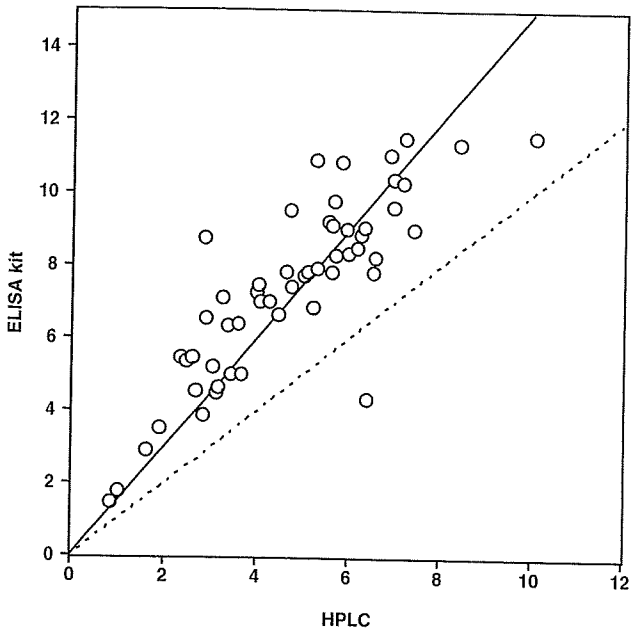

Fig. 6. Comparison of urinary 8-OH-dG levels (ng/ml) determined by ELISA (4°C) and HPLC methods. $y = 1.50x$, $r = 0.980$, $p < 0.0001$.

when the ELISA method is used for 8-OH-dG determination. All of those studies should be reevaluated by accurate measurements using HPLC, or at least the urea concentrations in these samples should be examined for their final conclusions. Hu et al. reported the analysis of 8-OH-dG in 140 urine samples collected from workers in a coke oven plant, by both LC-MS-MS and ELISA methods [18]. Only the LC-MS-MS measurements of 8-OH-dG showed a significant difference between the exposed and the control subjects. When we analyzed 120 urine samples, 10% of the samples showed an ELISA:HPLC ratio higher than 4 [10]. Those subjects might have a high urea concentration. Therefore, the ELISA method may generate a misleading conclusion and, at present, cannot be used for clinical analyses. This is in agreement with the current recommendations of the European Standards Committee on Urinary Lesion Analysis (ESCULA) [19].

In this study, when we measured the urinary urea concentration with a commercial kit, the urea accounted for only 34% of the overestimation by the ELISA (Fig. 4). However, based on the experiments with the urease treatment, 66% of the overestimation could be explained by urea (Fig. 5). One possible explanation for this discrepancy may be as follows. The measurement of urinary urea is

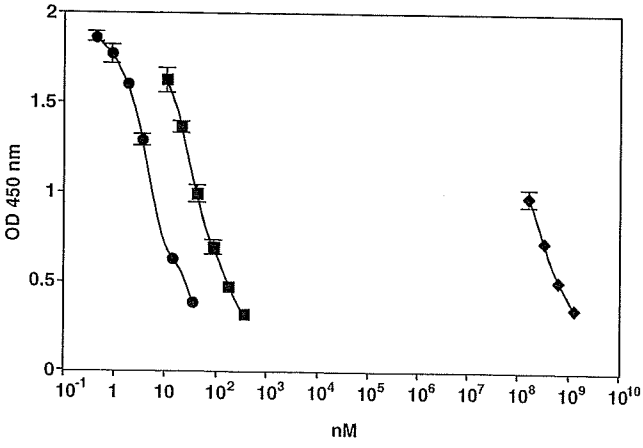

Fig. 7. Competitive inhibition of ELISA at 4 °C by 8-OH-dG (●), 8-OH-G (■), and urea (♦). Mean values ± SD are plotted ($n = 3$–6). Absence of SD bars means that the SD values were very low.

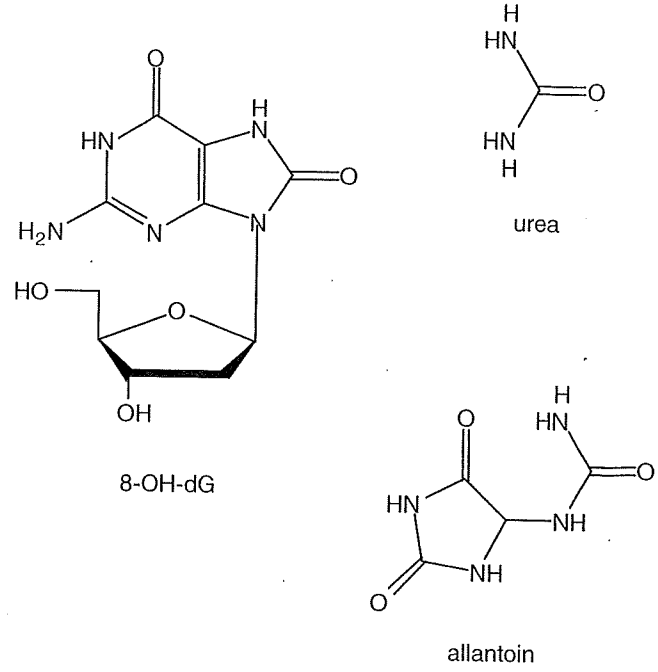

Fig. 8. Structures of 8-OH-dG (8-oxo form), urea, and allantoin.

underestimated, because a portion of the urea is weakly bound to other urinary components and is not detected by the analysis kit, whereas the urease treatment revealed the total amount of urea, including the bound form. In this hypothesis, the bound form of urea must be recognized in the ELISA.

It will be suspected that other urea derivatives, such as citrulline and biuret, cross-react with N45.1, in addition to urea. However, they may not contribute much to the overestimation of urinary 8-OH-dG, because their urinary contents may be very low. For example, the urinary citrulline level is on the order of micrograms per milliliter [20]. The methanol fraction (Fr. 27) in the HPLC analysis also contained a small amount of components that showed a positive reaction in the ELISA system (Fig. 1). This fraction may contain short DNA fragments containing 8-OH-dG, as suggested by Lunec and his collaborators [21].

It is possible that the concentrations of urea used in the competitive ELISA are denaturing the N45.1, or interfering with N45.1 binding, rather than acting as a competitive inhibitor. However, protein denaturation usually occurs with a high concentration of urea, such as 5–8 M, whereas the urinary urea concentration is below 1 M. Therefore, the denaturation of an antibody by urinary urea is unlikely. In addition, many compounds in urine are analyzed by ELISA without such problems. Therefore, we prefer the idea that urea is weakly recognized by N45.1 owing to structural similarity, which induces competitive inhibition. In either case, the recognition of urea by N45.1 and the denaturation of N45.1 would both have similar consequences when reading the ELISA plate, but perhaps this is outside the scope of this article.

We found that performing the ELISA at 4°C reduced the recognition of urea and improved the 8-OH-dG analysis by ELISA. However, at 4°C, the ribonucleoside 8-OH-G, which has a source different from that of 8-OH-dG, cross-reacted with the ELISA in addition to urea. Toyokuni et al. [7] reported that 8-OH-G needs to be present in a concentration 2 orders of magnitude higher than that of 8-OH-dG to compete to the same extent in the ELISA at 37°C. Although Evans et al. [14] reported that 8-OH-G needs to be present at 25 times the concentration of 8-OH-dG to exert equal competition at 4°C, in our experiments, an 8-OH-G concentration only 7-fold higher than that of 8-OH-dG showed the same competition. Considering that Weimann et al. reported that the 8-OH-G levels in human urine are about 2 times higher than the 8-

OH-dG levels [22], 8-OH-G may also contribute considerably to the overestimation of 8-OH-dG by the ELISA at 4°C.

In conclusion, urea, the most abundant component in urine, was found to cross-react with a commercial 8-OH-dG ELISA kit and to cause the overestimation of 8-OH-dG. Urea is present in high concentrations in the urine of humans and experimental animals, and its level is influenced by many factors other than oxidative stress. Therefore, improvements such as urea removal by pretreatment or its enzymatic decomposition by urease are required for the measurement of urinary 8-OH-dG by the ELISA at 37°C. Performing the ELISA at 4°C reduced the recognition of urea and improved the 8-OH-dG analysis. It is more desirable that a monoclonal antibody that specifically recognizes 8-OH-dG is developed in the future.

Acknowledgments

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Chronic alcohol consumption prevents 8-hydroxyguanine accumulation in 3′-methyl-4-dimethylaminoazobenzene-treated mouse liver

Takeshi Hirano [a,b,*], Akinori Sakai [c], Yuko Ootsuyama [b], Hiroshi Kasai [b]

[a] Department of Life and Environment Engineering, Faculty of Environmental Engineering, The University of Kitakyushu, Kitakyushu, Fukuoka 808-0135, Japan
[b] Department of Environmental Oncology, Institute of Industrial Ecological Sciences, University of Occupational and Environmental Health, Kitakyushu, Fukuoka 807-8555, Japan
[c] Department of Orthopedics, School of Medicine, University of Occupational and Environmental Health, Kitakyushu, Fukuoka 807-8555, Japan

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ABSTRACT

Alcohol consumption is known to have opposing effects on carcinogenesis: promotion and prevention. In this study, we examined the effects of 12% ethanol on oxidative DNA damage accumulation and its repair in mouse livers treated with 3′-methyl-4-dimethylaminoazobenzene (3′-MeDAB), a well-known hepatic carcinogen. We previously reported that 3′-MeDAB increased 8-hydroxyguanine (8-OH-Gua) accumulation and its repair activity, accompanied by the fragmentation of 8-oxoguanine DNA glycosylase 1 (OGG1), the main repair enzyme of 8-OH-Gua. The present results showed that 12% ethanol intake attenuated the 8-OH-Gua accumulation, but not the fragmentation of OGG1 induced by 3′-MeDAB. Additionally, no significant changes in oxidative status, as monitored by lipid peroxidation (LPO), were observed among the 3′-MeDAB-treated mouse livers with/without alcohol administration. These findings suggested that 12% ethanol consumption may reduce the risk of 3′-MeDAB-induced carcinogenesis by decreasing 8-OH-Gua accumulation.

Introduction

It is generally believed that alcohol consumption is related to carcinogenesis. Recent studies revealed that alcohol consumption was associated with an increase in breast cancer incidence in women [1,2], esophageal cancer [3], and colorectal cancer [4,5]. However, another report indicated that moderate alcohol consumption was not associated with ovarian cancer [6]. On the other hand, the cancer-preventive effect of alcohol drinking has also been reported. It is well-known that moderate consumption of wine may prevent some types of cancers [7–9]. Interestingly, the risk of rectal cancer induced by alcohol consumption was reduced when wine was included among the alcoholic beverages [10].

These studies concerning wine consumption suggested that anti-oxidant agents such as polyphenols, including catechin, quercetin and resveratrol, were responsible for the cancer-preventive effect of wine [11]. Thus, it seems that anti-oxidant agents play a key role in the cancer-preventive effects of wine, although the results have not been conclusive. Wang et al. suggested that resveratrol could reduce the localized estrogen production that plays a crucial role in the development of breast cancer [12]. In addition, for alcoholic beverages other than wine, a cohort study by Mahabir et al. suggested that alcohol (spirits and beer) consumption was associated with a decrease in the risk of renal cell carcinoma in male smokers [13]. A matched case–control study by Petti and Scully demonstrated a similar result, in which regular moderate alcohol (beer, wine, and spirits) consumption was associated with a decreased probability of leukoplakia occurrence, with respect to occasional or no alcohol consumption [14]. These results are not conclusive, but suggest that other components of alcoholic beverages (including wine) besides polyphenols may contribute to the beneficial effects on human health.

