14	52 ± 7	50 ± 6	55 ± 7	57 ± 14	66 ± 16	50 ± 17
Triglyceride (mg/dl)	56 ± 19	55 ± 22	50 ± 18	59 ± 24	14 ± 3	29 ± 12*	21 ± 11	14 ± 6
Phospholipid (mg/dl)	93 ± 8	92 ± 14	94 ± 10	93 ± 10	100 ± 11	108 ± 18	123 ± 20	95 ± 26
T. bilirubin (mg/dl)	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0
Glucose (mg/dl)	126 ± 13	121 ± 5	135 ± 18	141 ± 12	113 ± 11	106 ± 11	122 ± 8	123 ± 23
BUN (mg/dl)	13 ± 2	14 ± 3	12 ± 1	14 ± 2	16 ± 2	16 ± 2	15 ± 2	16 ± 2
Creatinine (mg/dl)	0.24 ± 0.02	0.24 ± 0.02	0.25 ± 0.02	0.25 ± 0.03	0.27 ± 0.02	0.26 ± 0.04	0.27 ± 0.02	0.28 ± 0.01
Sodium (mmol/l)	144 ± 1	144 ± 1	144 ± 1	143 ± 1	144 ± 1	143 ± 1	143 ± 1	143 ± 2
Potassium (mmol/l)	4.6 ± 0.1	4.6 ± 0.1	4.6 ± 0.1	4.7 ± 0.2	4.3 ± 0.2	4.4 ± 0.3	4.2 ± 0.2	4.2 ± 0.2
Chloride (mmol/l)	105 ± 1	106 ± 1	105 ± 1	106 ± 1	107 ± 1	107 ± 1	107 ± 2	107 ± 1
Calcium (mg/dl)	10 ± 0.2	9.8 ± 0.2	10.2 ± 0.3	9.8 ± 0.2	10 ± 0.3	10 ± 0.1	9.9 ± 0.1	10 ± 0.1
I. phosphorus (mg/dl)	7.8 ± 0.3	7.5 ± 0.4	7.9 ± 0.5	7.5 ± 0.6	7 ± 0.3	7 ± 0.6	6.9 ± 0.9	6.9 ± 0.8
T. protein (g/dl)	5.8 ± 0.1	6 ± 0.1	6 ± 0.1	5.9 ± 0.2	6.4 ± 0.3	6.2 ± 0	6.2 ± 0.2	6.1 ± 0.1
Albumin (g/dl)	3.2 ± 0	3.2 ± 0.1	3.2 ± 0.1	3.2 ± 0.1	3.6 ± 0.2	3.5 ± 0.1	3.6 ± 0.1	3.5 ± 0.1
Albumin/Globlin	1.21 ± 0.09	1.14 ± 0.07	1.15 ± 0.08	1.23 ± 0.06	1.31 ± 0.12	1.31 ± 0.09	1.43 ± 0.08	1.35 ± 0.07

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; LDH: lactate dehydrogenase; ALP: Alkaline phosphatase; BUN: Blood urea nitrogen

Table 6. Absolute and relative organ weight in rats dosed with SWCNT by gavage for 28 days

Dose (mg/kg bw/day)	Male				Female			
	0	0.125	1.25	12.5	0	0.125	1.25	12.5
Body weight [a]	406 ± 22	371 ± 33	390 ± 34	379 ± 21	232 ± 10	227 ± 13	212 ± 18	225 ± 16
Pituitary (mg) [b]	12.3 ± 1.5	11.3 ± 2	11.7 ± 1.2	10.6 ± 0.6	14.9 ± 1.1	12.5 ± 2	12.6 ± 1.9	13.2 ± 2.4
(mg%) [c]	3 ± 0.3	3.1 ± 0.5	3 ± 0.4	2.8 ± 0.2	6.4 ± 0.4	5.5 ± 0.7	6 ± 0.9	5.8 ± 0.7
Thyroid (mg)	21.9 ± 4.8	18 ± 2.4	20.2 ± 2.8	17.6 ± 2.1	14.2 ± 0.8	15.2 ± 2.5	13.2 ± 2.5	14.5 ± 1.7
(mg%)	5.4 ± 0.9	4.9 ± 0.6	5.2 ± 0.7	4.7 ± 0.4	6.1 ± 0.5	6.7 ± 1	6.3 ± 1.3	6.4 ± 0.6
Thymus (mg)	619 ± 131	538 ± 123	630 ± 96	541 ± 79	484 ± 45	480 ± 62	439 ± 112	450 ± 85
(mg%)	152 ± 25	144 ± 24	161 ± 12	143 ± 18	208 ± 13	212 ± 32	206 ± 36	199 ± 28
Heart (g)	1.34 ± 0.08	1.22 ± 0.11	1.32 ± 0.16	1.28 ± 0.11	0.82 ± 0.07	0.88 ± 0.06	0.78 ± 0.08	0.83 ± 0.08
(g%)	0.33 ± 0.01	0.33 ± 0.02	0.34 ± 0.03	0.34 ± 0.01	0.35 ± 0.02	0.39 ± 0.02*	0.37 ± 0.02	0.37 ± 0.02
Liver (g)	12.04 ± 1.17	11 ± 1.22	12.03 ± 1.54	11.52 ± 1.16	6.57 ± 0.43	6.59 ± 0.54	6.06 ± 0.66	6.42 ± 0.89
(g%)	2.97 ± 0.16	2.96 ± 0.16	3.08 ± 0.14	3.03 ± 0.15	2.83 ± 0.12	2.9 ± 0.13	2.86 ± 0.18	2.84 ± 0.19
Spleen (g)	0.81 ± 0.2	0.71 ± 0.12	0.71 ± 0.1	0.64 ± 0.01*	0.5 ± 0.03	0.48 ± 0.07	0.42 ± 0.07	0.44 ± 0.06
(g%)	0.20 ± 0.04	0.19 ± 0.02	0.18 ± 0.02	0.17 ± 0.01	0.22 ± 0.02	0.21 ± 0.03	0.2 ± 0.02	0.2 ± 0.02
Kidney (g)	2.9 ± 0.15	2.69 ± 0.18	2.84 ± 0.2	2.71 ± 0.1	1.81 ± 0.09	1.75 ± 0.16	1.67 ± 0.12	1.75 ± 0.14
(g%)	0.72 ± 0.03	0.73 ± 0.03	0.73 ± 0.03	0.72 ± 0.04	0.78 ± 0.03	0.77 ± 0.03	0.79 ± 0.03	0.78 ± 0.02
Adrenal (mg)	72 ± 12	59 ± 14	67 ± 9	62 ± 8	69 ± 11	61 ± 8	62 ± 7	71 ± 12
(mg%)	18 ± 3	16 ± 3	17 ± 3	16 ± 2	30 ± 4	27 ± 2	29 ± 2	32 ± 7
Testis (g)	3.35 ± 0.33	3.37 ± 0.33	3.29 ± 0.24	3.05 ± 0.42				
(g%)	0.82 ± 0.04	0.91 ± 0.08	0.85 ± 0.07	0.81 ± 0.11				
Epididymis (mg)	907 ± 66	911 ± 50	863 ± 67	809 ± 123				
(mg%)	224 ± 6	247 ± 22	223 ± 27	214 ± 32				
Ovary (mg)					87.4 ± 8.6	84.9 ± 4.5	81.3 ± 12.3	91.9 ± 11.6
(mg%)					37.7 ± 4.1	37.4 ± 1.6	38.5 ± 5.5	41.1 ± 6.7
Uterus (mg)					470 ± 143	334 ± 52	460 ± 107	407 ± 84
(mg%)					202 ± 56	148 ± 25	218 ± 45	180 ± 30

*: Significantly different from the control group ($p < 0.05$).
[a]: Values are given as the mean ± S.D.
[b]: Absolute organ weight.
[c]: Relative organ weight.

the end of the administration period (Table 5). In organ weight measurements, a significantly low absolute weight of the spleen was observed in males in the 12.5 mg/kg group at the end of the administration period (Table 6). However, there were no changes in the relative weight and histopathological examination of the spleen. A significantly high relative weight of the heart was observed in females in the 0.125 mg/kg group but not in the high dose groups at the end of the administration period. At necropsy, a dark red focus in the lung was observed sporadically in the control group and 12.5 mg/kg bw/day group at the end of the administration and recovery periods. At the histopathological examination, focal hemorrhage in the lung was observed in the animals showed a dark red focus. Blackish contents were observed in the cecum of animals in the treatment groups at the end of administration period. However, the histopathological examination revealed no effects in these organs. The contents in the intestines were not observed after the recovery period. Significant differences noted at the end of administration period all disappeared by the end of the recovery period.

A 28-day dose of MWCNT caused no death in both sexes. Black feces were observed in both sexes in all the treatment groups. No effects were found on detailed clinical observation, functional examination, body weight and food consumption (Table 7), organ weights, urinalysis and histopathology (Table 8). In the hematological examination, eosinophil counts were significantly low in females in the 5.0 and 50 mg/kg bw/day groups at the end of the administration period (Table 9) and in the 50 mg/ bw/day group at the end of recovery period ($1.13 \pm 0.38 \times 10^2/\mu l$ vs. $0.73 \pm 0.23 \times 10^2/\mu l$), but the changes were within the historical background data of the test facility ($0.9 \pm 0.86 \times 10^2/\mu l$). A few female animals incidentally showed high values for eosinophils in the control group, and a significant decrease in eosinophil counts was considered to be due to the high value of eosinophils in the control group. Therefore, it was not considered to be clinically

Table 7. Body weight and food consumption in rats dosed with MWCNT by gavage for 28 days

Dose	Male				Female			
(mg/kg bw/day)	0	0.5	5	50	0	0.5	5	50
Body weight (g)								
Day 1	183.7 ± 11.1	185.7 ± 9.7	186.7 ± 11.4	186.8 ± 8.4	149.7 ± 8.6	150.2 ± 10.8	150.5 ± 10.3	149.0 ± 10.3
Day 4	212.9 ± 13.3	213.2 ± 10.9	215.5 ± 14.0	215.0 ± 10.9	161.7 ± 9.9	161.8 ± 9.4	160.8 ± 10.7	159.1 ± 10.8
Day 7	242.0 ± 15.0	242.0 ± 14.4	244.5 ± 13.8	241.5 ± 13.0	172.0 ± 11.9	172.7 ± 15.4	172.0 ± 15.9	168.7 ± 13.3
Day 14	305.7 ± 18.5	304.5 ± 16.8	308.3 ± 17.5	305.3 ± 17.3	197.4 ± 12.3	193.0 ± 19.8	198.0 ± 19.9	194.0 ± 17.7
Day 21	362.4 ± 20.0	359.5 ± 17.2	367.2 ± 23.3	359.7 ± 24.1	219.7 ± 16.1	215.2 ± 22.1	218.8 ± 22.0	213.2 ± 19.9
Day 28	399.7 ± 24.1	392.2 ± 21.8	401.8 ± 30.0	394.5 ± 27.2	237.8 ± 18.7	227.7 ± 17.3	236.8 ± 23.2	230.4 ± 22.2
Food consumption (g/rat/day)								
Day 0 - 1	22.42 ± 2.02	22.00 ± 2.37	22.00 ± 1.67	22.58 ± 1.56	16.42 ± 2.19	16.83 ± 2.64	17.00 ± 1.26	17.50 ± 2.39
Day 1 - 4	23.33 ± 1.49	23.33 ± 1.45	23.90 ± 1.62	23.33 ± 1.12	16.76 ± 1.17	16.38 ± 0.98	16.17 ± 1.60	16.48 ± 1.68
Day 4 - 7	25.36 ± 1.75	24.95 ± 2.02	24.98 ± 1.55	24.65 ± 1.17	16.96 ± 1.39	16.77 ± 1.85	17.00 ± 2.31	17.10 ± 1.86
Day 7 - 14	26.94 ± 1.92	26.20 ± 1.62	26.53 ± 1.99	26.14 ± 1.18	18.27 ± 1.47	17.22 ± 1.02	17.92 ± 2.22	17.74 ± 1.82
Day 14 - 21	28.98 ± 1.42	28.25 ± 1.47	28.33 ± 3.34	27.69 ± 1.44	19.31 ± 1.96	18.50 ± 1.52	18.83 ± 2.33	18.62 ± 2.30
Day 21 - 28	29.09 ± 1.89	27.62 ± 2.14	28.18 ± 2.94	28.03 ± 2.26	19.66 ± 1.94	18.62 ± 1.86	19.20 ± 2.66	19.32 ± 2.51

