

# Increase of a Type of Oxidative DNA Damage, 8-Hydroxyguanine, and Its Repair Activity in Human Leukocytes by Cigarette Smoking<sup>1</sup>

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## ABSTRACT

To investigate the oxidative stress induced by cigarette smoking, the levels of a form of oxidative DNA damage, 8-hydroxyguanine (8-OH-Gua), and its repair activity in the leukocytes of current smokers, ex-smokers, and complete nonsmokers were measured. The mean level of 8-OH-Gua was 1.88-fold higher in smokers as compared to complete nonsmokers (the difference was statistically significant,  $P = 0.013$ ). The mean 8-OH-Gua repair activity was 1.6-fold higher in smokers than complete nonsmokers (the difference was statistically significant,  $P = 0.0053$ ). A positive association was observed for the 8-OH-Gua levels and its repair activity. Considerable interindividual variations in the 8-OH-Gua levels (current smokers, 3.5-fold; nonsmokers, 7.2-fold) and in its repair activity (current smokers, 7.5-fold; nonsmokers, 5.5-fold) were observed. These results demonstrate that not only smoking status but also life-style, environment, and genetic differences might have effects on the level of 8-OH-Gua and its repair activity in human leukocytes.

## INTRODUCTION

Epidemiological studies have shown a positive association between cigarette smoking and cancer of the respiratory tract, the upper digestive tract, the renal pelvis, and the bladder (1–4). Among the 3800 chemicals that have been identified in cigarette smoke, a large number of compounds show mutagenic and carcinogenic activity (5). Reactive oxygen species produced from cigarette smoke may also be involved in its carcinogenicity. Both the gas and tar phases of cigarette smoke are well-known sources of active oxygen species. Superoxide and  $H_2O_2$  have been identified in aqueous smoke solutions obtained by passing cigarette smoke into a buffer (6). The formation of hydroxyl radicals also has been demonstrated in aqueous extracts of cigarette tar (7) and in aqueous smoke solutions after the addition of iron sulfate or iron-containing crocidolite asbestos fibers (8). Such reactive oxygen species are thought to play an important role in cigarette carcinogenesis. Reactive oxygen species generated from cigarette smoke induce single-strand breaks and the formation of modified bases in the DNA of cultured human cells (9–11). These types of oxidative DNA damage may be important causes of cancer and would be useful markers for estimation of cancer risk. Among the various forms of oxidative DNA damage, 8-OH-Gua<sup>3</sup> (12) is most sensitively detected by high pressure liquid chromatography coupled with electrochemical detection (13, 14). The formation of 8-OH-Gua in DNA is likely to be involved in mutagenesis (15–17) and carcinogenesis (18). It also has been reported that cigarette smoking induces the formation of 8-OH-Gua in human leukocyte DNA (19), and the urinary excretion of 8-OH-Gua is increased in smokers (20). Highly specific repair processes for oxidative DNA lesions exist in mammalian cells (21). It has been reported that 8-OH-Gua repair activity was induced after cells were irradiated by gamma rays (22). Therefore, the

increase of 8-OH-Gua repair activity would also be a marker of cellular oxidative stress. In this study, we investigated the effect of cigarette smoking on the 8-OH-Gua levels and its repair capacity in human leukocytes.