The evidence that not only wine but also spirits and beer have cancer-preventive effects prompted us to investigate the molecular mechanisms of these effects of alcohol consumption due to factors other than polyphenols. In fact, recent evidence has suggested that the protective effects of red wine on cancer or cardiovascular diseases were not a consequence of the anti-oxidant capacity of alcohol [15,16]. Arendt et al. reported the reduction of DNA damage as another mechanism of the cancer-preventive function of wine [15].

8-Hydroxyguanine (8-OH-Gua) is the most abundant form generated by reactive oxygen species (ROS), and it is believed to have an important role in carcinogenesis [17], because 8-OH-Gua causes GC to TA transversion type point mutations in DNA [18,19]. In addition, there are repair systems to remove 8-OH-Gua. 8-Oxoguanine DNA glycosylase 1 (OGG1) is the main repair enzyme to remove 8-OH-Gua, as a base excision repair (BER) component [20,21].

* Corresponding author. Address: Department of Life and Environment Engineering, Faculty of Environmental Engineering, The University of Kitakyushu, Kitakyushu, Fukuoka 808-0135, Japan. Fax: +81 93 695 3299.
E-mail address: t-hirano@env.kitakyu-u.ac.jp (T. Hirano).

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It is noteworthy that the 8-OH-Gua accumulation level in DNA is the consequence of the balance between 8-OH-Gua generation and repair. Therefore, analyses of both the 8-OH-Gua level and its repair ability are important and useful for understanding carcinogenesis. We have studied the relationship between 8-OH-Gua accumulation/repair ability and health-related factors, such as chemical agents, X-rays, aging, physical exercise, and food [22–26]. Interestingly, we found that the 8-OH-Gua repair activity was inhibited by some chemical reagents [22,24]. Although the inhibition of the repair activity was likely to be associated with 8-OH-Gua accumulation in DNA, which leads to carcinogenesis, the significance and the exact mechanism remain unclear. Recently, we found that the fragmentation of the OGG1 protein accompanied the increase in 8-OH-Gua accumulation in the livers of 3′-MeDAB-treated mice [27] and in cultured mouse cells exposed to chemical reagents, such as etoposide [28], mitomycin C [28], and arsenic compounds [29]. Based on these studies, we speculated that OGG1 fragmentation might be related to the reduction in the 8-OH-Gua repair ability, leading to 8-OH-Gua accumulation.

In this study, we analyzed the 8-OH-Gua accumulation level in DNA and its repair ability (8-OH-Gua nicking activity and mOGG1 expression) in the livers of mice treated with carcinogens and/or ethanol, to examine the effects of alcohol consumption on carcinogenesis.

Materials and methods

Animal treatments and sampling. Male mice (ddY, 3-week-old) were purchased from Seac Yoshitomi, Ltd. (Fukuoka, Japan) and were fed a commercial diet (Clea, Tokyo, Japan) and tap water *ad libitum*. They were divided into six groups from A to F, as shown in Fig. 1. The mice in groups C, D, E, and F were free to drink a 12% ethanol solution instead of tap water during the period indicated in Fig. 1. The mice in all groups were sacrificed at 10 months after the beginning of the experiment, and the livers were removed and analyzed for the experiment.

Quantitation of 8-OH-Gua. The assay for 8-OH-Gua levels was described elsewhere. Briefly, the mouse livers were homogenized in a lysis solution, containing 1 mM desferal (deferoxamine mesylate, Sigma Chemical Co., MO). The DNA was isolated by the sodium iodide method, using a DNA Extraction WB Kit (Wako Pure Chemical Industries, Ltd, Osaka, Japan). The isolated DNA was digested with nuclease P1 (Yamasa Corp., Choshi, Japan) to obtain a deoxynucleoside mixture. The solution was filtered with an Ultrafree-Probind filter (Millipore, Bedford, MA) and was injected into a high-performance liquid chromatography (HPLC) column (Shiseido Capcell Pak C18 MG) equipped with an electrochemical detector (ECD) (Coulochem ESA).

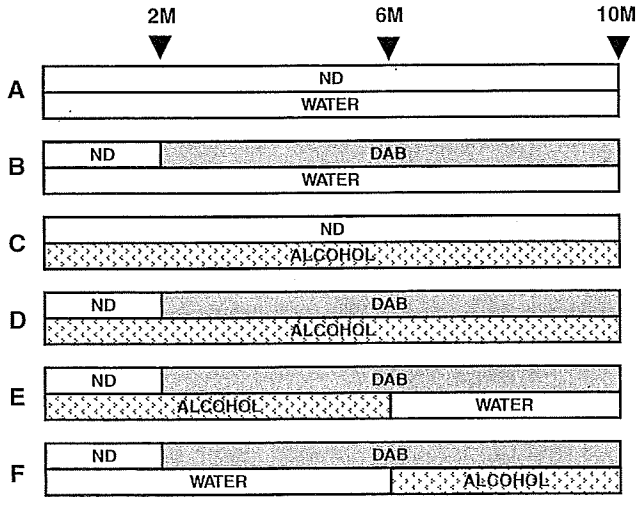

Fig. 1. The experimental protocol. (A) Control [ND/water] for 10 months; (B) [ND/water] for the first 2 months and [0.06% 3′-MeDAB/water] for the last 8 months; (C) [ND/alcohol] for 10 months; (D) [ND/alcohol] for the first 2 months and [0.06% 3′-MeDAB/alcohol] for the last 8 months; (E) [ND/alcohol] for the first 2 months, [0.06% 3′-MeDAB/alcohol] for the next 4 months, and [0.06% 3′-MeDAB/water] for the last 4 months; (F) [ND/water] for the first 2 months, [3′-MeDAB/water] for the next 4 months, and [3′-MeDAB/alcohol] for the last 4 months. ND, normal diet; DAB, 3′-MeDAB.

Endonuclease nicking assay. The BER activity was assayed by using a previously described method. Briefly, the tissue extracts were incubated with a 22-mer, fluorescently labeled, synthetic oligonucleotide containing an 8-OH-Gua residue in its sequence. These mixtures were electrophoresed on a 20% denaturing polyacrylamide gel. The excised fragments, generated as a consequence of BER activity, were analyzed by a Pharmacia ALF DNA sequencer (Fragment Manager, Ver. 1.1; Amersham Pharmacia Biotech, Uppsala, Sweden).

Western blotting. The Western blotting method was described elsewhere. The tissue extracts were fractionated on 4–12% SDS–polyacrylamide gels (NuPAGE, NOVEX, CA), and blotted onto PVDF membranes (Millipore, MA). The membranes were blocked with a buffer containing bovine serum albumin, incubated with anti-mOGG1 antiserum, and then incubated with an alkaline phosphatase-coupled secondary antibody (EY Laboratories, Inc., CA). The antigen–antibody complexes were visualized with a BCIP-NBT solution kit (Nacalai Tesque, Inc., Kyoto, Japan). The anti-mouse OGG1 antiserum was prepared as described in our previous work [27]. The amino acid sequence of the peptide used as an antigen was QSFRWKEOSP (aa 43–52).

Determination of LPO level. Tissues were homogenized with a Dounce-type homogenizer in 20 mM Tris–HCl (pH 7.4) containing 0.25 M sucrose and 1 mM EDTA. Homogenates were centrifuged at 10,000*g* for 30 min. The LPO levels in the supernatants were determined by using a spectrophotometric assay kit (LPO-586, BIOXYTECH, Bonneuil-sur-Marne, France) according to the manufacturer's instructions. The LPO levels are expressed as nanomoles of malon dialdehyde (MDA) produced per milligram of protein.

Statistical analysis. Values in the graphs and Table represent the means ± SD. The statistical significance was calculated using the Student *t* test. Probability values less than 0.05 were considered to indicate significant differences.

Results

Profiling of mice in the experiment

The body weights of the mice are shown in Table 1. Contrary to our prediction, the body weights of the mice treated with 3′-MeDAB were higher than those of the control mice. In addition, the significant effect of 3′-MeDAB and the minimal effect of 12% ethanol on the body weights were observed. The survival rates of the mice were 75% for group A (6/8), 63.6% for group B (14/22), 92.3% for group C (12/13), 57.1% for group D (8/14), 69.2% for group E (9/13), and 59.1% for group F (13/22). The survival rate of the mice in the alcohol administration group (group C) was relatively higher than those of the mice in the other groups. The etiological causes of mouse death were not investigated.

8-OH-Gua level

The 8-OH-Gua accumulation levels in the mouse liver DNA are shown in Fig. 2A. The 8-OH-Gua accumulation was increased in the livers of 3′-MeDAB-treated mice. This increase was dramatically

Table 1
Body weights of mice.

Weeks	A	B	C	D	E	F
Initial body weight: (3-week-old)			14.3 ± 1.5 (n = 84)			
4-week	35.6 ± 2.7*1 (n = 44)		33.9 ± 2.2 (n = 40)			
8-week	43.4 ± 4.0*2 (n = 44)		39.8 ± 3.0 (n = 40)			
12-week	47.8 ± 5.4*1 (n = 8)	49.0 ± 6.0*7 (n = 22)	42.8 ± 3.1*12 (n = 13)	46.0 ± 5.4 (n = 14)	43.8 ± 4.2*12 (n = 13)	48.5 ± 4.3 (n = 22)
16-week	50.7 ± 6.5 (n = 8)	52.6 ± 7.7*8 (n = 22)	46.0 ± 3.6*12 (n = 13)	49.2 ± 7.7 (n = 14)	46.3 ± 4.9*12 (n = 13)	51.8 ± 5.1 (n = 22)
20-week	51.7 ± 7.1 (n = 8)	57.3 ± 8.7*9 (n = 22)	49.0 ± 4.8*12 (n = 13)	52.6 ± 9.3 (n = 14)	49.0 ± 6.6*12 (n = 13)	56.0 ± 6.5 (n = 22)
24-week	53.9 ± 8.1 (n = 8)	59.5 ± 9.1*9 (n = 22)	50.1 ± 5.2*13 (n = 13)	54.4 ± 9.8 (n = 14)	49.9 ± 8.3*12 (n = 12)	58.8 ± 7.0 (n = 22)
28-week	54.4 ± 8.8 (n = 8)	60.2 ± 9.3*10 (n = 22)	51.6 ± 5.3*14 (n = 13)	55.9 ± 11.3 (n = 14)	52.3 ± 8.7*15 (n = 12)	57.9 ± 6.6 (n = 22)
32-week	54.0 ± 8.6*3 (n = 8)	62.5 ± 9.1*10 (n = 21)	52.9 ± 6.0*14 (n = 13)	57.2 ± 12.5 (n = 13)	54.4 ± 9.4 (n = 12)	59.5 ± 6.8 (n = 21)
36-week	52.7 ± 7.7*4 (n = 7)	63.0 ± 8.6*11 (n = 21)	55.6 ± 6.9*15 (n = 13)	59.8 ± 12.9 (n = 13)	54.8 ± 10.3 (n = 12)	60.5 ± 7.1 (n = 21)
40-week	51.7 ± 7.0*5 (n = 7)	63.7 ± 10.5*8 (n = 20)	56.5 ± 6.5*14 (n = 13)	60.6 ± 14.0 (n = 12)	53.5 ± 10.7*15 (n = 9)	63.2 ± 7.9 (n = 19)
44-week	51.7 ± 7.6*6 (n = 6)	62.2 ± 10.5*1 (n = 14)	53.2 ± 8.4*16 (n = 12)	65.8 ± 18.0 (n = 8)	56.1 ± 12.5 (n = 9)	62.9 ± 5.4 (n = 13)

*1 $p < 0.05$ vs. C.
*2 $p < 0.00005$ vs. C.
*3 $p < 0.05$ vs. B.
*4 $p < 0.01$ vs. B, $p < 0.05$ vs. F.
*5 $p < 0.01$ vs. B, $p < 0.005$ vs. F.
*6 $p < 0.05$ vs. B, $p < 0.005$ vs. F.
*7 $p < 0.01$ vs. C and E.
*8 $p < 0.05$ vs. C and E.
*9 $p < 0.005$ vs. C, $p < 0.01$ vs. E.
*10 $p < 0.005$ vs. C, $p < 0.05$ vs. E.
*11 $p < 0.01$ vs. C, $p < 0.05$ vs. E.
*12 $p < 0.005$ vs. F.
*13 $p < 0.001$ vs. F.
*14 $p < 0.01$ vs. F.
*15 $p < 0.05$ vs. F.
*16 $p < 0.05$ vs. D, $p < 0.005$ vs. F.

reduced by the administration of 12% ethanol. Although there were no significant differences in the 8-OH-Gua levels of the mouse livers among the ethanol-administered groups, ethanol intake was significantly effective in reducing the 8-OH-Gua accumulation induced by 3′-MeDAB treatment. Furthermore, long-term or earlier intake of ethanol was relatively effective in reducing 8-OH-Gua accumulation. Additionally, our results demonstrated that 12% ethanol itself had no effect on 8-OH-Gua accumulation (group A vs. group C).