Table 8. Histopathological findings of rats dosed with MWCNT by gavage for 28 days

Sex		Male				Female			
Dose (mg/kg bw/day)	Grade	0	0.5	5	50	0	0.5	5	50
Number of animals examined [a]		6	0	1	6	6	0	0	6
Lung	+								
Aggregation of alveolar macrophage	+	1	-	-	0	0	-	-	0
Mineralization of artery	+	0	-	-	0	1	-	-	0
Pancreas									
Atrophy of focal acinar cell	+	1	-	-	0	0	-	-	0
Ileum									
Diverticulum	+	0	-	1	0	1	-	-	0
Liver									
Microgranuloma	+	3	-	-	2	4	-	-	2
Kidney									
Tubular epithelium regeneration	+	2	-	-	1	0	-	-	0
Hyaline droplet in proximal tubular epithelium	+	0	-	-	1	0	-	-	0
Eosinophilic body in proximal tubular epithelium	+	0	-	-	1	0	-	-	0
Focal inflammatory cell infiltration in cortex	+	0	-	-	0	0	-	-	1

Grade; +: slight change. -: Not applicable.

[a] :The histopathological examination was carried out in 6 animals at 0 and 50 mg/kg bw/day and in animals which showed gross findings at 0.5 and 5 mg/kg bw/day.

or toxicologically important. Table 10 shows the results of serum biochemistry in rats dosed MWCNT. A significantly low γ-globulin fraction in females in the 50 mg/kg group was not accompanied by significant differences in other fractions, and no changes were noted in related parameters such as white blood cell counts. This was also considered to be due to a high value in the control group. Gamma-GTP in males in the 0.5 mg/kg group was significantly lower than that in the control group, but it was not dose-dependent. These changes in serum biochemistry were not observed at the end of the recovery period. Necropsy at the end of the administration period revealed swelling of the submandibular lymph node in one male in the 0.5 mg/kg group. This change was not dose-dependent and was not considered to be toxicologically important. Grayish green/dark green contents in the cecum, colon and/or rectum were observed in both sexes at 5.0 mg/kg bw/day and higher. These contents in the intes-

Table 9. Hematology in rats dosed with MWCNT by gavage for 28 days

Dose	Male				Female			
(mg/kg bw/day)	0	0.5	5	50	0	0.5	5	50
RBC ($\times 10^4/\mu l$)	878.8 ± 39.3	865.2 ± 27.7	862.8 ± 18.9	878.7 ± 35.5	879.3 ± 30.3	866.8 ± 48.2	855.8 ± 15.6	864.5 ± 24.0
Hemoglobin (g/dl)	17.20 ± 0.45	16.97 ± 0.56	17.12 ± 0.33	17.08 ± 0.28	16.77 ± 0.45	16.90 ± 0.82	16.50 ± 0.24	16.58 ± 0.43
Hematocrit (%)	48.57 ± 0.88	48.00 ± 1.56	48.72 ± 0.81	47.95 ± 1.09	46.13 ± 0.94	46.77 ± 1.94	45.98 ± 0.79	45.80 ± 1.45
MCV (fl)	55.33 ± 1.73	55.52 ± 1.89	56.47 ± 0.87	54.62 ± 1.86	52.48 ± 1.39	54.05 ± 2.16	53.73 ± 0.92	52.98 ± 1.12
MCH (pg)	19.58 ± 0.47	19.60 ± 0.41	19.85 ± 0.18	19.45 ± 0.68	19.07 ± 0.54	19.50 ± 0.68	19.30 ± 0.39	19.20 ± 0.37
MCHC (g/dl)	35.42 ± 0.40	35.35 ± 0.58	35.13 ± 0.30	35.63 ± 0.24	36.35 ± 0.38	36.12 ± 0.33	35.88 ± 0.40	36.22 ± 0.37
Reticulocyte (%)	3.683 ± 0.405	3.603 ± 0.448	3.545 ± 0.534	3.445 ± 0.434	2.907 ± 0.730	3.265 ± 0.431	3.013 ± 0.557	2.875 ± 0.644
WBC ($10^2/\mu l$)	128.57 ± 38.56	116.95 ± 15.35	116.48 ± 29.80	129.37 ± 27.96	91.07 ± 12.67	109.07 ± 27.58	88.83 ± 26.32	79.32 ± 18.52
Differential leukocyte counts ($\times 10^2/\mu l$)								
Lymphocyte	111.07 ± 35.91	100.13 ± 12.75	102.98 ± 27.80	111.30 ± 23.02	78.73 ± 11.18	95.97 ± 27.81	78.45 ± 26.20	68.18 ± 18.27
Neutrophil	12.63 ± 3.89	11.82 ± 2.65	9.95 ± 2.83	13.88 ± 5.17	9.32 ± 3.07	10.58 ± 4.12	8.48 ± 2.78	8.88 ± 4.68
Eosinophil	1.43 ± 1.02	1.67 ± 0.31	1.18 ± 0.64	1.07 ± 0.39	1.68 ± 0.89	1.08 ± 0.41	0.87 ± 0.33*	0.88 ± 0.34*
Basophil	0.07 ± 0.05	0.00 ± 0.00	0.03 ± 0.05	0.02 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.04	0.00 ± 0.00
Monocyte	3.37 ± 0.81	3.33 ± 0.76	2.33 ± 1.00	3.10 ± 0.88	1.33 ± 0.62	1.43 ± 0.48	1.02 ± 0.52	1.37 ± 1.18

RBC: Red blood cell; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; WBC: White blood cell.
*: Significantly different from the control group ($p < 0.05$).

Table 10. Serum biochemistry in rats dosed with MWCNT by gavage for 28 days

Dose (mg/kg bw/day)	Male				Female			
	0	0.5	5	50	0	0.5	5	50
T. protein (g/dl)	5.67 ± 0.18	5.65 ± 0.12	5.57 ± 0.23	5.70 ± 0.28	5.80 ± 0.32	5.65 ± 0.23	5.87 ± 0.21	5.75 ± 0.16
Glucose (mg/dl)	164.7 ± 8.2	162.2 ± 18.9	168.3 ± 16.8	173.5 ± 13.9	137.3 ± 17.3	134.5 ± 20.1	136.5 ± 13.4	138.8 ± 18.6
Triglyceride (mg/dl)	50.7 ± 11.5	48.7 ± 18.3	39.7 ± 13.5	44.7 ± 9.5	10.0 ± 2.5	11.2 ± 3.7	10.7 ± 4.1	11.2 ± 5.0
T. cholesterol (mg/dl)	59.5 ± 12.1	51.7 ± 9.3	56.8 ± 11.4	56.0 ± 10.1	56.0 ± 5.8	56.7 ± 9.0	60.8 ± 7.7	62.8 ± 13.7
BUN (mg/dl)	14.42 ± 2.12	13.53 ± 2.22	14.32 ± 1.57	15.22 ± 2.61	16.02 ± 1.36	17.22 ± 2.80	16.08 ± 4.45	15.77 ± 4.60
Creatinine (mg/dl)	0.497 ± 0.027	0.483 ± 0.023	0.502 ± 0.035	0.502 ± 0.022	0.505 ± 0.037	0.513 ± 0.043	0.507 ± 0.058	0.508 ± 0.045
T. bilirubin (mg/dl)	0.047 ± 0.008	0.038 ± 0.008	0.043 ± 0.008	0.040 ± 0.009	0.055 ± 0.012	0.063 ± 0.012	0.060 ± 0.021	0.057 ± 0.008
AST (IU/l)	67.3 ± 7.4	64.2 ± 7.7	66.3 ± 4.2	67.7 ± 10.0	69.3 ± 5.2	65.0 ± 4.7	61.5 ± 5.8	69.7 ± 11.6
ALT (IU/l)	26.2 ± 3.2	26.2 ± 2.6	25.7 ± 2.6	25.2 ± 2.6	20.3 ± 2.9	22.7 ± 2.4	21.2 ± 3.4	22.2 ± 4.4
ALP (IU/l)	688.8 ± 46.3	693.3 ± 122.5	614.2 ± 112.8	700.7 ± 171.2	423.8 ± 44.8	412.8 ± 91.3	333.2 ± 65.3	398.7 ± 138.4
γ-GTP (IU/l)	0.85 ± 0.21	0.62 ± 0.12*	0.68 ± 0.12	0.70 ± 0.14	1.22 ± 0.19	1.18 ± 0.12	0.95 ± 0.20	1.20 ± 0.27
Calcium (mg/dl)	10.18 ± 0.30	9.90 ± 0.14	9.93 ± 0.21	10.07 ± 0.27	9.88 ± 0.08	9.87 ± 0.46	10.00 ± 0.39	9.78 ± 0.43
I. phosphorus (mg/dl)	8.78 ± 0.49	8.67 ± 0.22	8.53 ± 0.48	8.77 ± 0.36	8.03 ± 0.42	8.02 ± 0.67	7.93 ± 0.38	7.70 ± 0.91
Sodium (mEq/l)	143.2 ± 1.2	143.2 ± 0.8	143.3 ± 0.8	143.8 ± 1.0	144.0 ± 1.4	143.5 ± 1.9	143.8 ± 1.5	144.3 ± 0.8
Potassium (mEq/l)	4.873 ± 0.268	4.923 ± 0.265	4.858 ± 0.117	4.762 ± 0.331	4.593 ± 0.372	5.108 ± 0.420	4.862 ± 0.351	4.755 ± 0.500
Chloride (mEq/l)	104.3 ± 1.9	105.0 ± 0.6	105.2 ± 1.2	105.0 ± 0.9	107.3 ± 2.1	107.2 ± 1.9	106.8 ± 1.9	106.7 ± 1.6
Albumin (%)	51.03 ± 2.11	51.93 ± 1.67	52.93 ± 1.25	53.50 ± 2.68	54.83 ± 2.31	55.53 ± 1.87	55.65 ± 1.94	55.88 ± 1.40
Alpha-1 globlin (%)	22.93 ± 3.10	23.35 ± 1.51	21.20 ± 2.81	21.47 ± 3.08	17.60 ± 2.54	17.93 ± 2.17	19.28 ± 2.38	19.32 ± 0.65
Alpha-2 globlin (%)	7.03 ± 0.34	6.65 ± 0.45	7.10 ± 0.51	6.98 ± 0.55	6.95 ± 0.77	7.45 ± 0.29	6.40 ± 0.75	6.57 ± 0.45
Beta globlin (%)	15.48 ± 0.98	14.72 ± 1.09	15.15 ± 1.1	14.48 ± 0.44	15.27 ± 0.85	14.57 ± 0.99	14.40 ± 1.11	14.35 ± 0.9
Gamma globlin (%)	3.52 ± 0.60	3.35 ± 0.63	3.62 ± 0.95	3.57 ± 0.43	5.35 ± 1.29	4.52 ± 0.76	4.27 ± 1.04	3.88 ± 0.67*
Albumin/Globlin	1.045 ± 0.089	1.083 ± 0.074	1.123 ± 0.057	1.153 ± 0.128	1.218 ± 0.115	1.252 ± 0.091	1.257 ± 0.101	1.268 ± 0.070

BUN: Blood urea nitrogen; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase.