## MATERIALS AND METHODS

**Materials.** The DNA Extractor WB Kit was purchased from Wako Biochemicals (Osaka). Nuclease P1 and acid phosphatase (type XA, P-1435) were from Sigma Chemical Co. The protein assay kit was purchased from Bio-Rad. The [ $\gamma$ -<sup>32</sup>P]ATP (specific activity, >5000 Ci/mmol) was from Amersham. T4 polynucleotide kinase was from Takara Shuzo Co. Other chemicals were of the highest purity commercially available. Milli-Q water was used for all experiments. The human peripheral blood samples were obtained from 30 healthy male volunteers, including 10 current smokers (who consumed an average of 34 cigarettes/day), 10 ex-smokers (the time since smoking cessation ranged from 10 months to 15 years), and 10 complete nonsmokers (a lifetime nonsmoker; it does not take into account any history of "passive smoking"). After informed consent was obtained from each subject, blood samples (20 ml) were collected and separated into two portions of 5 ml and 15 ml. The 5-ml portions of blood were centrifuged for 15 min at  $2000 \times g$ , and the buffy coat fractions were removed and frozen at  $-70^\circ\text{C}$  until used for the determination of the amount of 8-OH-Gua in the DNA. The 15-ml portions of blood were mixed with a Ficoll-Paque solution to separate the blood leukocyte fractions. The leukocyte fractions were homogenized in 50 mM Tris-HCl buffer (pH 7.5) containing protease inhibitors (5  $\mu\text{g}/\text{ml}$  each of pepstatin, leupeptin, antipain, and chymostatin) by a Potter-type homogenizer. The cellular debris was removed by centrifugation at  $12,000 \times g$  for 30 min, and the supernatant was aliquoted and stored at  $-70^\circ\text{C}$  until the measurement of 8-OH-Gua repair activity.

**Analysis of 8-OH-Gua Formation.** The samples were homogenized in lysis buffer with a Potter-type homogenizer, and the nuclear DNA in the homogenate was extracted using the DNA Extractor WB Kit (23). The extracted nuclear DNA was digested with nuclease P1 and acid phosphatase in a 10 mM sodium acetate solution ( $37^\circ\text{C}$  for 30 min). After the incubation, the mixture was treated with the iron exchange resin Muromac (Muromachi Kagaku, Tokyo, Japan) and was centrifuged at  $15,000 \times g$  for 5 min. The supernatant was transferred to a filter tube (Millipore; Samprep C; 0.2  $\mu\text{m}$ ), centrifuged at  $5000 \times g$  for 5 min, and injected onto a high-performance liquid chromatography column (Beckman; Ultrasphere-ODS; 5  $\mu\text{m}$ ,  $4.6 \times 250$  nm) equipped with an electrochemical detector (ESA Coulochem II: detector 1, 0.15V; detector 2, 0.30V). As standard samples, 20  $\mu\text{l}$  each of deoxyguanosine (0.5 mg/ml) and 8-hydroxydeoxyguanosine (5 ng/ml) solutions were injected. The value of 8-OH-Gua was calculated as the number per  $10^5$  guanine.

**Endonuclease Nicking Assay.** The total protein concentrations were determined with a protein assay kit (Bio-Rad) and were adjusted to 1 mg/ml. A 22-mer DNA containing 8-OH-Gua (5'-GGTGGCCTGACG<sup>\*</sup>CATTC-CCCAA-3'; G<sup>\*</sup>, 8-OH-Gua), prepared by the published method (24), was <sup>32</sup>P end-labeled, annealed with the complementary strand, and used as the substrate for this assay. The crude extract (10  $\mu\text{g}$  total protein) was incubated with 0.05 pmol of the double-stranded DNA substrate at  $25^\circ\text{C}$  for 1 h. After two ethanol precipitations, the pellet was dried, dissolved in 10  $\mu\text{l}$  of loading buffer (80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue), and was denatured by heating at  $90^\circ\text{C}$  for 3 min. Ten  $\mu\text{l}$  of the sample were applied to a 20% denaturing polyacrylamide gel for electrophoresis. The gel position of the cleaved fragment, generated as a consequence of the excision repair activity, was confirmed by comparison to the hot piperidine-treated oligonucleotide as a fragment marker (data not shown; Ref. 25). After electrophoresis, the autoradiograms were processed,

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<sup>3</sup> The abbreviation used is: 8-OH-Gua, 8-hydroxyguanine.

and the radioactivity was analyzed using a Bioimage analyzer system (Fujix BAS 2000). The repair activity was calculated from the ratio of the excised fragment to the total substrate (the remaining unexcised fragment plus excised fragment).