8-OH-Gua repair activity

The 8-OH-Gua endonuclease nicking activity is shown in Fig. 2B. The 8-OH-Gua repair activity was increased in the livers of mice treated with 3′-MeDAB. Although no significant change by ethanol intake was observed, there was tendency for the 8-OH-Gua repair activity to decrease in the livers of the mice in the ethanol-administered groups, D, E, and F, as compared to the livers of the mice treated with 3′-MeDAB (group B). Similar to the data for the 8-OH-Gua levels, there was no significant effect of 12% ethanol-alone administration on the 8-OH-Gua repair activity (group A vs. group C).

OGG1 expression

The Western blot analysis for mouse OGG1 expression is shown in Fig. 3C. We previously reported that various chemical reagents, such as 3′-MeDAB, etoposide, mitomycin C, and arsenic compounds, induced the fragmentation of the OGG1 protein [27–29]. In the present study, we detected fragments of mOGG1 in the livers of mice treated with 3′-MeDAB, and this fragmentation was detected in all groups of 3′-MeDAB-treated mice with/without ethanol administration (groups B, D, E, and F) (Fig. 2C).

LPO level

For the LPO level, there were no significant differences between the 3′-MeDAB-treated mouse groups and the control mouse group (group A) (Fig. 3). However, the LPO level was significantly lower in the alcohol-treated mouse liver (group C), as compared to the levels in the mice in other groups.

Discussion

We previously reported the effects of 3′-MeDAB on 8-OH-Gua accumulation and its repair activity [23,27]. In these reports, we showed that this carcinogen increased 8-OH-Gua accumulation and 8-OH-Gua endonuclease nicking activity. Based on these findings, we concluded that 8-OH-Gua accumulation might be involved in 3′-MeDAB-induced carcinogenesis. Furthermore, OGG1 fragmentation was also detected in the livers of mice treated with 3′-MeDAB [27]. Therefore, we speculated that 8-OH-Gua accumulation, due to disorder of the 8-OH-Gua repair systems, may be involved in the carcinogenesis mediated by 3′-MeDAB.

Our aim in this study was to examine whether ethanol had any effects on cancer risk or carcinogenesis by affecting the 8-OH-Gua level, its endonuclease nicking activity, and OGG1 expression. In this study, we found that 12% ethanol reduced the 3′-MeDAB-induced 8-OH-Gua accumulation (Fig. 2A). Moreover, the 8-OH-Gua repair activity showed a decreasing tendency in 3′-MeDAB-treated mouse livers by 12% ethanol administration, without any significant differences (Fig. 2B). The decrease in the 8-OH-Gua repair activity seems to be a consequence of the lower 8-OH-Gua levels.

Since we speculated that OGG1 fragmentation was a key event for 8-OH-Gua accumulation, we predicted that OGG1 fragmentation might be inhibited by ethanol intake, as in the inhibition of the increase in 8-OH-Gua levels. However, 12% ethanol intake failed to inhibit the 3′-MeDAB-induced fragmentation of OGG1 (groups D, E, and F). The observation that ethanol intake reduced 8-OH-Gua accumulation and showed a decreasing tendency in the endonuclease nicking activity, without inhibiting OGG1 fragmentation, suggests that OGG1 fragmentation might not be involved in 8-OH-Gua accumulation and repair. However, because

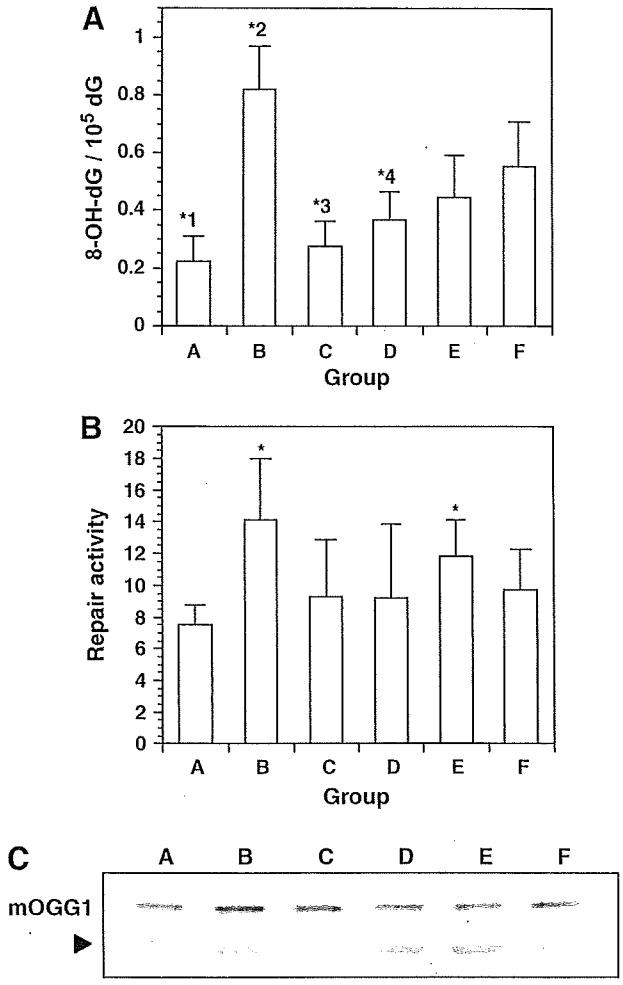

Fig. 2. (A) The levels of 8-OH-Gua in the DNA of mouse livers. The 8-OH-Gua value is expressed as the number of 8-OH-dG per 10^5 deoxyguanosine. 1: $p < 0.0005$ vs. group B, $p < 0.05$ vs. group E, $p < 0.01$ vs. group F; 2: $p < 0.0001$ vs. group C, $p < 0.005$ vs. group D, $p < 0.01$ vs. group E, $p < 0.05$ vs. group F; 3: $p < 0.05$ vs. group E, $p < 0.005$ vs. group F; 4: $p < 0.05$ vs. group F. (B) 8-OH-Gua nicking activity in the mouse livers. The activity was calculated as the ratio of the excised fragment intensity to the total substrate (unexcised substrate intensity plus excised fragment intensity). *: $p < 0.005$ vs. group A. (C) Western blotting of mOGG1 protein in the mouse livers. Mouse livers were removed and homogenized to produce crude extracts. The extracts were electrophoresed and blotted onto a PVDF membrane. A Western blot analysis was performed using an anti-mouse OGG1 antibody. The arrowhead indicates fragmented mOGG1. mOGG1, mouse OGG1.

other enzymes besides OGG1 reportedly act as repair enzymes for 8-OH-Gua [30], we speculate that 12% ethanol might induce these 8-OH-Gua repair systems to reduce the 8-OH-Gua level.

We also examined whether this effect of ethanol was due to the quenching of oxidative stresses. We found that the LPO level was not significantly different between them, except for the ethanol-alone treatment (group C). This result suggested that alcohol did not alter the oxidative status in the mouse liver treated with the carcinogen. However, we found that ethanol intake reduced the LPO level, as compared to the control liver (group C in Fig. 3). These results are different from those reported previously, in which alcohol drinking increased lipid peroxidation in both humans and animals [31,32]. This dissimilarity in the results might be derived from the different ethanol administration procedures. In addition, the reason why 3′-MeDAB increased the 8-OH-Gua level without inhibiting both the repair activity and ROS production is still unclear. In this study, we employed the LPO assay as an estimation of oxidative status. However, the measurement of F2-isoprostanes

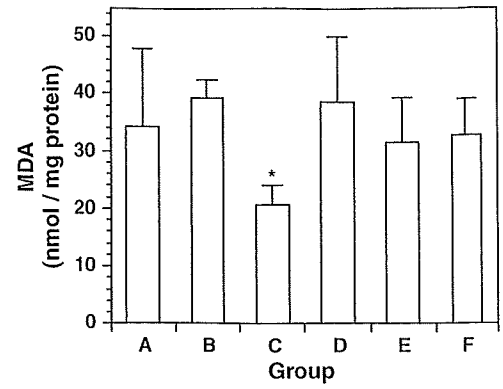

Fig. 3. LPO levels. The values are expressed as MDA (nmol/mg protein). *: $p < 0.05$ vs. group A, $p < 0.00001$ vs. group B, $p < 0.01$ vs. group D, $p < 0.05$ vs. group E, $p < 0.005$ vs. group F.

is more appropriate [33]. We will employ this assay in future experiments.

The relationship between alcohol consumption and oxidative DNA damage has been studied in several laboratories. Bradford et al. reported an increase in 8-OH-Gua and an induction of OGG1 expression in mice by ethanol-treatment for 4 weeks [34]. Another experiment using the Comet assay was performed to detect DNA damage induced by ethanol [35]. In the present study, we fed 12% ethanol to mice *ad libitum*. This administration procedure is different from those used in previous studies. It is noteworthy that several studies have shown a J-shaped relation between alcohol intake and mortality from all causes [36,37]. Therefore, the effect of alcohol depends on its dose and drinking pattern. Epidemiological research indicated that the type of alcoholic beverage is an important factor for a beneficial effect on human health [38]. In addition, although there have been no reports about the interaction of ethanol with 3′-MeDAB or its derivatives to our knowledge, we could not exclude either direct or indirect effects of alcohol on the metabolism of 3′-MeDAB. Taken together, to define the exact relationship between alcohol consumption and carcinogenesis, much more detailed information is required.

Our results demonstrate that alcohol consumption may not affect oxidative DNA damage accumulation, but instead it has an inhibitory effect on the increase in 8-OH-Gua accumulation induced by a carcinogen. It seems reasonable to expect that moderate alcohol consumption may also have a beneficial effect on carcinogenesis prevention in humans. We could even expect that moderate alcohol consumption would be positively used for the reduction of cancer risk in the future.