*: Significantly different from the control group ($p < 0.05$).

tines were not observed after the recovery period. Based on the absence of toxicological effects, the no observed adverse effect levels (NOAELs) of repeated dose toxicity of SWCNT and MWCNT were considered to be 12.5 mg/kg bw/day and 50 mg/kg bw/day in rats (the highest dose tested), respectively.

Our findings showed no toxicological effects of orally administered CNTs, but available data show CNTs, under some conditions, may induce harmful effects. Both SWCNTs and MWCNTs showed the capacity to induce toxicity such as inflammation and fibrosis in the lung when administered by pharyngeal aspiration, intratracheal instillation or inhalation (Shvedova *et al.*, 2008; Erdely *et al.*, 2009; Inoue *et al.*, 2008; Pauluhn, 2010; Warheit *et al.*, 2004; Lam *et al.*, 2004; Han *et al.*, 2008). The carcinogenic potential of intraperitoneally administered MWCNT was observed in p53 heterozygous mice (Takagi *et al.*, 2008) and intrascrotal injection of MWCNT in intact Fischer 344 rats (Sakamoto *et al.*, 2009). The cytotoxic potential of CNTs was also observed in skin and lung cells *in vitro* (Jia *et al.*, 2005; Monteiro-Riviere *et al.*, 2005) although cytotoxicity can be influenced by various factors such as material impurities, length and size distribution, surface area, and so on (Hussain *et al.*, 2009). Intravenously injected CNTs increased oxidative stress markers in the lung and liver in mice (Yang *et al.*, 2008) or induced slight hepatotoxicity by inflammation and oxidative damage in mice (Ji *et al.*, 2009).

Deng *et al.* (2007) showed that intratracheally dosed taurine-functionalized MWCNT accumulated mainly in the lung and remained there until 28 days following administration whereas an oral gavage dose led to distribution in the small/large intestines and stomach, and about 74% was excreted in the feces after 12 hr. Intravenously injected taurine-functionalized MWCNT accumulated and remained until 28 days following administration in the liver, lung and spleen. The authors of this study suggested that taurine-functionalized-MWCNT cannot enter the blood circulation or be absorbed by the intestine tracts, and that routes of exposure influence the distribution of CNTs although the distribution pattern was for functionalized MWCNT. The findings of a study by Deng *et al.* (2007) together with our study suggested that SWCNTs and MWCNTs dosed by gavage reached the gastrointestinal tract as agglomerates and were rapidly excreted via feces in rats.

However, there is a study in which rats were dosed with SWCNT by gavage once at 0.64 mg/kg bw, and the levels of oxidatively damaged DNA increased in the liver and lung tissue (Folkmann *et al.*, 2009). Furthermore, significant increases in the number of resorptions, and fetal morphological and skeletal abnormalities, were observed in fetuses of CD-1 mouse dams that were administered functionalized CNTs by gavage at 10 mg/kg bw/day on day 9 of gestation (Philbrook *et al.*, 2011). Surface functionalization increases solubility of CNTs, and therefore absorbability of CNTs could have been increased in this study. However, these two studies suggested that CNTs could be absorbed from the gastro-intestinal tract into the blood circulation.

The findings of a study by Philbrook *et al.* (2011) are in discord from a study by Lim *et al.* (2011), in which pregnant SD rats were given MWCNTs by gavage at 0-1,000 mg/kg bw/day on days 6-19 of gestation, and no adverse effects were found in fetuses. Although controversial findings were observed in oral dose studies, an intravenous study obviously showed the teratogenic potential of CNTs (Pietroiusti *et al.*, 2011). Pregnant CD-1 mice were intravenously injected with SWCNT, oxidized-SWCNT and ultra oxidized-SWCNT at 0-30 μg/animal on day 5.5 of gestation. Increases in incidence of early miscarriages and fetal malformations were observed in all treatment groups at 0.1 μg/animal and higher. This study suggested that low-dose SWCNTs may induce fetal malformations. Our study group will undertake further teratogenic studies to confirm whether CNTs cause developmental toxicity.

In conclusion, rats were dosed with SWCNT or MWCNT once daily by gavage for 28 days with a 14-day recovery period, and no toxicological effects were found up to the highest dose tested. However, the possibility that CNTs dosed by oral administration are absorbed from the gastro-intestinal tract and persistent in the body for the long term cannot be ruled out, and a further study for chronic oral toxicity will be helpful to confirm the safety of CNTs. Because of the small size of CNTs, particles may spread over the entire body, and causing adverse effects like observed in intravenous studies, once absorbed.

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REFERENCES

Deng, X., Jia, G., Wang, H., Sun, H., Wang, X., Yang, S., Wang, T. and Liu, Y. (2007): Translocation and fate of multi-walled carbon nanotubes in vivo. Carbon, **45**, 1419-1424.

Elgrabli, D., Abella-Gallart, S., Robidel, F., Rogerieux, F., Boczkowski, J. and Lacroix, G. (2008): Induction of apoptosis and absence of inflammation in rat lung after intratracheal instillation of multiwalled carbon nanotubes. Toxicology, **253**, 131-136.

Erdely, A., Hulderman, T., Salmen, R., Liston, A., Zeidler-Erdely, P.C., Schwegler-Berry, D., Castranova, V., Koyama, S., Kim, Y.A., Endo, M. and Simeonova, P.P. (2009): Cross-talk between lung and systemic circulation during carbon nanotube respiratory exposure. Potential biomarkers. Nano Lett., **9**, 36-43.

Folkmann, J.K., Risom, L., Jacobsen, N.R., Wallin, H., Loft, S. and Moller, P. (2009): Oxidatively damaged DNA in rats exposed by oral gavage to C60 fullerenes and single-walled carbon nanotubes. Environ. Health. Perspect., **117**, 703-708.

Han, S.G., Andrews, R., Gairola, C.G. and Bhalla, D.K. (2008): Acute pulmonary effects of combined exposure to carbon nanotubes and ozone in mice. Inhal. Toxicol., **20**, 391-398.

Hussain, M.A., Kabir, M.A. and Soood, A.K. (2009): On the cytotoxicity of carbon nanotubes. Current Science, **96**, 664-673.

Inoue, K., Koike, E., Yanagisawa, R., Hirano, S., Nishikawa, M. and Takano, H. (2009): Effects of multi-walled carbon nanotubes on a murine allergic airway inflammation model. Toxicol. Appl. Pharmacol., **237**, 306-316.

Inoue, K., Takano, H., Koike, E., Yanagisawa, R., Sakurai, M., Tasaka, S., Ishizaka, A. and Shimada, A. (2008): Effects of pulmonary exposure to carbon nanotubes on lung and systemic inflammation with coagulatory disturbance induced by lipopolysaccharide in mice. Exp. Biol. Med. (Maywood), **233**, 1583-1590.

Ji, Z., Zhang, D., Li, L., Shen, X., Deng, X., Dong, L., Wu, M. and Liu, Y. (2009): The hepatotoxicity of multi-walled carbon nanotubes in mice. Nanotechnology, **20**, 445101.

Jia, G., Wang, H., Yan, L., Wang, X., Pei, R., Yan, T., Zhao, Y. and Guo, X. (2005): Cytotoxicity of carbon nanomaterials: single-wall nanotube, multi-wall nanotube, and fullerene. Environ. Sci. Technol., **39**, 1378-1383.

Kobayashi, N., Ogura, I., Gamo, M., Kishimoto, A. and Nakanishi, J. (2009): Risk Assessment of Manufactured Nanomaterials -Carbon Nanotubes (CNTs)- Interim Report issued on October 16, 2009 Exective Summary. http://goodnanoguide.org/tiki-download_wiki_attachment.php?attId=31

Lam, C.W., James, J.T., McCluskey, R. and Hunter, R.L. (2004): Pulmonary toxicity of single-wall carbon nanotubes in mice 7 and 90 days after intratracheal instillation. Toxicol. Sci., **77**, 126-134.

Lim, J.H., Kim, S.H., Shin, I.S., Park, N.H., Moon, C., Kang, S.S., Kim, S.H., Park, S.C. and Kim, J.C. (2011): Maternal exposure to multi-wall carbon nanotubes does not induce embryo–fetal developmental toxicity in rats. Birth. Defects. Res. B Dev. Reprod. Toxicol., **92**, 69-76.

Magnuson, B.A., Jonaitis, T.S. and Card, J.W. (2011): A Brief Review of the occurrence, use, and safety of food-related nanomaterials. J. Food Sci., **76**, R126-R133.

MHLW, J. (2010): The results of "An Investigative Research on Consumer's Safety in Utilizing Nanomaterials (2009)". www.nihs.go.jp/mhlw/chemical/nano/nanopdf/H21houkoku/honbun/all.pdf

Monteiro-Riviere, N.A., Nemanich, R.J., Inman, A.O., Wang, Y.Y. and Riviere, J.E. (2005): Multi-walled carbon nanotube interactions with human epidermal keratinocytes. Toxicol. Lett., **155**, 377-384.

OECD (2010a): DRAFT DOSSIER DEVELOPMENT PLAN: Multi- walled Carbon Nanotubes (MWCNTs), April 28, 2010.

OECD (2010b): DRAFT DOSSIER DEVELOPMENT PLAN: Single- walled Carbon Nanotubes (SWCNTs), April 15, 2010.

OECD (2011): Sponsorship Programme for the Testing of Manufactured Nanomaterials. http://www.oecd.org/document/47/0,3746,en_2649_37015404_41197295_1_1_1_1,00.html

Pauluhn, J. (2010): Subchronic 13-week inhalation exposure of rats to multiwalled carbon nanotubes: toxic effects are determined by density of agglomerate structures, not fibrillar structures. Toxicol. Sci., **113**, 226-242.

Philbrook, N.A., Walker, V.K., Afrooz, A.R., Saleh, N.B. and Winn, L.M. (2011): Investigating the effects of functionalized carbon nanotubes on reproduction and development in Drosophila melanogaster and CD-1 mice. Reprod. Toxicol., **32**, 442-448

Pietroiusti, A., Massimiani, M., Fenoglio, I., Colonna, M., Valentini, F., Palleschi, G., Camaioni, A., Magrini, A., Siracusa, G., Bergamaschi, A., Sgambato, A. and Campagnolo, L. (2011): Low doses of pristine and oxidized single-wall carbon nanotubes affect mammalian embryonic development. ACS Nano, **5**, 4624-4633.

Sakamoto, Y., Dai, N., Hagiwara, Y., Satoh, K., Ohashi, N., Fukamachi, K., Tsuda, H., Hirose, A., Nishimura, T., Hino, O. and Ogata, A. (2010): Serum level of expressed in renal carcinoma (ERC)/ mesothelin in rats with mesothelial proliferative lesions induced by multi-wall carbon nanotube (MWCNT). J. Toxicol. Sci., **35**, 265-270.

Sakamoto, Y., Nakae, D., Fukumori, N., Tayama, K., Maekawa, A., Imai, K., Hirose, A., Nishimura, T., Ohashi, N. and Ogata, A. (2009): Induction of mesothelioma by a single intrascrotal administration of multi-wall carbon nanotube in intact male Fischer 344 rats. J. Toxicol. Sci., **34**, 65-76.

Shvedova, A.A., Kisin, E., Murray, A.R., Johnson, V.J., Gorelik, O., Arepalli, S., Hubbs, A.F., Mercer, R.R., Keohavong, P., Sussman, N., Jin, J., Yin, J., Stone, S., Chen, B.T., Deye, G., Maynard, A., Castranova, V., Baron, P.A. and Kagan, V.E. (2008): Inhalation vs. aspiration of single-walled carbon nanotubes in C57BL/6 mice: inflammation, fibrosis, oxidative stress, and mutagenesis. Am. J. Physiol. Lung Cell. Mol. Physiol., **295**, L552-565.