**Statistical Analysis.** The data is presented as mean  $\pm$  SD. Differences between groups were tested by the ANOVA factorial with Fisher's PLSD at the 5% significance level. Correlations between different variables were studied by regression analysis and the Spearman rank correlation test. All analyses were carried out using the Stat View 4.01 program (Berkeley, CA).

**RESULTS**

The mean age (years) of the subjects was  $44.6 \pm 5.8$  (mean  $\pm$  SD). The ages within the three subgroups of individuals, smokers, ex-smokers, and complete nonsmokers, were similar. All subjects were healthy, of normal weight for height, had usual dietary habits, and performed occasional sports activities. The mean 8-OH-Gua level and its repair activity for each of the three subgroups is shown in Table 1.

The individual levels of 8-OH-Gua in the peripheral leukocyte DNA are shown in Fig. 1. The mean 8-OH-Gua levels in smokers, ex-smokers, and complete nonsmokers were  $5.9 \pm 2.7/10^6$  guanine (mean  $\pm$  SD),  $4.0 \pm 1.3/10^6$  guanine, and  $3.1 \pm 1.6/10^6$  guanine, respectively. There were interindividual variations in all groups investigated. The largest interindividual difference, 7.2-fold, was found among the complete nonsmokers. The mean level of 8-OH-Gua in smokers was 1.88-fold higher than that of the complete nonsmokers (the difference was statistically significant,  $P = 0.013$ ) and 1.46-fold higher than ex-smokers ( $P = 0.023$ ). The 8-OH-Gua repair activity was measured by a nicking assay using an 8-OH-Gua containing DNA as the substrate. A typical example of an autoradiogram obtained by this assay is shown in Fig. 2. Higher levels of 8-OH-Gua repair activity were observed in current smokers as compared to complete nonsmokers. The individual 8-OH-Gua repair activities in the peripheral leukocyte samples are shown in Fig. 3. The mean repair activities of smokers, ex-smokers, and complete nonsmokers were  $0.11 \pm 0.04$  (mean  $\pm$  SD),  $0.09 \pm 0.03$ , and  $0.07 \pm 0.03$ , respectively. There were also interindividual variations in all groups investigated. Current smokers and complete nonsmokers showed 7.5- and 5.5-fold interindividual variations in the 8-OH-Gua repair activity, respectively. The mean 8-OH-Gua repair activity was 1.6-fold higher in smokers than in complete nonsmokers (the difference was statistically significant,  $P = 0.0053$ ). The large variations of the 8-OH-Gua levels (7.2-fold) and its repair activity (7.5-fold) are not due to experimental error, because the variation of three independent analyses of the same subject was within a narrow range ( $\pm \sim 30\%$ ). A positive association was observed between the 8-OH-Gua levels and the 8-OH-Gua repair enzyme activities in the analysis of all subjects ( $r = 0.379$ ;  $P < 0.05$ ;