Acknowledgment

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Involvement of NAD(P)H:Quinone Oxidoreductase 1 and Superoxide Dismutase Polymorphisms in Ulcerative Colitis

Toshihito Kosaka,[1] Junji Yoshino,[1] Kazuo Inui,[1] Takao Wakabayashi,[1] Takashi Kobayashi,[1] Shinya Watanabe,[1] Shigekazu Hayashi,[2] Yoshifumi Hirokawa,[3] Taizo Shiraishi,[3] Takayuki Yamamoto,[4] Mayumi Tsuji,[5] Takahiko Katoh,[5] and Masatoshi Watanabe[6]

Inflammatory bowel disease is a multifactorial disease. Oxidative stress has been thought to be one of etiologic factor for inflammatory bowel disease. The genes superoxide dismutase (*SOD2*) and NAD(P)H:quinone oxidoreductase 1 (*NQO1*) are involved in inflammation and oxidative stress. The purpose of the present case–control study with 134 patients with ulcerative colitis (UC) and 125 healthy controls was to determine whether polymorphisms of these genes, the *NQO1* C609T and the *SOD2* Ala-9Val, are associated with the risk of UC and influence the clinical characteristics. These polymorphisms were examined by polymerase chain reaction–restriction fragment length polymorphisms and direct sequencing. In patients showing steroid resistance, the number with the *NQO1* T/T genotype was significantly higher than other genotypes (odds ratio 9.45, 95% confidence interval 2.46–41.6, $p = 0.002$). In the patients whose onset of UC was age 20 years or younger, more patients had *SOD2* T/T genotype than the other genotypes (odds ratio 6.46, 95% confidence interval 0.82–51.0). No association between these polymorphisms and UC risk was apparent. The *NQO1* C609T polymorphism may influence steroid resistance of UC patients, while the *SOD2* Ala-9Val polymorphism may influence age of onset of UC. Oxidative stress may influence the clinical features of UC.

Introduction

INFLAMMATORY BOWEL DISEASE (IBD) includes two common forms, Crohn's disease (CD) and ulcerative colitis (UC), and is recognized as a multifactorial disease (Koutroubakis *et al.*, 1996). Factors that may affect IBD include diet, infantile environment, and immune defense abnormalities limited to the intestinal tract. Recently, oxidative stress has been proposed to be a factor that influences IBD (Kruidenier *et al.*, 2003). Koutroubakis *et al.* (2004) showed that serum total antioxidant capacity was lower in both CD and UC patients compared with healthy controls. This finding suggests that decreased antioxidant defenses may be a primary phenomenon severely compromising the mucosa and increasing its susceptibility to oxidative tissue damage. Genetic factors have also been examined in IBD, but despite the many studies that have searched for susceptibility genes to IBD, there is currently no consensus (Orchard *et al.*, 2000). However, a recent genome-wide study for UC susceptibility genes was performed in 1052 individuals with UC (Silverberg *et al.*, 2009). In this study, UC loci attaining genome-wide significance levels were identified on chromosome 1p36 and 12q15. In this current study, we investigated the effect of NADP(H):quinone oxidoreductase 1 (NQO1) and manganese-containing superoxide dismutase (SOD2) on UC. NQO1 is an obligate two-electron reductase that catalyzes the reduction of quinines, quinine-imines, and nitro-compounds (Ross *et al.*, 2000). NQO1 may also play an antioxidant role via the reduction of endogenous quinones. The reduction of endogenous quinones helps to protect cellular membranes against oxidative damage.

SOD plays an important role in the protection of cells from the products of oxidative stress. SOD is an enzyme that catalyzes the dismutation of superoxide radicals to hydrogen

[1]Department of Internal Medicine, Second Teaching Hospital Fujita Health University School of Medicine, Nagoya, Japan.
[2]Medical Examination Center, Yachiyo Hospital, Anjou, Japan.
[3]Department of Pathologic Oncology, Mie University Graduate School of Medicine, Tsu, Japan.
[4]Inflammatory Bowel Disease Center and Department of Surgery, Yokkaichi Social Insulance Hospital, Yokkaichi, Japan.
[5]Department of Public Health, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan.
[6]Graduate School of Engineering, Yokohama National University, Yokohama, Japan.

peroxide. Two types of SOD lead to the requirement of the metal species at the active site: copper- and zinc-containing SOD (CuZn-SOD or SOD1, cytoplasmic) and manganese-containing SOD (Mn-SOD or SOD2, mitochondrial) (Crapo *et al.*, 1992; Guidot *et al.*, 1993). The other form is recently discovered. The forms containing Cu and Zn also have an extracellular (EC-SOD or SOD3) location (Zelko *et al.*, 2002). SOD2 is a mitochondrial enzyme that quenches free radicals and protects against oxidative stress by converting superoxide radicals to H2O2.

In this study, we conducted the study to investigate whether *NQO1* and *SOD2* polymorphisms influence the clinical characteristics of UC. In addition, the allele and genotype frequencies of the *NQO1* and *SOD2* polymorphisms were compared between UC patients and controls because of the possible influence these two polymorphisms might have on the incidence and clinical features of UC.

Materials and Methods

Subjects

We investigated 134 patients with UC (77 males and 57 females) and 125 healthy controls (67 males and 58 females). The UC patients were recruited between September 2004 and April 2007. The diagnosis of UC was based on conventional clinical, radiologic, endoscopic, and pathologic criteria. The characteristics of the UC patients are shown in Table 1. We analyzed the effect of *NQO1* and *SOD2* polymorphisms on the clinical characteristics. To assess the *NQO1* and *SOD2* polymorphisms in terms of influence on the incidence of UC, we compared the genotype frequencies of both polymorphisms in UC patients with those in controls.

Blood samples

Blood samples were collected from patients and controls after they had given informed consent to participate in this study. This study was approved by the Fujita Health University Ethics Committee. DNA was extracted from blood samples using a PUREGENE DNA isolation kit (Gentra Systems, Minneapolis, MN).

Table 1. Characteristics of Patients with Ulcerative Colitis

Age of onset (years)	36.7 ± 15.1
Colitis duration (years)	9.6 ± 7.8
Extension	
Proctitis	23 (17.2%)
Left-sided	58 (43.3%)
Pancolitis	53 (39.5%)
Type of clinical course	
First episode	16 (11.9%)
Chronic relapse	79 (59.0%)
Chronic persistent	39 (29.1%)
Severity	
Mild	40 (29.9%)
Moderate	60 (44.8%)
Severe	34 (25.3%)

The total number of patients with ulcerative colitis was 134. First episode, patients presenting with a first episode of colitis; chronic relapse, patients repeated relapse and remission; chronic persistent, patients continued active colitis over 6 months from first episode; severity is classified by Truelove and Witts' classification.

Genotypes

The *NQO1* C609T and *SOD2* Ala-9Val polymorphisms were recognized using polymerase chain reaction (PCR)–restriction fragment length polymorphisms. The primer sets were as follows (Olson *et al.*, 2004): for *NQO1*, 5′-ATTCTC TAGTGTGCCTGAG-3′ (forward) and 5′-AATCCTGCCTG GAAGTTTAG-3′ (reverse); for *SOD2*, 5′-ACCAGCAGGCA GCTGGCGCCGG-3′ (forward) and 5′-GCGTTGATGTGAG GTTCCAG-3′ (reverse). The PCR amplifications were performed in a 30-μL aliquot containing 50 ng of genomic DNA, 12 pmol of each primer, 3.0 μL of 10× buffer solution, 20 nmol/μL of dNTP, and 1 U of Taq polymerase. The PCR conditions for *NQO1* included an initial denaturation step at 95°C for 5 min, followed by 35 amplification cycles, each cycle containing denaturation at 95°C for 30 s, primer annealing at 60°C for 30 s, and extension at 72°C for 30 s, and then a final extension at 72°C for 7 min. PCR conditions for *SOD2* included an initial denaturation step at 95°C for 5 min, followed by 35 amplification cycles, each cycle containing denaturation at 95°C for 30 s, primer annealing at 63°C for 30 s, and extension at 72°C for 30 s, and then a final extension at 72°C for 7 min. The PCR products were subjected to overnight digestion with *Hin*fIV for *NQO1* at 37°C (Phillips *et al.*, 2004) and *Ngo*MIV for *SOD2* at 37°C (Akyol *et al.*, 2004) and then electrophoresed in 2% agarose gels. The digestion products gave the following band patterns: for *NQO1*, 318 bp for the wild-type C/C; 164 and 154 bp for the variant T/T; and 318, 164, and 154 bp for the heterozygous C/T (Fig. 1). For *SOD2*, the digestion products gave the following band patterns: 107 bp for the wild-type T/T; 89 and 18 bp for the variant C/C; 107, 89, and 18 bp for the heterozygous C/T (Fig. 2). Some of the digested *NQO1* and all of the *SOD2* products were examined on 12.5% polyacrylamide gels (Gene Gel Excel 12.5/24 kit from GE Healthcare Bio-Sciences, Tokyo, Japan) and stained with a DNA silver staining kit (GE Healthcare Bio-Sciences). The *NQO1* T/T genotype was expected to show two DNA bands of 164 and 154 bp; however, in this study, the T/T genotype resolved as a single band on an agarose gel. The two bands were clearly resolved on a polyacrylamide gel. The 164 and 154 bp bands showed a similar electrophoretic pattern for the C/T genotype. The band of 18 bp for the *SOD2* C/T and C/C genotypes was not clearly identified because the band was very small; however, the 107 and 89 bp bands were clearly recognized on a polyacrylamide gel. The results of PCR–restriction fragment length polymorphisms were confirmed by direct sequencing. DNA was extracted from the agarose gels using an extraction kit (Qiagen, Hilden, Germany). The genotypes were confirmed by sequence analysis using an auto sequencer (data not shown).

Statistical analysis

All data were analyzed by STATA10.0 (Stata Corporation, College Station, TX). The clinical features of UC, allele frequency, and genotype distribution were evaluated by a logistic regression test. Each polymorphism was tested to ensure that it did not deviate from Hardy–Weinberg equilibrium by the χ^2-test.

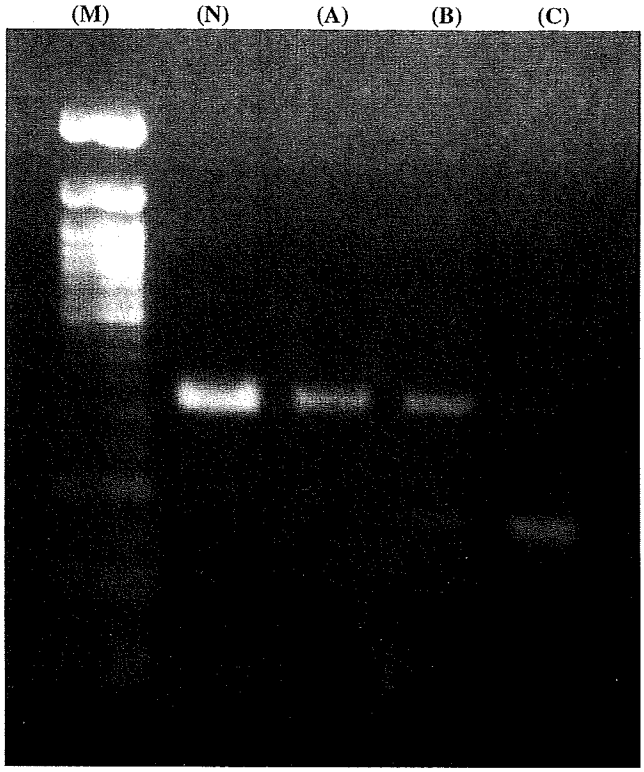

FIG. 1. Electrophoresed pattern of *NQO1* polymorphism. (**M**), marker; (**N**), no digestion (318 bp); (**A**), C/C genotype (318 bp); (**B**), C/T genotype (318 and 164 bp); (**C**), T/T genotype (164 bp). This figure shows the electrophoresed pattern of *NQO1* polymorphisms on 2% agarose gel stained with ethidium bromide. (**A**) This pattern was identified to be 318 bp for C/C wild type. (**B**) This pattern was identified to be a heterozygous type for C/T. (**C**) This pattern was identified to be a variant type for T/T. The *NQO1* T/T band genotype was expected to show two DNA bands of 164 bp and 154 bp; however, in this study, the T/T genotype resolved as a single band on agarose gel. The two bands were clearly resolved on a polyacrylamide gel.