Takagi, A., Hirose, A., Nishimura, T., Fukumori, N., Ogata, A., Ohashi, N., Kitajima, S. and Kanno, J. (2008): Induction of mesothelioma in p53+/- mouse by intraperitoneal application of multi-wall carbon nanotubes. J. Toxicol. Sci., **33**, 105-116.

Takahashi, M., Kato, H., Doi, Y., Hagiwara, A., Hirata-Koizumi, M., Ono, A., Kubota, R., Nishimura, T. and Hirose, A. (2012) Sub-acute oral toxicity study with fullerene C60 in rats. J. Toxicol. Sci., **37**, 353-61.

Warheit, D.B., Laurence, B.R., Reed, K.L., Roach, D.H., Reynolds, G.A. and Webb, T.R. (2004): Comparative pulmonary toxicity assessment of single-wall carbon nanotubes in rats. Toxicol. Sci., **77**, 117-125.

Yang, S.T., Wang, X., Jia, G., Gu, Y., Wang, T., Nie, H., Ge, C., Wang, H. and Liu, Y. (2008): Long-term accumulation and low toxicity of single-walled carbon nanotubes in intravenously exposed mice. Toxicol. Let., **181**, 182-189.

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Original Article

Sub-acute oral toxicity study with fullerene C60 in rats

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ABSTRACT — To obtain initial information on the possible repeated-dose oral toxicity of fullerene C60, Crl:CD(SD) rats were administered fullerene C60 by gavage once daily at 0 (vehicle: corn oil), 1, 10, 100, or 1,000 mg/kg/day for 29 days, followed by a 14-day recovery period. No deaths occurred in any groups, and there were no changes from controls in detailed clinical observations, body weights, and food consumption in any treatment groups. Moreover, no treatment-related histopathological changes were found in any organs examined at the end of the administration period and at the end of the recovery period. Blackish feces and black contents of the stomach and large intestine were observed in males and females at 1,000 mg/kg/day in the treatment group. There were no changes from controls in the liver and spleen weights at the end of the administration period, but those weights in males in the 1,000 mg/kg/day group increased at the end of the recovery period. Using liquid chromatography-tandem mass spectrometry, fullerene C60 were not detected in the liver, spleen or kidney at the end of the administration period and also at the end of the recovery period. In conclusion, the present study revealed no toxicological effects of fullerene C60; however, the slight increases in liver and spleen weights after the 14-day recovery period may be because of the influence of fullerene C60 oral administration. In the future, it will be necessary to conduct a long-term examination because the effects of fullerene C60 cannot be ruled out.

Key words: Fullerene C60, Gavage, Rat, Repeated dose toxicity

INTRODUCTION

Since the publication of a paper on fullerenes in 1985 (Kroto *et al.*, 1985), the application of fullerenes has been considered due to their fascinating properties, such as substituent modification, endohedrality, and superconductivity. The production and use of fullerenes in the market is limited at present, but is expected to grow significantly (Aschberger *et al.*, 2010), and the potential of general public exposure as well as occupational exposure at manufacturing sites to pristine fullerene (fullerene C60) will increase in the future.

The main exposure routes of fullerene C60 in the occupational setting are considered to be inhalation and dermal contact. Aschberger *et al.* (2010) summarized as follows; fullerenes have low acute and sub-chronic inhalation toxicity, and as for dermal toxicity, fullerenes did not induce acute toxic effects to the skin, and no long term dermal studies were available.

In the general population, the possible exposure routes of concern include oral exposure; there is a possibility of oral intake by contamination of food and drinking water with fullerene C60 and from fullerene C60-containing products that the consumer touches directly. Moreover, in workers who inhale fullerene C60, it could also be taken up via the gastrointestinal tract because nanosized particles cleared from the respiratory tract via the mucociliary escalator can subsequently be ingested into the GI tract (Oberdörster *et al.*, 2005).

There are three acute oral dose toxicity tests for fullerenes available. In an acute oral toxicity test of fullerene C60 using an *in vivo* micronucleus test carried out with male and female mice at doses of 20-78 mg/kg, no mice died and no abnormalities were detected (Shinohara *et*

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al., 2009); in an acute oral toxicity test of the mixture of fullerenes C60 and C70 with male and female rats at a dose of 2,000 mg/kg, no deaths or abnormalities were observed in any rats and the body weights of both sexes in the treated group increased in a similar pattern to the control group (Mori *et al.*, 2006); and in an acute oral toxicity test of water-soluble polyalkylsulfonated C60 with female rats at a dose of 2,500 mg/kg, no deaths occurred (Chen *et al.*, 1998). From these outcomes of acute oral studies, it can be concluded that the acute oral toxicity of fullerenes is very low; however, no information on repeated oral dosing tests of fullerenes is available.

In the present study, an oral repeated dose toxicity study of pristine fullerene C60 was conducted according to the test guidelines. In addition, we measured the amount of fullerene C60 in the liver, spleen, and kidney using liquid chromatography-tandem mass spectrometry (LC-MS/MS) after administration of fullerene C60. We report and discuss the results of the study.

MATERIALS AND METHODS

The present study was conducted in 2010-2011 at DIMS Institute of Medical Science, Inc. (Aichi, Japan). The study design complied with the Test Guideline of the Japanese Chemical Control Act (law concerning examination and regulation of manufacture, etc., of chemical substances), "Twenty-eight-day Repeated Dose Toxicity Test in Mammalian Species" (EA *et al.*, 1986). All procedures involving the use and care of animals were performed in accordance with the principles for Good Laboratory Practice (MOE *et al.*, 2003) and "Standards Relating to the Care, Management of Laboratory Animals and Relief of Pain" (MOE, 2006). This experiment was approved by the institutional animal care and use committee of DIMS Institute of Medical Science.

Chemicals and reagents

Fullerene C60 (Nanom Purple SU, 0.71 nm in diameter, black powder. CAS No. 99685-96-8) was obtained from Frontier Carbon Corp. (Fukuoka, Japan). The fullerene C60 (lot no. 10B0098-A) used in the present study was 99.9% pure and was kept at room temperature (17-22°C) in a dark place. Corn oil, as a vehicle, was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). All other reagents used in present study were specific purity grade.

Animals

Crl:CD(SD) male and female rats (4 weeks old) were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). All animals were maintained in an air-conditioned room at 20.0-22.5°C, with a relative humidity of 48-62%, a 12-hr light/dark cycle, and ventilation with at least 10 air changes per hour. They were housed one or two of the same sex per cage in plastic cages with stainless steel covers.

A basal diet (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water were provided *ad libitum*. Male and female rats were assigned to each dose group by stratified random sampling based on body weight. The initial numbers of rats were 10/sex in the control and the highest dose group, and 5/sex in other dose groups. After 7-day acclimation, they were subjected to treatment at 5 weeks of age.

Administration

The dosage levels were determined based on the guideline and the maximum dose was 1,000 mg/kg/day. The lowest dose was set at 1 mg/kg/day (concentration of fluid: 0.1 mg/ml) based on the solubility of fullerene C60 in olive oil being approximately 0.1 mg/ml (Yamakoshi, 1999). The intermediate doses were selected as 100 and 10 mg/kg/day with a proportional factor of 10.

Fullerene C60 was weighed for each dosing level and the vehicle (corn oil) was added. Each dosing fluid including that for the vehicle control was sonicated 3 times (for 5 min each) in a beaker cooled with ice. Sonication was performed at 5- to 10-min intervals after it was confirmed that the fluid was sufficiently cool. The dosing fluids were prepared from 1 p.m. to 5 p.m. on the day before each administration day, and mixed using a stirrer at room temperature (17-22°C) in a dark place until just before administration.

For each fluid dose, samples collected from the upper, central, and lower parts of the glass container were observed and photographed under an optical microscope on the first and last day of the administration period. All doses, even the lowest dose of 0.1 mg/ml, did not completely dissolve in corn oil, and included visible and invisible residues which could be seen at 40 x magnification, although we assumed the lowest dose as completely soluble. Photographs of samples of each dosage looked similar between the first and last day. Typical microscopic photographs of samples on the last day are shown in Fig. 1. Black particles shown in Fig. 1 are aggregated fullerene C60. Administration was by oral dosage at 10 ml/kg using a disposable syringe and a disposable gastric tube. The dosing volume was adjusted by the latest body weight of each rat.

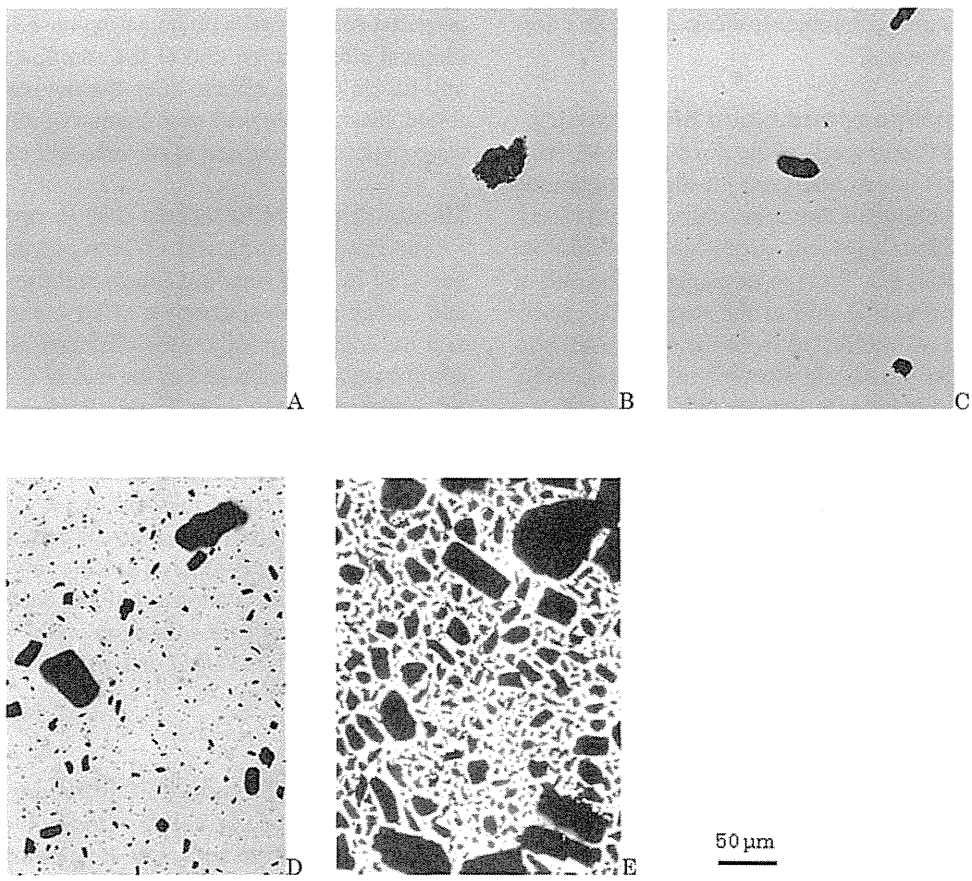

Fig. 1. Typical microscopic photographs of each dosage fluid on the last day of the administration period, with a single scale bar for all microscopic photographs. (A) 0 mg/kg/day, vehicle, (B) 1 mg/kg/day, (C) 10 mg/kg/day, (D) 100 mg/kg/day, and (E) 1,000 mg/kg/day. Black particles are aggregated fullerene C60. No staining, original magnification × 40.

Experimental design

Rats were given fullerene C60 by gavage once daily at 0 (vehicle control), 1, 10, 100, or 1,000 mg/kg/day for 29 days. On the day after the last dosing, five males and five females from each group were euthanized for the assessment of hematology, blood biochemistry, organ weights, and macroscopic and microscopic findings. The respective remaining five rats/sex at 0 and 1,000 mg/kg/day were kept without treatment for 14 days as a recovery period and then fully examined.