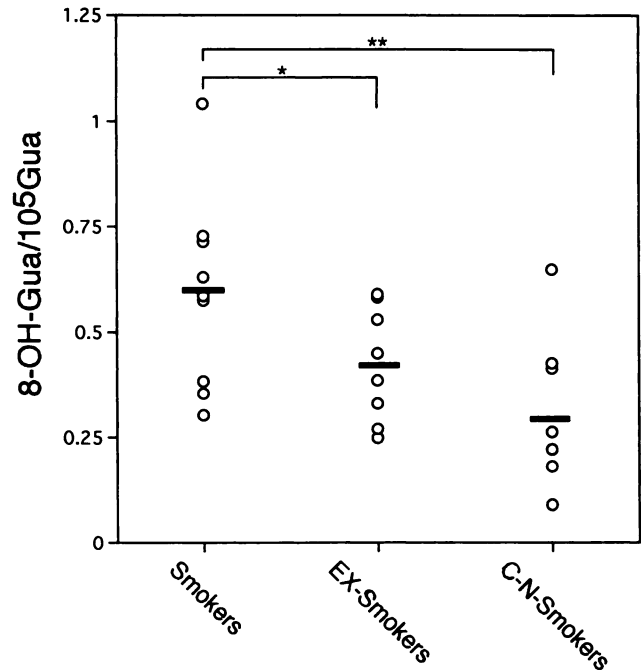


Fig. 1. Smoking status and 8-OH-Gua levels in human peripheral leukocyte DNA. Each data point (○) represents one individual, and the horizontal line indicates the mean value of the group. Differences between groups were tested by the ANOVA factorial with Fisher's PLSD at the 5% significance level. \*,  $P = 0.023$ ; \*\*,  $P = 0.013$ . C-N-Smokers, complete nonsmokers.

Table 1 8-OH-Gua levels and repair activities in the peripheral leukocytes of human smokers, ex-smokers, and complete nonsmokers

	Smokers <sup>a</sup>	Ex-smokers <sup>b</sup>	Complete nonsmokers
Age			
Mean $\pm$ SD	44 $\pm$ 4.2	44.5 $\pm$ 4.6	45.5 $\pm$ 8.2
Range	40-53	41-57	34-51
Number	10	10	10
8-OH-Gua/ $10^5$ Gua			
Mean $\pm$ SD	0.59 $\pm$ 0.27	0.40 $\pm$ 0.13	0.31 $\pm$ 0.16
Range	0.30-1.04	0.25-0.59	0.09-0.65
Number	10	10	10
8-OH-Gua repair activity			
Mean $\pm$ SD	0.11 $\pm$ 0.04	0.09 $\pm$ 0.03	0.07 $\pm$ 0.03
Range	0.02-0.15	0.05-0.14	0.02-0.11
Number	10	10	10

<sup>a</sup> Smokers consumed an average of 34 cigarettes per day, and all subjects had smoked for over 20 years.

<sup>b</sup> For the ex-smokers, the time since smoking cessation ranged from 10 months to 15 years.

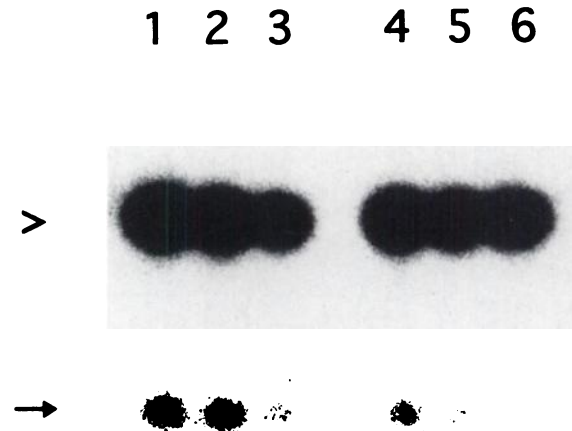


Fig. 2. Difference in the 8-OH-Gua repair enzyme activity associated with smoking status. This autoradiogram shows the 8-OH-Gua repair activity of two typical examples from each of the three subgroups. The gel position of the cleaved fragment, generated as a consequence of the excision repair activity, was confirmed by comparison to the hot piperidine treated oligonucleotide as a fragment marker (data not shown). Lanes 1 and 4, smokers; Lanes 2 and 5, ex-smokers; Lanes 3 and 6, complete nonsmokers. >, substrate DNA; ->, excised fragment.

Fig. 4). Regression analysis of the data for all subjects revealed a linear relationship between the number of cigarettes smoked per day and the 8-OH-Gua levels ( $r = 0.616$ ;  $P = 0.002$ ; Fig. 5A). A linear relationship was also obtained between the 8-OH-Gua repair activity and the number of cigarettes smoked per day ( $r = 0.468$ ;  $P = 0.0084$ ; Fig. 5b). A positive correlation between the 8-OH-Gua levels and the Brinkman index was also obtained in smokers and ex-smokers ( $r = 0.651$ ;  $P = 0.0014$ ; Fig. 6). Therefore, as the lifetime cigarette consumption increased, the oxidative DNA damage would increase.