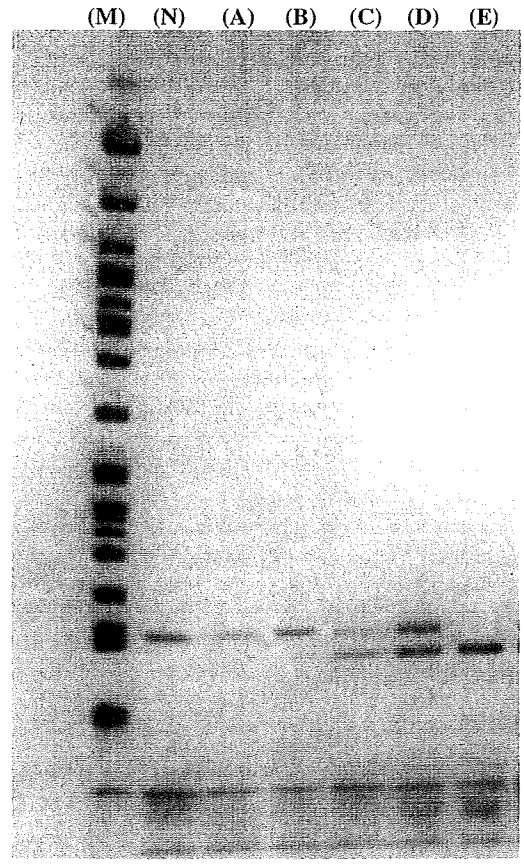

FIG. 2. Electrophoresed pattern of *SOD2* polymorphism on polyacrylamide gel. (**M**), marker; (**N**), no digestion (107 bp); (**A**) and (**B**), T/T genotype (107 bp); (**C**) and (**D**), C/T genotype (107 and 89 bp). (**E**), C/C genotype (89 bp). This figure showed the electrophoresed pattern of *SOD2* polymorphism on 12.5% polyacrylamide gel. (**A**) and (**B**) are patterns that were identified to be wild type for T/T. (**C**) and (**D**) are patterns that were identified to be a heterozygous type for C/T. (**E**) is a pattern that was identified to be a variant type for C/C. The band of 18 bp for C/T and C/C genotypes was not clearly identified because the band was very small; however, the 107 and 89 bp bands were clearly recognized on a polyacrylamide gel.

Results

NQO1 *and* SOD2 *polymorphism genotypes*

The allele frequencies of the *NQO1* and *SOD2* polymorphisms and the genotype frequencies are shown in Table 2. Three control samples could not be genotyped for the *SOD2* polymorphism. The genotype data were consistent with the Hardy–Weinberg equilibrium (*NQO1*—patient group: $\chi^2 = 2.79$, $p = 0.10$; control group: $\chi^2 = 0.01$, $p = 0.90$) (*SOD2*—patient group: $\chi^2 = 1.82$, $p = 0.18$; control group: $\chi^2 = 3.59$, $p = 0.06$). In the control group, the frequencies of the C/C, C/T, and T/T genotypes for the *NQO1* polymorphism were 39.2%, 46.4%, and 14.4%, respectively, whereas the frequencies of the C/C, C/T, and T/T genotype for the *SOD2* polymorphism were 0.8%, 34.4%, and 64.8%, respectively. In the UC patients, the genotype and allele frequencies of neither the *NQO1* nor the *SOD2* polymorphisms differed significantly from the controls.

Influence of NQO1 *and* SOD2 *polymorphisms on the clinical characteristics of UC*

We attempted to identify an association between the *NQO1* and *SOD2* polymorphisms and the clinical features of

TABLE 2. GENOTYPE FREQUENCY OF NAD(P)H:QUINONE OXIDOREDUCTASE 1 AND SUPEROXIDE DISMUTASE

	UC (n = 134)/ *control* (n = 125)	*OR (95% CI)*	p
NQO1			
C/C/ + C/T	109/107	1.00 (referent)	
T/T	25/18	0.73 (0.37–1.42)	0.354
SOD2			
C/C + C/T	39/44[a]	1.00 (referent)	
T/T	95/78	0.73 (0.43–1.24)	0.243

The genotype frequency of *NQO1* and *SOD2* is compared between UC and control. In the UC patients, the genotype and allele frequencies of neither the *NQO1* nor the *SOD2* polymorphisms differed significantly from the controls.

[a]Three control samples could not be genotyped for *SOD2* polymorphism.

OR, odds ratio; CI, confidence interval; *NQO1*, NAD(P)H:quinone oxidoreductase 1; *SOD2*, superoxide dismutase.

the UC patients (age of onset, sex, nature of clinical course, severity of colitis, and steroid resistance). The severity of colitis was classified using the Truelove and Witts' classification (Truelove *et al.*, 1978). We defined the steroid-resistant group as patients who did not have remission with a conventional steroid dose, and required surgery or other therapy, such as plasma exchange. The association between the clinical characteristics of the UC patients and the two polymorphisms are shown in Tables 3 and 4. Regarding the severity of colitis, more patients with the *NQO1* T/T genotype developed severe colitis compared to the other genotypes (odds ratio [OR] 2.20, 95% confidence interval [CI] 0.69–6.95). In Table 5, the data of steroid resistance in the UC patients and the two polymorphisms are summarized. A significantly greater number of patients with the *NQO1* T/T genotype showed steroid resistance than the other genotypes (OR 9.20, 95% CI 2.19–38.7, $p = 0.002$).

For the patients whose onset of UC was age 20 years or younger, more patients had the *SOD2* T/T genotype than the other genotypes (OR 6.56, 95% CI 0.83–51.7, $p = 0.074$). Considering the other factors investigated, such as the severity of colitis, there was no difference in the *SOD2* polymorphisms among the patients.

Discussion

In this study, we investigated the association between polymorphisms related to oxidative stress and UC. The *NQO1* gene is located on 16q22 (Jaiswal, 1991). The *NQO1* C609T polymorphism encoded by codon 187 in exon 6 (Traver *et al.*, 1997) includes a single C-to-T substitution of the *NQO1* cDNA that causes a Pro187Ser amino acid change. This substitution leads to a reduction in the activity of the enzyme C/T heterozygotes and no activity in T/T homozygotes (Siegel *et al.*, 1999). The T allele of *NQO1* may be associated with an increased risk of various type of malignancy, including leukemia (Larson *et al.*, 1999; Naoe *et al.*, 2000), esophageal cancer (Zhang *et al.*, 2003), and colorectal cancer (Lafuente *et al.*, 2000).

Table 3. Clinical Features of Ulcerative Colitis and NAD(P)H:Quinone Oxidoreductase 1 Polymorphism

Clinical feature	NQO1 *genotype* C/C+ C/T/T/T	*OR (95% CI)*	p
Age			
<20	12/3	1.00 (referent)	
≥20	97/22	0.89 (0.23–3.46)	0.871
Sex			
Male	65/12	1.00 (referent)	
Female	44/13	1.55 (0.65–3.72)	0.329
Extension			
Proctititis	21/2	0.57 (0.11–2.95)	0.499
Left-sided	45/14	1.83 (0.68–5.04)	0.228
Pancolitis	43/8	1.00 (referent)	
Type of clinical course			
First episode	11/5	1.00 (referent)	
Chronic relapse	67/12	0.38 (0.11–1.34)	
Chronic persistent	31/8	0.56 (0.15–2.17)	0.321
Severity			
Mild	34/6	1.00 (referent)	
Moderate	51/9	0.93 (0.32–3.10)	
Severe	24/10	2.20 (0.75–7.51)	0.203

The table shows the relationship between clinical features of UC and *NQO1* genotype. Regarding the severity of colitis, more patients with the *NQO1* T/T genotype developed severe colitis compared to the other genotypes (OR 2.20).

Table 4. Clinical Features of Ulcerative Colitis and Superoxide Dismutase Polymorphism

Clinical feature	SOD2 *genotype* T/T/C/C+C/T	*OR (95% CI)*	p
Age			
<20	14/1	1.00 (referent)	
≥20	81/38	6.56 (0.83–51.7)	0.074
Sex			
Male	56/22	1.00 (referent)	
Female	39/17	1.09 (0.51–2.31)	0.826
Extension			
Proctititis	15/7	0.95 (0.32–2.78)	0.925
Left-sided	45/14	0.58 (0.25–1.33)	0.197
Pancolitis	34/18	1.00 (referent)	
Type of clinical course			
First episode	11/5	1.00 (referent)	
Chronic relapse	26/14	0.75 (0.23–2.45)	
Chronic persistent	58/20	1.19 (0.34–4.15)	0.746
Severity			
Mild	27/11	1.00 (referent)	
Moderate	46/14	0.72 (0.29–1.83)	
Severe	22/14	1.50 (0.57–3.99)	0.489

The table shows the relationship between clinical features of UC and *SOD2* genotype. For the patients whose onset of UC was age 20 years or younger, a higher proportion had the *SOD2* T/T genotype than the other genotypes (OR 6.56, $p = 0.074$).

Table 5. Steroid Resistance with Ulcerative Colitis and Two Polymorphisms

Steroid resistance	*Genotype*	*OR (95% CI)*	p
NQO1	C/C+C/T/T/T		
Steroid effective ($n = 65$)	58/7	1.00 (referent)	
Steroid resistance ($n = 18$)	11/7	9.20 (2.19–38.7)	0.002
SOD2	T/T/C/C+C/T		
Steroid effective ($n = 65$)	44/21	1.00 (referent)	
Steroid resistance ($n = 17$)	13/5	0.81 (0.25–2.61)	0.727

In this table, the data of steroid resistance in the UC patients and the two polymorphisms are summarized. A significantly greater number of patients with the *NQO1* T/T genotype showed steroid resistance than the other genotypes ($p = 0.002$).

The *SOD2* gene is located on 6q25 (Church *et al.*, 1992). The *SOD2* Ala-9Val polymorphism has a structural mutation of a T-to-C substitution in the coding sequence that changes the amino acid codon at the −9 position in the signal peptide from valine to alanine (Shimoda-Matsubayashi *et al.*, 1996). The C allele may confer an advantage to the SOD2 protein, rendering C/C genotype and C/T genotype than the T/T genotype (Sutton *et al.*, 2003). The Ala-9Val polymorphism has been associated with various diseases, such as Parkinson's disease (Shimoda-Matsubayashi *et al.*, 1996), nonfamilial idiopathic cardiomyopathy (Hiroi *et al.*, 1999), breast cancer (Ambroson *et al.*, 1999), and colorectal cancer (Stoehlmacher *et al.*, 2002).