Daily observation and a functional observation battery

All animals were observed at least twice daily for clinical signs of toxicity in their cage. A functional observation battery (FOB), including observations in hands: ease of removal, respiration, salivation, nose secretion, lacrimation, exophthalmos, ptosis, eyeball opacity, skin, soiled perineal region, handling reactivity, and open field observations: exploration, gait, behavior, posture, fur, twitch, convulsion, tremor, rearing, defecation, urination, was conducted once a week during treatment, and sensory reactivity to stimuli of different types (reactivity to sensory stimulation: visual, auditory, tactile, and nociceptive; cranial nerve reflexes: palpebral reflex, pinna reflex, and papillary reflex; spinal reflexes: flexor reflex and extensor thrust reflex; postural reaction: proprioceptive positioning reaction; righting reactions: surface righting reaction and aerial righting reaction; and landing foot splay), grip strength (fore/hind limb), and motor activity (DAS system, model DAS-008; Neuroscience, Inc., Tokyo, Japan), once during the fourth week of treatment. Body weight was recorded on days 0, 7, 14, 21, and 28 of the dosing period and days 6 and 13 of the recovery period. Food

consumption was measured once a week during the dosing and recovery periods.

Urinalysis, hematology and blood biochemistry

One day in the fourth week of the dosing period, urine was collected for 4 hr and analyzed for dipstick parameters, such as the volume of the urine, color, occult blood, ketone bodies, glucose, protein, urobilinogen, bilirubin, specific gravity, and pH. Prior to necropsy at the end of the administration period and at the end of the recovery period, blood was collected from the abdominal aorta under deep ether anesthesia after overnight starvation. One portion of the blood was treated with EDTA-2K and examined for hematologic parameters such as red blood cell count, hemoglobin, hematocrit, white blood cell count, platelet count, and differential leukocyte count. Another blood sample was treated with sodium citrate, and blood clotting parameters, such as prothrombin time and activated partial thromboplastin time, were examined. Serum from one remaining portion of blood was analyzed for blood biochemistry [total protein, albumin, albumin-globulin ratio, glucose, total cholesterol, triglycerides, total bilirubin, urea nitrogen, creatinine, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma-glutamyl transpeptidase, lactate dehydrogenase, phospholipid, calcium, inorganic phosphorus, sodium, potassium, chlorine]. Serum from the remaining portion of blood was analyzed for levels of triiodothyronine, thyroxine, and thyroid stimulating hormone at Bozo Research Center Inc. (Shizuoka, Japan).

Organ weights and histopathological analysis

After blood collection, all animals were sacrificed by exsanguination, and the surface and cavity of the body and the organs and tissues of the entire body were observed macroscopically. The pituitary, thymus, thyroids (including parathyroids), heart, liver, spleen, kidneys, adrenals, testes, epididymides, uterus, and ovaries were then removed and weighed (after formalin fixation of the pituitary and thyroids). The trachea, lungs (including bronchus), lymph nodes (mandibular, mesenteric, and axillary), stomach, duodenum, jejunum, ileum, cecum, colon, rectum, urinary bladder, eyeballs, mammary gland (male), brain, spinal cord (cervical, pectoral, and lumbar part), sciatic nerve, prostates, bone marrow (femur) as well as the above organs were fixed in 10% neutral-buffered formalin phosphate (after Bouin fixation for testes and epididymides). Histopathological examination was conducted for all of these organs of the control and the highest dose groups at the end of the administration period. Paraffin sections for microscopic examination were routinely prepared and stained with hematoxylin-eosin. If any pathological effects at the end of the administration period or any toxicological effects after the recovery period were found, histopathological examination of related organs was also conducted at the end of the recovery period.

Measurement of fullerene C60 in organs

For the determination of concentrations of fullerene C60 in liver (median lobe), right and left kidneys, and spleen samples, samples of all males in the control and the highest groups were obtained at the end of the administration period and at the end of the recovery period, weighed, frozen with liquid nitrogen, and stored in a deep freezer (-80 to -74°C) until used. The mean values of the wet weight of organs were 0.1 g (spleen) to 0.4 g (liver). The analytical method of LC-MS/MS and the extraction procedure from tissues of experimental animals were as reported previously (Kubota *et al.*, 2009, 2011), and C70 was used as an internal standard for quantification. The detection limits for each organ were 0.102 μg/g wet wt. (liver), 0.146 μg/g wet wt. (kidneys), and 0.587 μg/g wet wt. (spleen).

Data analysis

Parametric data, such as FOB findings, body weight, food consumption, urinalysis findings (except for the results of qualitative analysis), hematological and blood biochemical findings, serum hormone level, and organ weights, were analyzed by Bartlett's test (Bartlett, 1937) for homogeneity of distribution. If homogenous, Dunnett's test (Dunnett, 1964) was conducted and, if not homogenous, Steel's multiple comparison test (Steel, 1959) was conducted to compare control and individual treatment groups. For two groups, parametric data were analyzed by the F-test (Snedecor and Cochran, 1967) for homogeneity of distribution. If homogenous, Student's t-test (Steel and Torrie, 1980) was conducted and, if not homogenous, Aspin-Welch's t-test (Snedecor and Cochran, 1967) was conducted for comparison. For significant differences in the incidences of FOB, urinalysis, and histopathological findings, Fisher's exact test (Fisher, 1973) was performed, and the grade of lesions was compared using the Mann-Whitney U-test (Mann and Whitney, 1947). A 5% level of probability was used as the criterion for significance.

RESULTS

No deaths or clinical signs of toxicity occurred in any groups. In general appearance, blackish feces were observed in males and females at 1,000 mg/kg/day from

dosing day 4 to the end of the administration period, and from day 0 to day 1 of the recovery period. In the detailed clinical observation, the number of urinations was significantly increased at 1 mg/kg/day in females on one day in week 2, and the number of defecations was significantly decreased at 1,000 mg/kg/day in males on one day in week 3, but these were not persistent changes. There were no changes from controls in the manipulation test, grip strength, motor activity, body weight, and food consumption.

In urinalysis at the end of the administration period, only an increase in the number of positive incidences of ketone bodies was observed at 10 and 1,000 mg/kg/day in males (data not shown). In the hematological examination, a decrease in the differential lymphocyte ratio and an increase in the differential eosinophil ratio were observed at 10 mg/kg/day in males at the end of the administration period, but not at the end of the recovery period (data not shown). Blood chemistry results are shown in Table 1. An increase in creatinine at 100 mg/kg/day in males, and a decrease in albumin at 1,000 mg/kg/day in males were observed only at the end of the administration period, and an increase in total protein was observed in females only at the end of the recovery period. No changes from controls were found in serum levels of triiodothyronine, thyroxine, and thyroid stimulating hormone.

At necropsy, black contents of the stomach and large intestine were observed in all animals at 1,000 mg/kg/day at the end of the administration period, but not at the end of the recovery period. No other macroscopic changes were observed in all treated animals at the end of the administration period and at the end of the recovery period. Body and organ weights at the end of the administration period or the recovery period are shown in Table 2. An increase in relative thymus weight at 100 mg/kg/day in females and a decrease in relative kidney weight at 1,000 mg/kg/day in males were observed at the end of the administration period, but not at the end of the recovery period. Increases in absolute and relative liver weights and absolute spleen weight were observed in males in the treatment group only at the end of the recovery period. Histopathological findings are shown in Table 3. There were no changes from controls in all organs examined at the end of the administration period and also no changes in the liver and spleen of males examined in the recovery period. In the analysis using LC-MS/MS, the contents of fullerene C60 were under the detection limit in all samples of the liver, kidneys, and spleen at the end of the administration period and at the end of the recovery period (data not shown).

DISCUSSION

The present study was conducted to obtain initial information on the possible repeated-dose toxicity of fullerene C60 in rats. There were no treatment-related effects during and at the end of the administration period. Although there were statistically significant differences in the following findings at the end of the administration period, they were not considered to be toxicological because there was no dose-dependency; an increase in the number of positive incidences of urine ketone bodies in males, a decrease in the differential lymphocyte ratio and an increase in the differential eosinophil ratio in males in hematology, an increase in serum creatinine in males, and an increase in relative thymus weight in females. As for decreases in serum albumin and the relative kidney weight at 1,000 mg/kg/day in males at the end of the administration period, and also an increase in total protein at 1,000 mg/kg/day in females at the end of the recovery period, their toxicological significance remains to be elucidated in the present study.

Blackish feces and black contents of the stomach and large intestine observed in the present study were considered to result from the administered fullerene C60 itself. Blood clots cannot be included in these blackish feces and black contents because there were no necropsy and histopathological findings including bleeding and erosion in the gastrointestinal tract. It would appear that a large amount of fullerene C60 passed through the gastrointestinal tract without significant absorption. Fullerene C60 which had not dissolved in vehicle was considered to mix with food in the gut, and to have been excreted outside of the body.

In a recent solubility study of fullerenes in natural oils and animal fats (Semenov *et al.*, 2009), the solubility of fullerene C60 in corn oil was 0.6 mg/ml at 20°C. In the present study, however, 0.1 mg/ml fullerene C60 did not completely dissolve in corn oil. This difference in the dissolution amount of fullerene C60 may be due to differences in the materials and methods used.

Regarding the absorption of water-soluble fullerene synthesized using dipolar trimethylenemethane administered orally to male rats, virtually all radioactivity (97%) was excreted in the feces, and trace amounts of fullerene derivatives were identified in the urine (less than 3%) (Yamago *et al.*, 1995). This result shows at least that dissolved fullerene can be absorbed from the intestines. Moreover, absorption of pristine fullerene C60 administered orally to female rats was suggested because levels of 8-oxo-2'-deoxyguanosine, one of the products of DNA oxidation, in the liver and lung are higher than those of

Table 1. Principal blood biochemical values in male and female rats given fullerene C60 by gavage