DISCUSSION

Many investigators have reported that carcinogen-DNA adduct levels in human lung tissues and human peripheral lymphocytes are increased in smokers (26, 27). We investigated the levels of 8-OH-Gua and its repair activity in human leukocytes obtained from 10 current smokers, 10 ex-smokers, and 10 complete nonsmokers. Our study revealed that in smokers, both the levels of 8-OH-Gua and its repair activity are significantly increased ( $P < 0.05$ ). This indicates that cigarette smoke induces oxidative stress in peripheral leukocytes. In particular, a positive correlation between the number of cigarettes smoked per day and the 8-OH-Gua levels and its repair activity was observed. We also found a positive correlation between the 8-OH-Gua levels and the Brinkman index in smokers and ex-smokers. These results suggest that oxidative DNA damage may be associated with the total number of cigarettes smoked per lifetime. Therefore, long-term use of tobacco may be harmful to health (28). In this study,

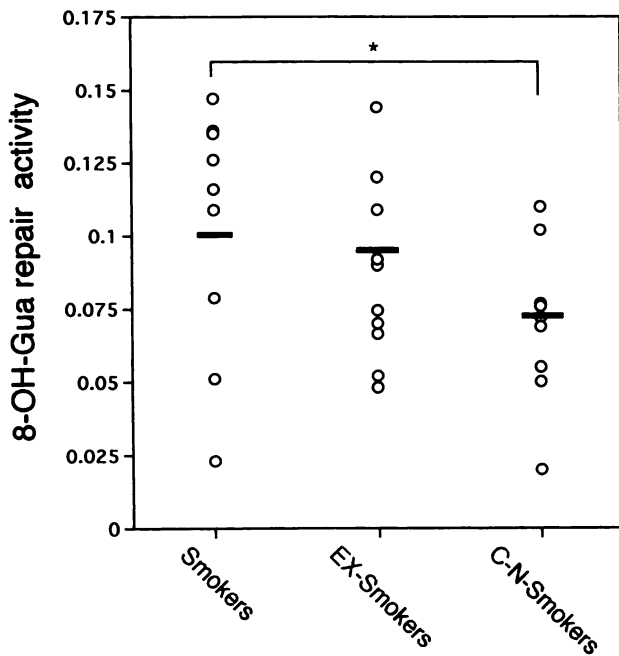


Fig. 3. Smoking status and the 8-OH-Gua repair activity in human peripheral leukocytes. Differences between groups were tested by the ANOVA factorial with Fisher's PLSD at the 5% significance level. \*,  $P = 0.0053$ . C-N-Smokers, complete nonsmokers.

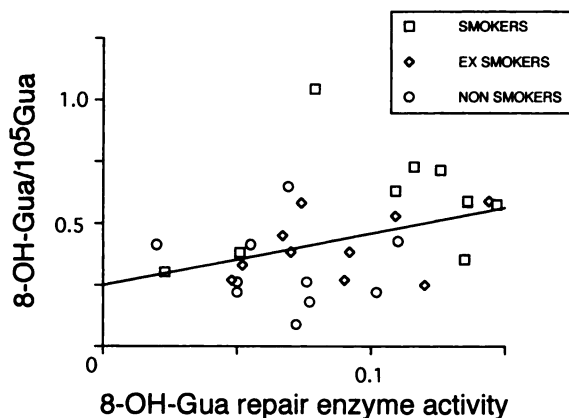


Fig. 4. Relationship between the 8-OH-Gua levels and its repair activities in human peripheral leukocytes from all subjects. Correlations between different variables were studied by regression analysis and the Spearman rank correlation test. Linear correlation coefficient,  $r = 0.379$ ;  $P < 0.05$ .