This study provided two interesting findings of the *NQO1* polymorphism associated with UC. First, a significantly greater number of patients with the *NQO1* T/T genotype showed steroid resistance than the other genotypes. Second, more patients with the *NQO1* T/T genotype contracted severe colitis than the other genotypes. According to a previous report, the T/T genotype for *NQO1* C609T polymorphism has a null phenotype. NQO1 plays a role as a superoxide scavenger. Because of its role, NQO1 protects against superoxide-induced toxicity. Kruidenier *et al.* (2003) reported that intestinal inflammation was accompanied by excessive production of reactive metabolites. In addition, nitric oxide synthase and xanthine oxidase were both associated with oxidative stress in IBD. No studies have yet shown that NQO1 directly influences oxidative stress in IBD. However, patients with the *NQO1* T/T genotype may not prevent oxidative stress in the intestinal mucosa because the T/T genotype suppresses the activity of this enzyme. The suppression of NQO1 may influence the severity of UC.

The precise mechanism of steroid resistance in UC remains unclear. About 20–30% of the patients with severe acute UC make a poor response to corticosteroids (Hyde and Jewell, 1997). Involvement of genetic polymorphisms has been previously reported. Farrell and Kelleher (2003) reviewed glucocorticoid resistance in IBD. This review focused on the molecular mechanism of glucocorticoid resistance for the multidrug resistance gene (*MDR1*). The *MDR1* gene codes for a drug efflux pump, P-glycoprotein (Hoffmeyer *et al.*, 2000). MDR1 expression was elevated in UC and CD patients who required bowel resection for failed medical therapy (Farrell *et al.*, 2000). This suggests that IBD patients who failed to respond to medical therapy might escape effective immunosuppression by steroids and other immunosuppressive agents, because these drugs were MDR substrates and were pumped out of the target cells due to P-glycoprotein–mediated efflux. The association between *MDR1* polymorphism and cyclospoline A failure was investigated in patients with steroid resistant UC (Daniel *et al.*, 2007). The TT genotype in exon 21 of *MDR1* was associated with a higher risk of cyclospoline A failure in these patients.

Single-nucleotide polymorphisms of Solute Carrier Family (*SLC*) *22A4/5* have received greater clinical attention because they can modulate their transporter functions, causing individual variations in drug responsiveness (Newman *et al.*, 2005). In a Japanese study, the association of *SLC22A4/5* with steroid responsiveness of IBD was investigated (Nakahara *et al.*, 2008). The CG haplotype, comprising the C allele of the −446C > T and −368T > G in *SLC22A5* appeared to be a predictor of steroid resistance in Japanese patients with CD.

The previous report proposed that the criteria to predict patients with severe UC would respond poorly to intensive medical treatment and require colectomy (Travis *et al.*, 1996). According to the criteria, patients with frequent stools (>8/day), or raised C-reactive protein (CRP) levels (>450 mg/L) after 3 days of intensive treatment, need to be identified, as most will require colectomy on that admission. The criteria suggested that patients with severe UC applied to the criteria would respond poorly to intravenous corticosteroid therapy and require colectomy. Severe colitis of UC may induce steroid resistance (Esteve *et al.*, 2008). However, severe colitis does not necessarily resulted in steroid resistance; it is thought that severe colitis is assumption of steroid resistance.

Several factors cause steroid resistance, including oxidative stress. It has been reported that oxidative stress affected steroid resistance because of reduced Histone deacetylase 2 (HDAC-2) activity (Marwick *et al.*, 2007). Chromatin is subjected to a variety of posttranslational modifications known as the histone code that ultimately affect gene transcription. This modification such as acetylation and deacetylation are programmed by two families of enzymes referred to as histone acetyltransferases and HDACs. HDAC-2 plays a pivotal role in corticoid action, and recruits corticosteroid through the glucocorticoid receptor. Under condition of oxidative stress, HDAC-2 is inactivated and cannot be recruited into the corepressor by glucocorticoid receptor. This mechanism results in steroid resistance. Patients with *NQO1* T/T genotype have insufficient prevention of oxidative stress. The insufficient prevention of oxidative stress causes steroid resistance in patients with *NQO1* T/T genotype.

In the patients with an age of onset at age 20 years or younger, more had the *SOD2* T/T genotype than the other genotypes, suggesting that the T/T genotype might be associated with the age of onset of UC. As for the significance of the relatively early disease onset, the *SOD2* gene might influence the onset of disease directly or by acting upon another gene or factor. However, Taufer *et al.* (2005) showed that the *SOD2* polymorphism was associated with DNA damage in association with oxidative stress. These authors assumed that SOD2 supported the free radical theory of aging. Another author suggested that the T allele resulted in less effective targeting of SOD2 (Shimoda-Matsubayashi *et al.*, 1996). Our results may indicate that this genotype is associated with insufficient prevention of oxidative stress, and the resulting increase in oxidative stress may cause the development of colitis at a younger age.

In conclusion, the *NQO1* C609T polymorphism may influence the severity of UC and steroid resistance in UC patients, while the *SOD2* Ala-9Val polymorphism may influence the age at onset of UC. Further studies are needed to investigate the effect of *NQO1* and *SOD2* polymorphisms on UC.

Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Junji Yoshino, M.D.
Department of Internal Medicine
Second Teaching Hospital Fujita Health University
School of Medicine
3-6-10 Otobashi, Nakagawaku
Nagoya 454-8509
Japan

E-mail: jyoshino@fujita-hu.ac.jp

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Genotoxicity of nano/microparticles in *in vitro* micronuclei, *in vivo* comet and mutation assay systems

Yukari Totsuka*[1], Takashi Higuchi[1], Toshio Imai[2], Akiyoshi Nishikawa[3], Takehiko Nohmi[4], Tatsuya Kato[5], Shuich Masuda[5], Naohide Kinae[5], Kyoko Hiyoshi[6], Sayaka Ogo[7], Masanobu Kawanishi[7], Takashi Yagi[7], Takamichi Ichinose[8], Nobutaka Fukumori[9], Masatoshi Watanabe[10], Takashi Sugimura[1] and Keiji Wakabayashi[1]

Address: [1]Cancer Prevention Basic Research Project, National Cancer Center Research Institute, 1-1 Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan, [2]Central Animal Laboratory, National Cancer Center Research Institute, 1-1 Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan, [3]Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan, [4]Division of Genetics and Mutagenesis, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan, [5]Department of Food and Nutritional Sciences, Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, 52-1, Yada, Shizuoka, 422-8526, Japan, [6]Fundamental Nursing, School of Nursing, University of Shizuoka, 52-1, Yada, Shizuoka, 422-8526, Japan, [7]Environmental Genetics Laboratory, Frontier Science Innovation Center, Osaka Prefecture University, 1-2 Gakuen-cho Naka-ku, Sakai-city, Osaka, 599-8570, Japan, [8]Department of Health Sciences, Oita University of Nursing and Health Sciences, 2944-9 Megusuno, Oita-city, Oita, Japan, [9]Department of Environmental Health and Toxicology, Tokyo Metropolitan Institute of Public Health, 24-1, Hyakunin-cho 3-Chome, Shinjuku-ku, Tokyo, 169-0073, Japan and [10]Division of Materials Science and Engineering, Yokohama National University, Graduate School of Engineering, 79-5, Tokiwadai, Hodogaya-ku, Yokohama, 240-8501, Japan

Email: Yukari Totsuka* - ytotsuka@gan2.res.ncc.go.jp; Takashi Higuchi - tahiguch@ncc.go.jp; Toshio Imai - toimai@ncc.go.jp; Akiyoshi Nishikawa - nishikaw@nihs.go.jp; Takehiko Nohmi - nohmi@nihs.go.jp; Tatsuya Kato - p7107@mail.u-shizuoka-ken.ac.jp; Shuich Masuda - masudas@u-shizuoka-ken.ac.jp; Naohide Kinae - kinae@u-shizuoka-ken.ac.jp; Kyoko Hiyoshi - hiyoshi@u-shizuoka-ken.ac.jp; Sayaka Ogo - es04509@riast.osakafu-u.ac.jp; Masanobu Kawanishi - kawanisi@riast.osakafu-u.ac.jp; Takashi Yagi - yagi-t@riast.osakafu-u.ac.jp; Takamichi Ichinose - ichinose@oita-nhs.ac.jp; Nobutaka Fukumori - Nobutaka_Fukumori@member.metro.tokyo.jp; Masatoshi Watanabe - mawata@ynu.ac.jp; Takashi Sugimura - tsugimur@ncc.go.jp; Keiji Wakabayashi - kwakabay@ncc.go.jp

* Corresponding author

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Abstract

Background: Recently, manufactured nano/microparticles such as fullerenes (C_{60}), carbon black (CB) and ceramic fiber are being widely used because of their desirable properties in industrial, medical and cosmetic fields. However, there are few data on these particles in mammalian mutagenesis and carcinogenesis. To examine genotoxic effects by C_{60}, CB and kaolin, an *in vitro* micronuclei (MN) test was conducted with human lung cancer cell line, A549 cells. In addition, DNA damage and mutations were analyzed by *in vivo* assay systems using male C57BL/6J or *gpt* delta transgenic mice which were intratracheally instilled with single or multiple doses of 0.2 mg per animal of particles.

Results: In *in vitro* genotoxic analysis, increased MN frequencies were observed in A549 cells treated with C_{60}, CB and kaolin in a dose-dependent manner. These three nano/microparticles also induced DNA damage in the lungs of C57BL/6J mice measured by comet assay. Moreover, single or multiple instillations of C_{60} and kaolin, increased either or both of *gpt* and Spi$^-$ mutant frequencies in the lungs of *gpt* delta transgenic mice. Mutation spectra analysis showed transversions were

predominant, and more than 60% of the base substitutions occurred at G:C base pairs in the *gpt* genes. The G:C to C:G transversion was commonly increased by these particle instillations.

Conclusion: Manufactured nano/microparticles, CB, C_{60} and kaolin, were shown to be genotoxic in *in vitro* and *in vivo* assay systems.

Background

Nano/microparticles are widely used because of their desirable properties in industrial, medical and cosmetic fields [1-6]. Accordingly, these particles can be released into the human environment and then can be inhaled. Most exposure to airborne nano/micromaterials occurs in the work place. Nano/microparticles can be classified into three groups: natural, anthropogenic and man-made (or artificial). The natural kind, for example, is produced during forest fires or volcanic eruptions. Anthropogenic particles are quite often a by-product of industrial activities such as welding or polishing. Diesel exhaust products, PM10 and PM2.5, well known as combustion nanoparticles, also belong to this group. The man-made group includes engineered nanomaterials [5].

Among these nano/mocroparticles, diesel exhaust particles have been well documented, in their general toxicity, mutagenicity and carcinogenicity [7-10]. In addition, asbestos, a naturally occurring nano-sized silicate mineral fiber, has been considered to be a human carcinogen [11-13]. Animal experiments and epidemiological studies have already demonstrated that pulmonary fibrosis, bronchogenic carcinomas and malignant mesotheliomas are closely associated with asbestos exposure. Another mineral fiber, titanium dioxide (TiO_2) has also been subjected to extensive research, and TiO_2 has already been shown to be carcinogenic [14]. Moreover, man-made vitreous fibres, including glass fibres, refractory ceramic fibres, and rock wool, have been sorted as carcinogens [15]. Kaolin/kaolinite is a clay mineral with the chemical composition $Al_2Si_2O_5(OH)_4$, and is used in ceramics, medicines, food additives, toothpaste and cosmetics. The largest use of kaolin is in the production of paper [3]. In 1993, W. B. Bunn 3rd *et al.* reported that increased incidences of lung tumors and mesotheliomas were observed in long-term inhalation studies of rats and hamsters treated with micro-sized refractory ceramic fibres containing kaolin as the main component [16]. However, other genotoxic and carcinogenic potentials of kaolin have not been studied *in vitro* and *in vivo*. In addition, the mechanism of cancer development by kaolin is still unclear.