Dose (mg/kg/day)	At the end of the administration period					At the end of the recovery period	
	0	1	10	100	1000	0	1000
Male							
No. of animals	5	5	5	5	5	5	5
AST (U/l)	88.4 ± 20.2	92.8 ± 29.4	72.8 ± 13.8	80.6 ± 10.5	64.6 ± 11.6	127.8 ± 29.2	100.6 ± 34.9
ALT (U/l)	33.8 ± 4.1	33.4 ± 4.2	33.4 ± 6.1	33 ± 8.4	32.6 ± 7.4	33.6 ± 5.9	31 ± 4.9
ALP (U/l)	588.2 ± 114.1	634.8 ± 67	649.6 ± 100.3	564 ± 101.4	610.4 ± 134.2	423.4 ± 75.9	410 ± 43.7
γ-GTP (U/l)	0.58 ± 0.28	0.42 ± 0.13	0.5 ± 0.19	0.6 ± 0.07	0.66 ± 0.34	0.58 ± 0.22	0.4 ± 0.34
Lactate dehydrogenase (U/l)	127.6 ± 32.6	142.2 ± 34.4	121.8 ± 47.2	133.6 ± 50.2	113.2 ± 15.8	184.4 ± 40.4	161.4 ± 66.5
Urea nitrogen (mg/dl)	9.44 ± 1.21	10.22 ± 1.14	9.78 ± 1.67	10.8 ± 2.16	9.6 ± 1.44	13.52 ± 1.32	12.84 ± 0.67
Creatinine (mg/dl)	0.232 ± 0.044	0.282 ± 0.022	0.268 ± 0.035	0.32 ± 0.049**	0.27 ± 0.019	0.286 ± 0.029	0.27 ± 0.016
Glucose (mg/dl)	145.8 ± 21.4	150.6 ± 15.8	149.4 ± 15.9	155.8 ± 30.7	165.2 ± 10.7	138.6 ± 8	145 ± 27.3
Total cholesterol (mg/dl)	50.8 ± 10.5	48.8 ± 11	55.4 ± 6.2	59.8 ± 7.6	55 ± 12.1	55.4 ± 10.7	65.8 ± 17
Phospholipid (mg/dl)	93.2 ± 15.3	88 ± 13.8	101.2 ± 8.8	106 ± 7.6	101 ± 16.2	92 ± 13.4	106 ± 20.5
Triglycerides (mg/dl)	59.4 ± 22.5	47.6 ± 9.2	54.2 ± 5.2	36.6 ± 9.3	66.4 ± 28.2	38.6 ± 16.4	64.6 ± 34.2
Total protein (g/dl)	5.78 ± 0.08	5.72 ± 0.11	5.52 ± 0.13	5.76 ± 0.27	5.68 ± 0.13	5.94 ± 0.23	6.06 ± 0.17
Albumin (g/dl)	2.52 ± 0.13	2.46 ± 0.05	2.38 ± 0.11	2.42 ± 0.11	2.34 ± 0.11*	2.42 ± 0.08	2.44 ± 0.11
A/G	0.774 ± 0.054	0.758 ± 0.036	0.76 ± 0.06	0.73 ± 0.064	0.704 ± 0.057	0.69 ± 0.023	0.674 ± 0.027
Female							
No. of animals	5	5	5	5	5	5	5
AST (U/l)	115.6 ± 35.4	119.4 ± 26.5	122 ± 28.6	108.6 ± 30.2	99.4 ± 40.2	109.8 ± 28.9	130.4 ± 58.9
ALT (U/l)	31.6 ± 6.8	35.8 ± 6.3	34.8 ± 10.5	30.4 ± 9.4	33.4 ± 11	29 ± 4	35 ± 21.8
ALP (U/l)	533.2 ± 238.9	399.4 ± 99	432.4 ± 86.1	343.4 ± 48.6	379.4 ± 45.9	270.8 ± 14	301 ± 42.3
γ-GTP (U/l)	0.7 ± 0.32	0.78 ± 0.16	0.8 ± 0.14	0.64 ± 0.15	0.66 ± 0.25	0.74 ± 0.18	0.8 ± 0.48
Lactate dehydrogenase (U/l)	177.6 ± 73	143.2 ± 41.4	169.6 ± 47.5	150.6 ± 15.3	168.6 ± 11.7	144.6 ± 31.7	129.2 ± 44.9
Urea nitrogen (mg/dl)	10.88 ± 1.29	11.98 ± 2.08	11.32 ± 1.88	12.66 ± 1.27	12.84 ± 1.44	16.78 ± 2.47	17.9 ± 1.67
Creatinine (mg/dl)	0.31 ± 0.029	0.322 ± 0.041	0.316 ± 0.029	0.326 ± 0.032	0.318 ± 0.029	0.352 ± 0.022	0.352 ± 0.013
Glucose (mg/dl)	132.6 ± 13	124.6 ± 21.1	133.2 ± 16.1	139 ± 15.5	138.8 ± 18	124 ± 10.4	130.4 ± 5.9
Total cholesterol (mg/dl)	56.4 ± 15.8	61.4 ± 6	56 ± 8.2	65.8 ± 12.4	60.4 ± 8.8	78 ± 8.2	75.6 ± 13.6
Phospholipid (mg/dl)	107.8 ± 25.6	111.4 ± 11.1	104.6 ± 13.3	125.6 ± 18.6	116.2 ± 14.4	141.2 ± 15.1	142 ± 25.2
Triglycerides (mg/dl)	25.8 ± 14.9	21.2 ± 12.6	19 ± 7	20.8 ± 6.9	16.2 ± 11.2	26 ± 8.4	35.6 ± 14.8
Total protein (g/dl)	5.76 ± 0.21	5.82 ± 0.31	5.78 ± 0.24	5.94 ± 0.15	5.92 ± 0.11	6.06 ± 0.21	6.32 ± 0.11*
Albumin (g/dl)	2.56 ± 0.05	2.58 ± 0.18	2.54 ± 0.11	2.7 ± 0.19	2.72 ± 0.11	2.64 ± 0.09	2.72 ± 0.13
A/G	0.802 ± 0.037	0.8 ± 0.089	0.784 ± 0.03	0.836 ± 0.081	0.85 ± 0.05	0.776 ± 0.081	0.756 ± 0.062

Values are expressed as the mean ± S.D. Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γ-GTP, gamma-glutamyl transpeptidase; A/G, albumin-globulin ratio. *: Significantly different from control group at $p < 0.05$. **: Significantly different from control group at $p < 0.01$.

Table 2. Principal organ weights of male and female rats given fullerene C60 by gavage

Dose (mg/kg/day)	At the end of the administration period					At the end of the recovery period	
	0	1	10	100	1000	0	1000
Male							
No. of animals	5	5	5	5	5	5	5
Body weight[a] (g)	415.0 ± 39.0	426.6 ± 36.8	408.6 ± 32.8	424.4 ± 50.8	422.2 ± 39.4	454.4 ± 49.4	485.6 ± 19.3
Thymus (g)	0.54 ± 0.16	0.45 ± 0.09	0.50 ± 0.10	0.51 ± 0.13	0.50 ± 0.13	0.46 ± 0.08	0.46 ± 0.11
	(0.130 ± 0.032)[b]	(0.104 ± 0.017)	(0.121 ± 0.024)	(0.119 ± 0.016)	(0.117 ± 0.025)	(0.100 ± 0.015)	(0.095 ± 0.026)
Liver (g)	12.80 ± 1.93	13.33 ± 1.64	11.93 ± 0.71	13.96 ± 2.95	12.98 ± 1.39	12.17 ± 1.27	13.99 ± 1.15*
	(3.076 ± 0.288)	(3.123 ± 0.253)	(2.926 ± 0.136)	(3.274 ± 0.385)	(3.073 ± 0.121)	(2.681 ± 0.113)	(2.878 ± 0.137*)
Kidneys (g)	2.84 ± 0.40	2.83 ± 0.26	2.71 ± 0.20	2.71 ± 0.17	2.59 ± 0.16	2.91 ± 0.20	3.15 ± 0.34
	(0.684 ± 0.046)	(0.663 ± 0.017)	(0.664 ± 0.035)	(0.643 ± 0.036)	(0.615 ± 0.031*)	(0.644 ± 0.045)	(0.646 ± 0.047)
Spleen (g)	0.56 ± 0.08	0.65 ± 0.08	0.61 ± 0.07	0.66 ± 0.15	0.61 ± 0.10	0.67 ± 0.09	0.82 ± 0.05*
	(0.134 ± 0.009)	(0.153 ± 0.017)	(0.149 ± 0.005)	(0.153 ± 0.021)	(0.144 ± 0.019)	(0.148 ± 0.017)	(0.169 ± 0.015)
Female							
No. of animals	5	5	5	5	5	5	5
Body weight[a] (g)	217.6 ± 20.5	222.4 ± 12.2	216.8 ± 12.8	211.6 ± 16.8	218.2 ± 7.4	235.0 ± 16.7	234.8 ± 22.8
Thymus (g)	0.36 ± 0.07	0.45 ± 0.11	0.39 ± 0.05	0.47 ± 0.09	0.40 ± 0.09	0.41 ± 0.08	0.41 ± 0.11
	(0.163 ± 0.021)	(0.200 ± 0.042)	(0.180 ± 0.032)	(0.222 ± 0.032*)	(0.185 ± 0.041)	(0.172 ± 0.026)	(0.176 ± 0.053)
Liver (g)	6.43 ± 0.91	6.89 ± 0.57	6.66 ± 0.34	6.48 ± 0.54	6.85 ± 0.56	6.28 ± 0.42	6.19 ± 0.98
	(2.950 ± 0.203)	(3.097 ± 0.129)	(3.080 ± 0.252)	(3.066 ± 0.131)	(3.142 ± 0.236)	(2.675 ± 0.109)	(2.626 ± 0.214)
Kidneys (g)	1.48 ± 0.12	1.41 ± 0.12	1.43 ± 0.07	1.51 ± 0.12	1.52 ± 0.17	1.67 ± 0.13	1.51 ± 0.28
	(0.682 ± 0.076)	(0.637 ± 0.045)	(0.662 ± 0.041)	(0.717 ± 0.050)	(0.697 ± 0.061)	(0.714 ± 0.048)	(0.640 ± 0.068)
Spleen (g)	0.40 ± 0.04	0.46 ± 0.06	0.41 ± 0.04	0.43 ± 0.06	0.42 ± 0.05	0.45 ± 0.07	0.44 ± 0.07
	(0.185 ± 0.012)	(0.206 ± 0.027)	(0.187 ± 0.011)	(0.200 ± 0.018)	(0.195 ± 0.028)	(0.190 ± 0.020)	(0.189 ± 0.020)

Values are expressed as the mean ± S.D. Values in parentheses are relative organ weights (organ weight per body weight, %). a: The values presented were obtained after the animals were fasted overnight. *: Significantly different from control group at $p < 0.05$.

Table 3. Number of animals with histopathological findings in male and female rats given fullerene C60 by gavage

	Male		Female	
Dose (mg/kg/day)	0	1000	0	1000
No. of animals	5	5	5	5
At the end of the administration period				
Liver				
Normal	2	1	1	0
Granuloma, minimal	1	3	3	1
Granuloma, slight	0	0	0	1
Granuloma, moderate	0	0	0	1
Tension lipidosis, slight	0	1	0	0
Vacuolation, cytoplasmic, minimal	3	3	3	4
Vacuolation, cytoplasmic, slight	0	0	1	1
Kidney				
Normal	4	5	4	2
Mineralization, minimal	0	0	1	3
Scar, mininal	1	0	0	0
Prostate				
Normal	4	5		
Cellular infiltration, lymphocyte, minimal	1	0		
Uterus				
Normal			3	5
Dilatation, lumen, slight			2	0
At the end of the recovery period				
Liver				
Normal	3	3		
Granuloma, minimal	2	1		
Vaculoation, cytoplasmic, minimal	0	1		
Spleen				
Normal	5	5		

controls (Folkmann *et al.*, 2009), although the presence of fullerenes in the liver and lungs was not demonstrated. In three acute oral dose toxicity tests (Shinohara *et al.*, 2009; Mori *et al.*, 2006; Chen *et al.*, 1998), there was no discussion about absorption of the fullerene used.

In the study, when intravenously injected into female rats, ^{14}C-labeled fullerene C60 was rapidly (within 1 min) cleared from the circulation and the majority accumulated in the liver (about 92%), followed by the spleen (about 4%), 2-hr post-injection, and the 14C-labeled fullerene C60 was not eliminated from the liver, but from the spleen, 120-hr post-injection (Bullard-Dillard *et al.*, 1996). In a study (Kubota *et al.*, 2011) of tail vein injection of fullerene C60 into rats using liposomes as a carrier, burdens of fullerene C60 were widely distributed in five tissues, the liver, lungs, spleen, kidneys, and brain (in descending order) although no fullerene C60 was detected in the blood on day 1 after completion of the injections. Fullerene C60 accumulated in the liver did not decrease until 14 days, and for up to 28 days after the completion of injections, and a time-dependent decrease in fullerene C60 concentration was not observed in the spleen until 28 days (Kubota *et al.*, 2011).

Because these above-mentioned studies suggested that fullerene C60 could be absorbed via the gastrointestinal tract (Folkmann *et al.*, 2009; Yamago *et al.*, 1995) and distribute in the spleen and liver (Bullard-Dillard *et al.*, 1996; Kubota *et al.*, 2011), increased liver and spleen weights after the recovery period in the present study may relate to the oral administration of fullerene C60. However, it was clear that there was no accumulation of fullerene C60 which could change the weights of the liver and spleen directly. The causal relationships between the possible absorption of fullerene C60 and those weight changes were unknown because the pathological findings as indirect influences, such as swelling and congestion, were not also observed in the liver and spleen.