On the other hand, carbon black (CB), fullerenes (C_{60}) and carbon nanotubes (CNTs) are developed as engineered nanoproducts [1,2,6,17]. Despite their highly desirable structures, their toxicity and carcinogenicity are concerns because these engineered nanoproducts are considered to be very stable and could lead to continuous inflammation when deposited in tissues. CNTs especially have received much attention from the aspect of toxicity due to their asbestos-like rod-shaped particles, and iron content [17-19]. Recently Takagi *et al.* demonstrated that multi-wall carbon nanotubes induced mesothelioma in *p53*+/- mice by a single i.p. injection [20]. In contrast, C_{60} is a spherical molecule consisting entirely of carbon atoms, and various derivatives have been reported [6,21,22]. C_{60} has widely different properties, such as scavenging of reactive oxygen species, direct interaction with biomolecules and radical formation; however, clear genotoxic and carcinogenic effects have not yet been demonstrated.

The present study aims to examine the genotoxicity/clastogenicity of widely distributed nano/microparticles such as C_{60}, CB and kaolin by an *in vitro* micronucleus test. Moreover, we analyzed the genotoxic effects of these particles by an *in vivo* comet assay and mutation assay system using *gpt* delta transgenic mice. In this mouse model, point mutations and deletions are separately analyzable by *gpt* and Spi$^-$ selections, respectively [23,24]. The mutation assay using the *gpt* delta mouse was validated and so far is widely used in the field of environmental mutagenicity.

Results

Size distribution and agglomeration state in suspensions of nano/microparticles

Figure 1 shows representative transmission electron microscope (TEM) images for the state of test materials

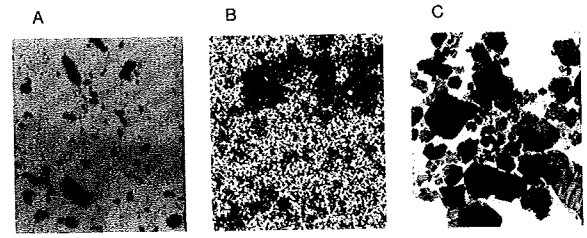

Figure 1
Representative TEM images of the presently used nano/microparticles within the suspensions. C_{60} (PanelA), CB (Panel B) and kaolin (Panel C) were suspended in saline containing 0.05% Tween 80 at a concentration of 2 mg/mL with a 10 min sonication. All images are shown at the original magnification of × 10,000.

dispersed in saline containing 0.05% Tween 80. These were commonly observed to be a mixture of well dispersed fine particles and agglomerates. C_{60} was frequently agglomerated, but fine particles were also observed either individually or within pear-shaped agglomerates. In contrast, CB was relatively well dispersed, and agglomerates were occasionally present. In the case of kaolin, low-density tabular structures with rectangular or hexagonal shape were characteristically observed. The size distribution of materials used in the present study was analyzed by dynamic light scattering (DLS). C_{60} demonstrated a wide distribution with ranges of 10.5 to 12913.9 nm, and most abundant sizes were two peaks at 234.1 ± 48.9 and 856.5 ± 119.2 nm, respectively. CB particles formed a normal distribution with ranges of 13.6 to 337.4 nm and major peak average was at around 232.0 nm. In the case of kaolin, a major peak average was 357.6 ± 199.4 nm belonging to a range of 5.1 to 4846.9 nm. Although the primary particle size of kaolin was 4.8 μm, it is likely that sonication might lead to size reduction.

In vitro *micronucleus test*

To examine the genotoxicity of particles, we analyzed the micronucleus inducing activity of C_{60}, CB and kaolin using human lung cancer cell line, A549. A six-hour treatment of 200 μg/mL CB and kaolin caused growth inhibition of 60% in A549 cells; however, C_{60} did not inhibit growth of cells at any concentrations (between 0.02 - 200 μg/mL, data not shown). As shown in Figure 2, C_{60} and kaolin particles increased the number of micronucleated cells in a dose-dependent manner. On the other hand, CB increased the number of micronucleated cells up to 2 μg/mL, and thereafter seemed to plateau. The background frequency of micronucleated cells was 0.7% to 1.0%, and the frequency rose to 10% and 5% at 200 μg/mL of C_{60} and kaolin, respectively, and 3.3% at 2 μg/mL of CB treatment. The increase of the frequency from that of the control cells was statistically significant in all particle-treated cells. C_{60} demonstrated the most strong genotoxic/clastogenic potencies among these three particles.

In vivo *genotoxicity analyzed by alkaline comet assay*

DNA damage induced by particles was evaluated using comet assay under alkaline conditions. Figure 3 shows the mean values of DNA tail moment in the lungs with or without single-particle treatment at 0.2 mg/body for 3 hr. In the case of particle exposure, DNA damage was significantly increased as compared with the vehicle control up to 2 - 3 fold, and its intensity was C_{60} > CB > kaolin. On the other hand, we examined the genotoxicity of nano/microparticles at a dose of 0.05 mg/animal. DNA damage observed in the lung of mice was almost the same as those of the vehicle control (data not shown). Moreover, we examined the effects of different exposure times for 3 and 24 hr. While DNA damages induced by CB or kaolin were not changed either for 3 or 24 hr, DNA damage caused by C_{60} was decreased for 24 hr compared with 3 hr (data not shown). It seems that DNA damage repair enzymes might affect the result of comet assay.

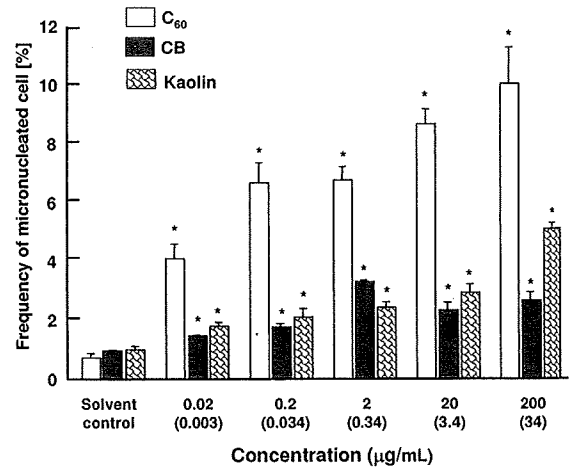

Figure 2
Frequency of micronucleated A549 cells incubated with C_{60} CB or kaolin. The values represent the mean of three experiments ± SD. An asterisk (*) represents that each frequency is significantly different ($p < 0.01$) from that of control cell in a Student's t-test. Concentrations in μg/cm² are given in parenthesis.

General observations of gpt *delta transgenic mice administrated with particles*

Body weights of *gpt* delta mice receiving a single dose of vehicle control reached 31.1 ± 1.8 g at 12 weeks after instillation. Values for *gpt* delta mice which received a single dose of particles at 0.2 mg/body were 30.0 ± 2.4 g for C_{60}, 32.6 ± 1.1 g for CB and 30.8 ± 2.3 g for kaolin, respectively, at 12 weeks after instillation. The average consumption of diet per day per mouse was 3.6 g, with no effects from particle instillation. No body weight and diet consumption changes were also observed with multiple doses of particles. All mice used for the single dose study survived to the end of the study, although, in the case of multiple doses, one fullerene- and one kaolin-administrated mouse died within two weeks after the last instillation, probably due to respiratory disturbances.

gpt *Mutations in the lungs of* gpt *transgenic mice with particle treatment*

To determine the mutagenic effects of particles in the lungs, *gpt* delta transgenic mice were exposed to C_{60}, CB and kaolin at doses of 0.2 mg/body by single intratracheal instillation, and mutations were analyzed. Figure 3 shows the mutant frequencies (MFs) of the lungs. The back-

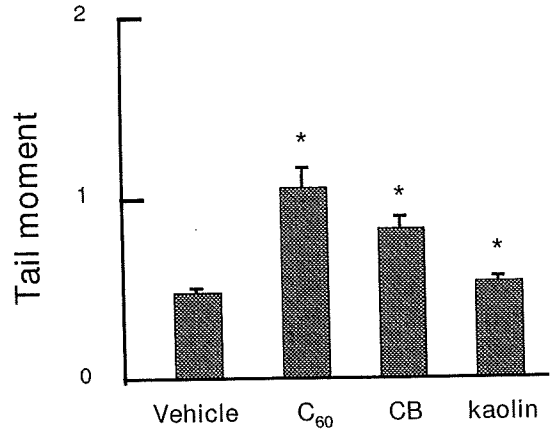

Figure 3
DNA damage in lungs of C57BL/6J mice intratracheally instilled with particles. DNA damage was measured by comet assay. Male mice were treated at a dose of 0.2 mg per animal of particles, and mice were sacrificed 3 hr after particle administrations. The values represent the mean of five animals ± SE. An asterisk (*) denotes $p < 0.01$ in a Dunnett's test after one-way ANOVA of Tail Moment of particle-treated vs. corresponding vehicle-control mice.

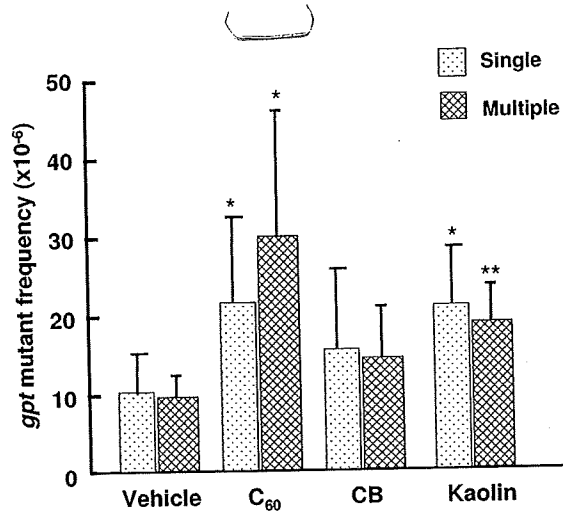

Figure 4
***gpt* MFs in the lungs of mice singly and multiply intratracheally instilled with particles.** Male mice were treated with single (0.2 mg per animal) or multiple (0.2 mg per animal × 4) doses of particles, and mice were sacrificed 12 (single) and 8 (multiple) weeks after particle administrations. Mean values ± SD are shown. An asterisk (*, **) denotes $p < 0.05$ (*) and $p < 0.01$ (**) in a Student's *t*-test of MF of particle-treated vs. the corresponding vehicle-control mice.

ground MF of lungs was 10.30 ± 0.53 × 10^{-6}. MFs in the lungs induced by C_{60} and kaolin were significantly increased by 2-fold compared with vehicle-instilled animals. CB showed increasing tendency for MF in the lungs, but not statistically significant.

Next, we examined the mutagenic effects of consecutive exposure of particles. The *gpt* MFs in the lungs obtained from mice multiply exposed (4 times) to 0.2 mg/body each of C_{60}, CB or kaolin are shown in Figure 4. In cases of C_{60} and kaolin, MFs of the lungs were significantly higher as compared to those of control animals, and their values were 2 - 3 fold increased. In the case of CB exposure, MFs were slightly increased but not statistically significant.

To analyze the mutational characteristics induced by particles, we examined PCR and DNA sequencing analysis of 6-thioguanine (6-TG)-resistant mutants. More than 40 independent 6-TG resistant mutants derived from multiple particle instillation (0.2 mg × 4), and 25 mutants from vehicle instilled animals were identified. Classes of mutations found in the *gpt* gene are listed in Table 1. Base substitutions predominated with both particle-induced and spontaneous cases. No A:T to T:A and G:C to C:G transversions were detected in vehicle control groups, indicating that these types of mutations are rare events in the spontaneous mutations. Interestingly, G:C to C:G transversion commonly increased in all three particle treatments compared to the vehicle control. G:C to A:T transition also significantly increased in CB and kaolin instillation but not in C_{60}. In addition, the numbers of A:T to T:A transversion were slightly increased in the treatment with C_{60} and CB. Other types of mutations, including deletions and insertions, were also observed in both particle-treated and vehicle control groups, but these were of minor significance.

The distribution of spontaneous and particle-induced mutations in the *gpt* gene is shown in Figure 5. Base substitutions were spread throughout the coding region with a preference for some sites. However, clear mutational hotspots for each particle could not be seen except deletion mutations occurring at a run of 5 adenines (positions 8 to 12) and at position 244 for C_{60} treatment. The distribution of base substitutions along the *gpt* gene did not vary with the particle types. Twelve out of 200 particle-induced mutations occurred at position 64, eighteen at position 110, ten at position 115. All of the base substitutions occurring at positions 110 and 115 were G to A transitions, and at position 64 were C to T transitions, which were common among spontaneous mutants. In contrast, four to eight mutations occurred at positions 116, 143,

Table 1: Classification of *gpt* mutations from the lungs of control and particle multiply (0.2 mg × 4) treated mice[a)]

	Control		C_{60}		CB		Kaolin	
Type of mutation in gpt	No.	%	No.	%	No.	%	No.	%
Base substitutions								
Transitions	10	40	35	41	18	45	37	50
A:T->G:C	2	8	11	13	2	5	5	7
G:C->A:T	8	32	24	28	16	40	32	43
Transversions	10	40	35	40	17	43	30	41
A:T->T:A	0	0	2	2	1	3	0	0
A:T->C:G	2	8	3	3	4	10	5	7
G:C->T:A	8	32	25	29	8	20	17	23
G:C->C:G	0	0	5	6	4	10	8	11
Deletions	4	16	12	14	4	10	6	8
Insertions	1	4	3	4	0	0	1	1
Others	0	0	1[b)]	1	1[c)]	3	0	0
Total	25	100	86	100	40	101	74	100

[a)]Independent mutations were isolated no more than once from any individual mouse.
[b)]Multiple mutation (Four base substitutions)
[c)]Tandem mutation (GG->TT)

189, 320, 406 and 418 were only seen in the particle-treated mice, therefore it is suggested that these mutations can be considered as particle-induced mutations. Among these, five out of six mutations at position 406 were found in C_{60} instillation, and all mutation patterns were G to T transversions. Four out of 7 and five out of 8 at positions 189 and 418 were detected in kaolin instillation, and the majorities of the mutations were G to A and C to A, respectively. Moreover, these hotspots induced by particles occurred at G or C residues in the *gpt* gene without association for specific sequences.

Spi⁻ MFs in the lungs of gpt *transgenic mice with particle treatment*

We also measured the Spi⁻ MFs in the lungs of *gpt* delta mice instilled with multiple doses (0.2 mg × 4) of particles (Figure 6). Spi⁻ MFs of the vehicle control was 4.85 ± 2.04 × 10^{-6}, in contrast, particle-administrated groups were 4.91 ± 3.03 × 10^{-6} for C_{60}, 6.87 ± 4.06 × 10^{-6} for CB and 8.12 ± 3.32 × 10^{-6} for kaolin. As shown in Figure 6, Spi⁻ MFs in the lungs of the CB- and kaolin-treated, but not C_{60}-treated groups were increased, and in particular, the values of the kaolin-treated groups were significantly elevated up to 2-fold.

gpt *Mutations in the kidneys of* gpt *transgenic mice with particle treatment*

To determine the tissue distribution and specificity of particles with intratracheal instillation, *gpt* MFs of the kidney were analyzed. *gpt* MFs of the vehicle control versus particle-multiple administrated groups (0.2 mg × 4) were 1.33 ± 0.51 × 10^{-5} versus 1.67 ± 0.66 × 10^{-5} for C_{60}, 1.03 ± 0.39 × 10^{-5} for CB and 1.32 ± 0.32 × 10^{-5} for kaolin. From these observations, it is suggested that these particles did not induce mutation in the kidneys under these conditions.

Histopathological evaluation

Histopathological analyses of lung tissues of *gpt* delta mice consecutively instilled particles, C_{60}, CB and kaolin, at 0.2 mg/body per week for 4 weeks each are shown in Figure 7. Test substances-phagocytized alveolar macrophages were diffusely found in the lungs, but not in the vehicle group. Focal granulomatous formation accompanied with or without the test substance-phagocytized macrophages were also frequently observed in the lungs of particle-multiply-instilled mice. Similar findings, but a slight degree of particle accumulation and granuloma formation, were also observed in lungs of mice with particle single-instillations (data not shown). The degree of granuloma formation in the lungs of multiple C_{60}- or CB-exposed mice appeared more severe than those in multiple kaolin-exposed mice. No abnormalities were observed in the kidneys obtained from mice multiply instilled with particles (data not shown).

Discussion and conclusion

This study demonstrated the genotoxicity of nano/micro-particles widely used for industrial, cosmetic and medical fields. In *in vitro* genotoxic analysis, increased MN frequencies were observed in A549 cells treated with C_{60}, CB and kaolin in a dose-dependent manner. On the other hand, these three particles also induced DNA damage in the lungs of C57BL/6J mice measured by comet assay. Furthermore, we found that C_{60} and kaolin demonstrated mutagenicity either or both of *gpt* and Spi⁻ mutations in the *gpt* delta transgenic mice systems. The *gpt* gene MFs were significantly increased in the lungs of *gpt* delta mice with C_{60} and kaolin, but not CB administrations. A dose-dependent MF increase was observed in the lungs of C_{60}, but not kaolin treated groups. The reason is still unclear, but suggesting that the single dose of kaolin already repre-

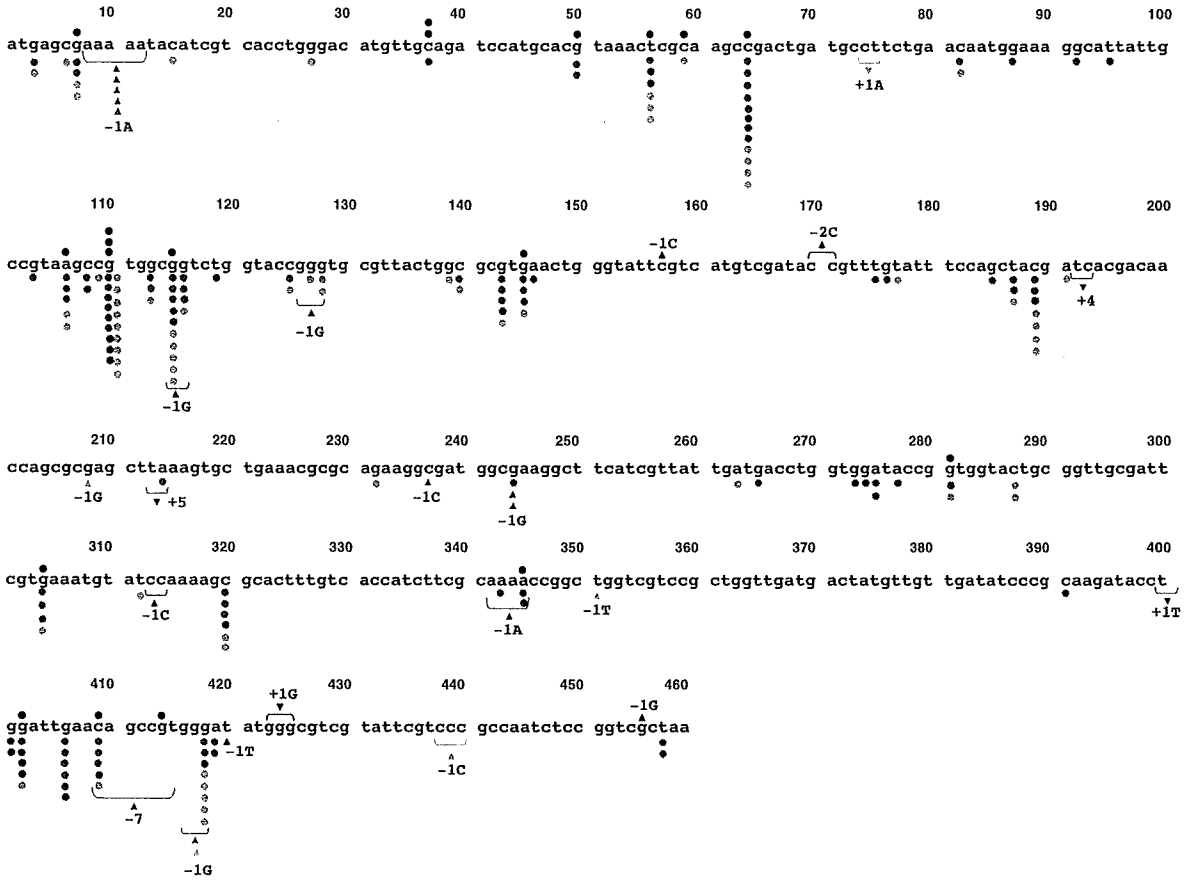

Figure 5

Spontaneous and particle-induced mutations in the coding region of the *gpt* gene. Mutations obtained from the control mice are shown above the wild type sequence, and mutations obtained from the particle-treated mutant clone are shown below the wild type sequence. The types of particles are indicated by color coding: red for C_{60}, blue for CB and sky blue for kaolin. Mutation types, base substitution, and deletion and insertion are indicated by circle, triangle, and inverted triangle, respectively.

sented the maximum response. On the other hand, kaolin demonstrated significantly increased Spi- MFs; however, C_{60} showed similar values compared with the vehicle control of the lungs. Spi- selection detects deletions in size more than 1 bp and 10 kb [24]; therefore, additional DNA damages involved in deletion mutations might be induced by kaolin. It, it is also suggested that C_{60} does not prefer to induce such kinds of DNA damages under these conditions. In contrast to the present study, Xu *et al.* have reported that C_{60} dramatically increases large deletion mutations in *gpt* delta transgenic mouse primary embryo fibroblast cells [25]. The observed difference of mutational signatures of C_{60} between a cell line and lung tissue might be related to differences between *in vitro* and *in vivo* assay systems in DNA damage formations, DNA repair or translesion DNA synthesis.

To further elucidate the mechanisms behind the increase in mutant frequency observed in this study, we analyzed mutation spectra using a PCR-direct sequencing method. Most mutations induced by three particles in the present study, occurred at G:C base pairs (52/76, 68%). Among these, 13 G:C base pairs were located in the G or C runs. The most prominent hot spots were at base pairs 143, 189, 320, 406 and 418, and there were no significant differences in the distributions of mutation hot spots in the three particles. This may reflect the distribution of DNA damage sites caused by particles. The most prominent mutation type induced by particles was G:C to C:G transversion. Since these mutations were commonly increased regardless of the constituents of particles (i.e. C_{60} and CB were graphite and kaolin was aluminum silicate), it is suggested that mechanisms leading to the induction of such