In conclusion, the results of this study of no marked change after 29-day repeated dosing of fullerene C60 by gavage indicated that its toxicity by oral administration is relatively low; however, increased liver and spleen weights observed after the recovery period may be asso-

ciated with fullerene C60 administration although no histopathological changes were found and absorbed fullerene C60 was under the detection limits in these organs. Therefore, with the prospective exposure by increased uses in future because of low toxic substance, more long-term exposure study is necessary to clarify the effects of fullerene C60 via oral exposure.

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REFERENCES

Aschberger, K., Johnston, H.J., Stone, V., Aitken, R.J., Tran, C.L., Hankin, S.M., Perers, S.A.K. and Christensen, F.M. (2010): Review of fullerene toxicity and exposure--appraisal of a human health risk assessment, based on open literature. Regul. Toxicol. Pharmacol., **58**, 455-473.

Bartlett, M.S. (1937): Properties of sufficiency and statistical tests. Proceedings of the royal society of London. Series A, Mathematical and Physical Sciences (1934-1990), **160**, 268-282.

Bullard-Dillard, R., Creek, K.E., Scrivens, W.A. and Tour, J.M. (1996): Tissue sites of uptake of 14C-labeled C60. Bioorganic Chemistry, **24**, 376-385.

Chen, H.H., Yu, C., Ueng, T.H., Chen, S., Chen, B.J., Huang, K.J. and Chiang, L.Y. (1998): Acute and subacute toxicity study of water-soluble polyalkylsulfonated C60 in rats. Toxicol. Pathol., **26**, 143-151.

Dunnett, C.W. (1964): New tables for multiple comparisons with a control.

EA, MHW and MITI (1986): Partial Amendment of the Testing Methods for New Chemical Substances. Planning and Coordination Bureau, Environment Agency (EA) No. 700, Pharmaceutical Affairs Bureau, Ministry of Health and Welfare (MHW) No.1039 and Basic Industries Bureaus, Ministry of International Trade and Industry (MITI) No. 1014, dated December 5, 1986.

Fisher, R.A. (1973): Statistical methods for research workers. Hapner publishing, New York.

Folkmann, J.K., Risom, L., Jacobsen, N.R., Wallin, H., Loft, S. and Moller, P. (2009): Oxidatively damaged DNA in rats exposed by oral gavage to C60 fullerenes and single-walled carbon nanotubes. Environ. Health Perspect., **117**, 703-708.

Kroto, H.W., Heath, J.R., O'Brien, S.C., Curl, R.F. and Smalley, R.E. (1985): C60: Buckminsterfullerene. Nature, **318**, 162-163.

Kubota, R., Tahara, M., Shimizu, K., Sugimoto, N., Hirose, A. and Nishimura, T. (2009): Development of a liquid chromatography-tandem mass spectrometry method for the determination of fullerenes C60 and C70 in biological samples. Kokuritsu Iyakuhin Shokuhin Eisei Kenkyusho Hokoku, 65-68.

Kubota, R., Tahara, M., Shimizu, K., Sugimoto, N., Hirose, A. and Nishimura, T. (2011): Time-dependent variation in the biodistribution of C60 in rats determined by liquid chromatography-tandem mass spectrometry. Toxicol. Lett., **206**, 172-177.

Mann, H.B. and Whitney, D.R. (1947): On a test of whether one of two random variables is stochastically larger than the other. Ann. Math. Stat., **18**, 50-60.

MOE (2006): Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain, Ministry of the Environment (MOE), Japan (Notice No.88), dated April 28, 2006.

MOE, METI and MHLW (2003): Standard concerning testing laboratories implementing tests for new chemical substances etc., Joint notification by director generals of Environmental Policy Bureau, Japan, Ministry of the Environment (MOE) (Kanpokihatsu No. 031121004) and Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry (METI) (Seikyokuhatsu No. 3), dated November 17, 2003 and by director general of Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare (MHLW) (Yakusyokuhatsu No. 1121003), dated November 21, 2003.

Mori, T., Takada, H., Ito, S., Matsubayashi, K., Miwa, N. and Sawaguchi, T. (2006): Preclinical studies on safety of fullerene upon acute oral administration and evaluation for no mutagenesis. Toxicology, **225**, 48-54.

Oberdörster, G., Oberdörster, E. and Oberdörster, J. (2005): Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environ. Health Perspect., **113**, 823-839.

Semenov, K., Charykov, N. and Arapov, O. (2009): Temperature dependence of the light fullerenes solubility in natural oils and animal fats. Fullerenes Nanotubes and Carbon Nanostructures, **17**, 230-248.

Shinohara, N., Matsumoto, K., Endoh, S., Maru, J. and Nakanishi, J. (2009): In vitro and in vivo genotoxicity tests on fullerene C60 nanoparticles. Toxicol. Lett., **191**, 289-296.

Snedecor, G.W. and Cochran, W.G. (1967): In statistical methods. The Iowa State University Press Ames, Iowa.

Steel, R.G.D. (1959): A multiple comparison rank sum test: treatments versus control. Biometrics, **15**, 560-572.

Steel, R.G.D. and Torrie, J.H. (1980): Principles and procedures of statistics. McGraw-Hill Book Company, New York.

Yamago, S., Tokuyama, H., Nakamura, E., Kikuchi, K., Kananishi, S., Sueki, K., Nakahara, H., Enomoto, S. and Ambe, F. (1995): In vivo biological behavior of a water-miscible fullerene: 14C labeling, absorption, distribution, excretion and acute toxicity. Chem. Biol., **2**, 385-389.

Yamakoshi, Y. (1999): Study on the biological actions of photoexcited fullerenes (in Japanese). Doctoral thesis at the Graduate School of Pharmaceutical Sciences in the University of Tokyo.

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Dose-dependent mesothelioma induction by intraperitoneal administration of multi-wall carbon nanotubes in p53 heterozygous mice

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Among various types of multi-wall carbon nanotubes (MWCNT) are those containing fibrous particles longer than 5 μm with an aspect ratio of more than three (i.e. dimensions similar to mesotheliomagenic asbestos). A previous study showed that micrometer-sized MWCNT (μm-MWCNT) administered intraperitoneally at a dose of 3000 μg/mouse corresponding to 1×10^9 fibers per mouse induced mesotheliomas in p53 heterozygous mice. Here, we report a dose-response study; three groups of p53 heterozygous mice ($n = 20$) were given a single intraperitoneal injection of 300 μg/mouse of μm-MWCNT (corresponding to 1×10^8 fibers), 30 μg/mouse (1×10^7) or 3 μg/mouse (1×10^6), respectively, and observed for up to 1 year. The cumulative incidence of mesotheliomas was 19/20, 17/20 and 5/20, respectively. The severity of peritoneal adhesion and granuloma formation were dose-dependent and minimal in the lowest dose group. However, the time of tumor onset was apparently independent of the dose. All mice in the lowest dose group that survived until the terminal kill had microscopic atypical mesothelial hyperplasia considered as a precursor lesion of mesothelioma. Right beneath was a mononuclear cell accumulation consisting of CD45- or CD3-positive lymphocytes and CD45/CD3-negative F4/80 faintly positive macrophages; some of the macrophages contained singular MWCNT in their cytoplasm. The lesions were devoid of epithelioid cell granuloma and fibrosis. These findings were in favor of the widely proposed mode of action of fiber carcinogenesis, that is, frustrated phagocytosis where the mesotheliomagenic microenvironment on the peritoneal surface is neither qualitatively altered by the density of the fibers per area nor by the formation of granulomas against agglomerates. (*Cancer Sci* 2012; 103: 1440–1444)

Unique properties such as persistency and electric conductivity promise a high potential for technology applications of carbon nanotubes (e.g. in lithium ion batteries). Immediately after the invention of the carbon nanotube, its persistency and fibrous shape have posed a challenge for toxicology known as "fiber carcinogenesis".[1] A recent study showed that a particular type of multi-wall carbon nanotube (Mitsui MWCNT-7, designated in general here as micrometer-sized MWCNT or μm-MWCNT) contains a considerable percentage of particles similar to asbestos in length and diameter.[2] To investigate its mesotheliomagenic potential, we used an intraperitoneal injection (i.p.) method that was extensively used in the 1970s and 1980s for the elucidation of key dimensions of the fiber (e.g. length and diameter) and for toxicity assessment of various man-made fibers.[3–6] Although the route of exposure is not realistic for humans, the i.p. injection method has been considered appropriate to assess the mesotheliomagenic potential of fibers,[7] and the least potent fibers were found to induce a positive result at a dose of 10^9 fibers i.p. in rats.[6–8]

Our first study identified the mesotheliomagenic potency of Mitsui MWCNT-7 at a single maximum dose (i.e. 10^9 fibers) in the peritoneal cavity of p53 heterozygous (p53+/−) mice[2] (data shown as a reference in Fig. 1). Marsella *et al.*[9] has shown that development of mesothelioma by crocidolite asbestos was accelerated in this mutant mouse. We have bred this mouse and tested it as an alternative model to replace the wild-type mouse carcinogenicity test of the National Toxicology Program of the National Institute of Environmental Health Sciences/NIH of the United States.[10] As a result, spontaneous neoplastic lesions of this model have been well characterized.[11]

Here, we applied the same fiber to p53+/− mice at doses of 1/10, 1/100, and 1/1000 of the dose used in the previous study (i.e. 300, 30 and 3 μg/mouse), which corresponds to approximately 1×10^8, 1×10^7, and 1×10^6 fibers per mouse, respectively, and monitored the mice for 1 year.

Materials and Methods

Experimental animals. The p53+/− mice were generously supplied by Dr S. Aizawa,[12] and back crossed with normal wild-type C57BL/6 females (SLC, Shizuoka, Japan) for more than 20 generations at the National Institute of Health Sciences (NIHS), Tokyo. Eighty male p53+/− mice aged 9–11 weeks were divided into four groups of 20 mice, and housed individually under specific pathogen-free conditions with a 12-h light-dark cycle at a NIHS animal facility. They were given tap water and autoclaved CRF-1 pellets (Oriental Yeast Co. Ltd., Tokyo, Japan) *ad libitum*. Experiments were humanely conducted under the regulation and permission of the Animal Care and Use Committee of the NIHS.

Histology. Liver, kidney, spleen, lung, digestive tract and macroscopic tumors (*en bloc* in the case of severe peritoneal adhesion) were fixed in 10% neutral buffered formalin. After conventional processing, paraffin-embedded sections were stained with hematoxylin–eosin (HE) and examined histopathologically under a light microscope. A pair of polarizing filters was set to a light microscope to detect birefringent particles.

For the selected atypical mesothelial hyperplasia lesions, serial sections were stained for CD45R(B220), CD3 and F4/80 using anti-mouse CD45R (eBioscience, San Diego, CA, USA), anti-rat CD3 (AbD Serotec, Kidlington, UK), anti-mouse F4/80 antibodies (eBioscience), which were diluted at 1:100, 1:50 and 1:50, respectively. The slides were incubated at 4°C overnight

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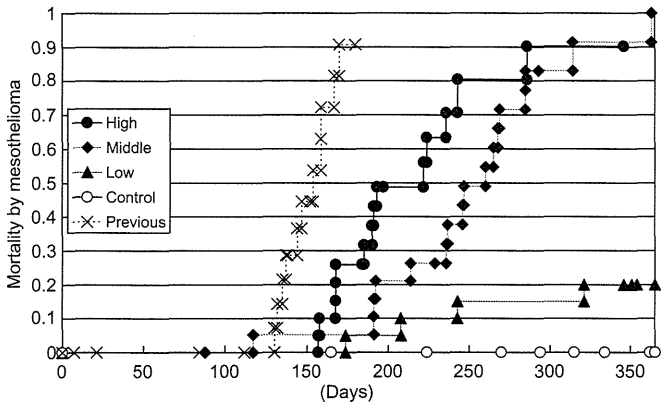

Fig. 1. Dose-dependent induction of mesotheliomas by micrometer-sized multi-wall carbon nanotubes (μm-MWCNT). Mice with lethal mesotheliomas are plotted using the Kaplan–Meier method. High: 300 μg/mouse, corresponding to 1×10^8 fibers/mouse; middle: 30 μg/mouse, corresponding to 1×10^7 fibers/mouse; low: 3 μg/mouse, corresponding to 1×10^6 fibers/mouse); previous: data from a previous study (i.e. 3 mg/mouse, corresponding to 1×10^9 fibers/mouse). No mesothelioma was observed in the vehicle control group.

and then incubated for 1 h with biotinylated species-specific secondary antibodies diluted 1:500 (Vector Laboratories, Burlingame, CA, USA) and visualized using avidin-conjugated alkaline phosphatase complex (ABC kit; Vector Laboratories).

Test material. Multi-wall carbon nanotube (MITSUI MWCNT-7, Lot No. 060125-01k), the same lot used in our previous study[2] was used. As reported in our previous paper, one gram of MWCNT corresponded to 3.55×10^{11} particles. The length ranged from 1 to 20 μm with a median of 2 μm. More than 25% of the particles were longer than 5 μm; their width ranged from 70 to 170 nm with a median of 90 nm. The approximate average content of iron was 3500 ppm (0.35%) and that of sulfur was 470 ppm. The concentration of chlorine in the fibers was 20 ppm and that of fluorine and bromine was below the limits of detection (5 and 40 ppm, respectively).[2]

Multi-wall carbon nanotubes was suspended at a concentration of 3 mg/mL to 0.5% methyl cellulose (Shin-Etsu Chemical Co. Ltd, Tokyo, Japan) solution and autoclaved (121°C, 15 min). After addition of Tween 80 (Tokyo Chemical Industry Co. Ltd, Tokyo, Japan; final 1.0% concentration), the solution was subjected to sonication at 150 watt for 5 min using an ultrasonic homogenizer (VP30s; TAITEC Co., Saitama, Japan).

Treatment. Eighty male p53+/− mice aged 9–11 weeks were randomly divided into four groups of 20. The high-dose group mice were given a single i.p. injection of 300 μg/mouse of MWCNT particles (corresponding to 1×10^8 fibers) in 1 mL suspension. The middle-dose group mice received 30 μg/mouse (1×10^7) and the low-dose group mice received 3 μg/mouse (1×10^6), respectively. The control group mice received vehicle solution (1 mL). Treated mice were monitored for 1 year. To minimize stress to the animals and re-aggregation of suspension, the injection was promptly performed without anesthesia.

Results

Peritoneal mesotheliomas were induced in a dose-dependent manner shown by an increase in the cumulative incidence of the tumors (Fig. 1). In the high-dose group, 14/20 mice had single or multiple lethal mesotheliomas up to 2 × 2 cm in size located within the peritoneal cavity, invading adjacent organs and structures with or without peritoneal dissemination. The remaining mice died of ileus due to severe peritoneal adhesion and fibrosis, and among them five had small incidental (non-lethal) mesotheliomas. The total incidence of mesothelioma was 19/20 (95%) among the animals. These lesions were qualitatively identical to our previous study.[2] In the middle-dose group, 17/20 (85%) mice had lethal mesothelioma. Three mice without lethal mesothelioma died or became moribund due to other reasons including leukemia. In the low-dose group, 4/20 mice had lethal mesothelioma (Fig. 2) and 1/20 had a non-lethal mesothelioma (found at the terminal kill on day 365), which makes the overall incidence of mesothelioma 5/20 (25%). The other 15 mice that survived until the terminal kill showed focal mesothelial atypical hyperplasia.[13] These lesions, up to 0.5 mm in diameter, consisted of a single layer of mesothelium characterized by cuboidal or hobnail appearance with slight to moderate nuclear atypia. Right beneath the

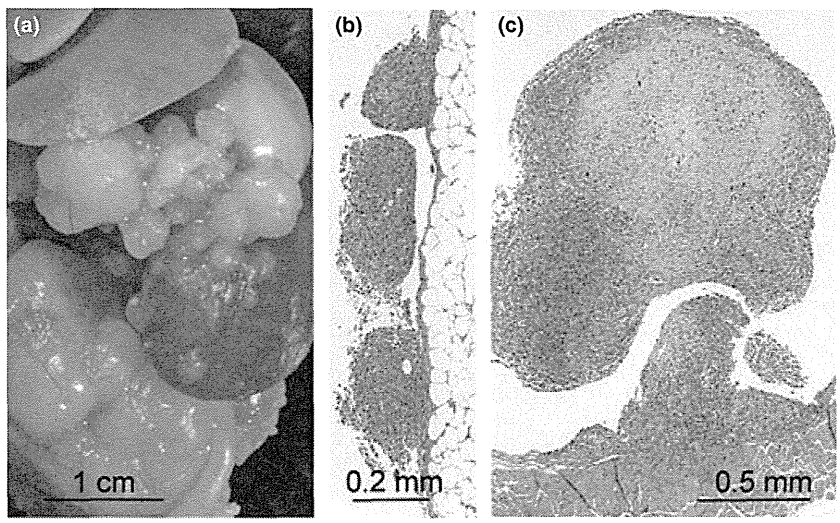

Fig. 2. Morphology of the induced mesotheliomas in the low-dose group. (a) Macroscopic view of the abdominal cavity of a mouse in the low-dose group. Multiple nodules are seen on the surface of the peritoneal serosa. This mouse died on day 243 with multiple nodules up to size 1 × 1 × 1 cm. (b) Low-power light microscopy view of the multiple nodules on the peritoneal surface of the mesentery. Granulomas and fibrous scars are minimal in the low-dose group. (c) Histology of a small nodule compatible with a diagnosis of moderately to poorly differentiated epithelioid mesothelioma. Larger nodules tended to be composed of undifferentiated sarcomatous components.[2]

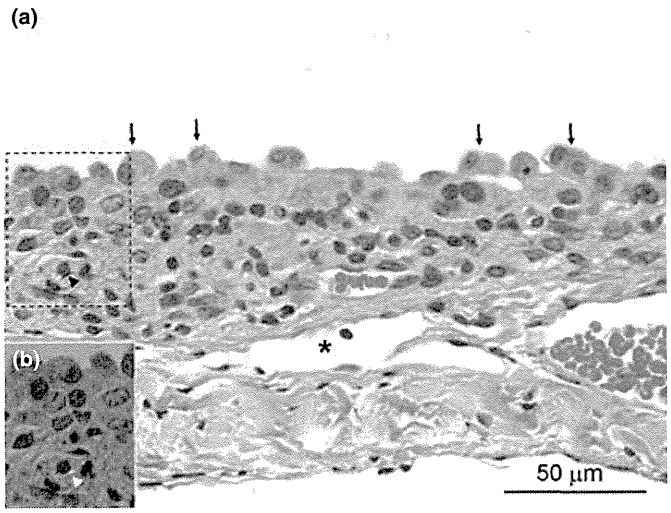

Fig. 3. Atypical mesothelial hyperplasia. (a) Atypical mesothelial hyperplasia of the tendinous portion of the diaphragm of a mouse in the low-dose group (sampled at terminal kill, that is, 365 days after i. p. inoculation of the multi-wall carbon nanotubes [MWCNT]). Arrows: hobnail appearance of the atypical hyperplastic mesothelial cells; asterisk: lymphatic drainage of the peritoneal cavity. (b) Polarized image of the dotted area in (a). Arrowhead: a MWCNT fiber in a macrophage-like cell (birefringent).

atypical mesothelium was a lentiform accumulation of mononuclear inflammatory cells up to 0.1 mm in thickness (Fig. 3). The accumulation is a combination of ill-demarcated zones of CD45-positive lymphocytes, CD3-positive lymphocytes and CD45/CD3-negative F4/80-negative or CD45/CD3-negative F4/80 weakly positive macrophage-like cells (Fig. 4). Single MWCNT fiber was often found in the cytoplasm of the macrophage-like cells. These lesions were devoid of epithelioid cell granuloma and fibrous scars.

Peritoneal fibrosis, peritoneal adhesion and formation of foreign body granulomas towards agglomerated MWCNT were dose dependent and minimal in the low-dose group. In the control group, mesotheliomas were not found (0%). There were eight mice with lethal or incidental thymic lymphoma, leukemia or reticulum cell sarcoma, osteosarcoma of the cranial bone, and 12/20 were tumor free. These tumors are known to develop spontaneously in p53+/− mice with increasing age[10] and none of these tumors were treatment dependent.

Histology of the mesotheliomas ranged from a differentiated epithelioid type to an undifferentiated sarcomatous type. Osteoid and rhabdoid differentiations, both known in human cases,[14–16] were found in nine mice (two in the low dose, three in the middle dose, and four in the high dose group, respectively) among a total of 41 mesothelioma cases in the present study.

An additional finding was the dissemination of singular fibers to systemic organs such as the liver, mesenteric lymph nodes, pulmohilar lymph nodes, choroid plexus of the brain, glomeruli of the kidney and lung alveoli (Fig. 5). Because the brain, including the choroid plexus, lacks afferent lymphatics,[17,18] it is probable that the fibers were distributed systemically via the blood stream.

Discussion

The present study showed a dose-dependent induction of mesothelioma by the μm-MWCNT from 1/1000 of the dose of our previous study (i.e. 3 μg/mouse corresponding to 1×10^6 fibers).

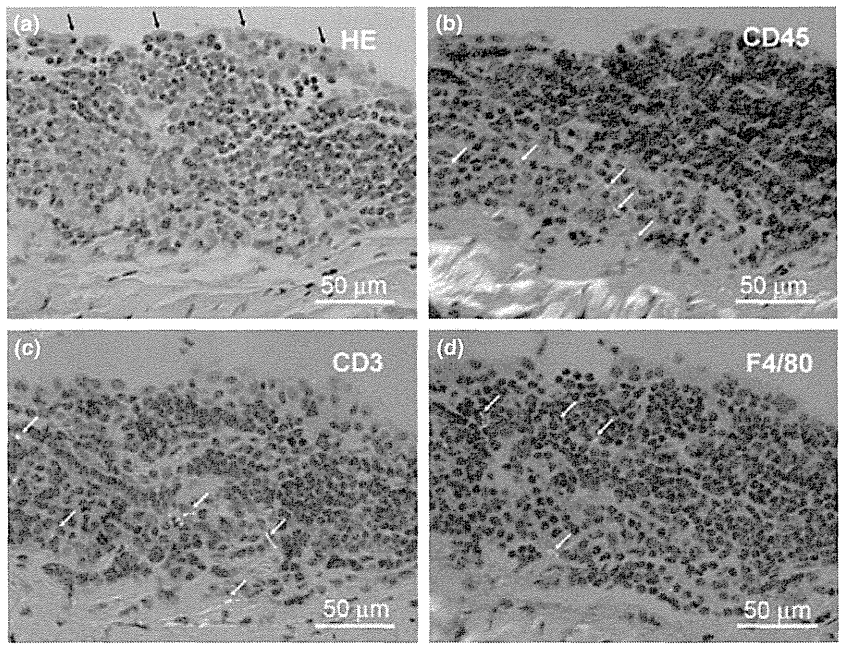

Fig. 4. Immunohistochemistry of lentiform mononuclear cell accumulation underlying the atypical mesothelial hyperplasia. (a) Serial section of an atypical mesothelial hyperplasia of the tendinous portion of the diaphragm of a mouse in the low-dose group (sampled at terminal kill). (a) Hematoxylin–eosin staining. Black arrows: hobnail appearance of the hyperplastic mesothelial cells. (b–d) Polarized image of the serial sections immunohistochemically stained for CD45, CD3 and F4/80. Multi-wall carbon nanotubes (birefringent; white arrows) are seen in the macrophage-like CD45/CD3-negative, F4/80-faintly positive cell cytoplasm. It is noted that epithelioid cell granuloma and fibrous scars are absent in this type of lesion.

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