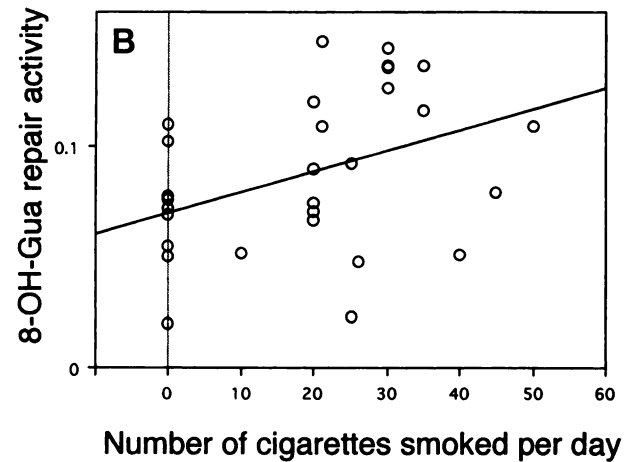
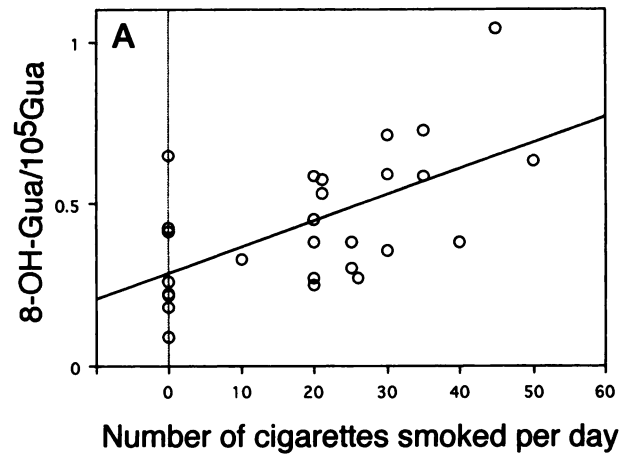


Fig. 5. A, relationship between the 8-OH-Gua levels in human peripheral leukocyte DNA and the number of cigarettes smoked per day. Correlations between different variables were studied by regression analysis and the Spearman rank correlation test. Linear correlation coefficient,  $r = 0.616$ ;  $P = 0.002$ . B, relationship between the 8-OH-Gua repair activities in human peripheral leukocytes and the number of cigarettes smoked per day. Correlations between different variables were studied by regression analysis and the Spearman rank correlation test. Linear correlation coefficient,  $r = 0.468$ ;  $P = 0.0084$ .

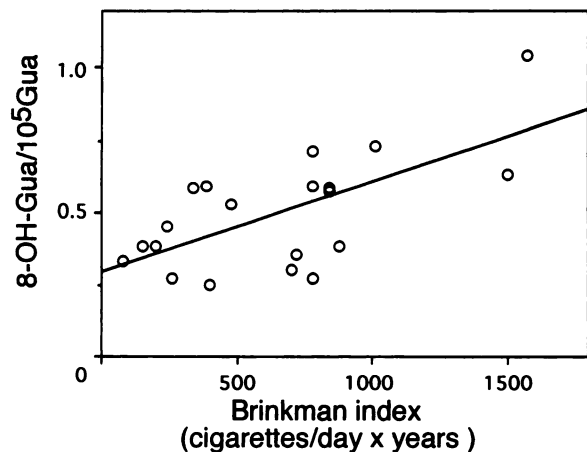


Fig. 6. Relationship between the 8-OH-Gua levels and the Brinkman index. Complete nonsmokers have been excluded from this analysis. Correlations between different variables were studied by regression analysis and the Spearman rank correlation test. Linear correlation coefficient,  $r = 0.651$ ;  $P = 0.0014$ .

current smokers and complete nonsmokers showed a 7.5- and a 5.5-fold interindividual variation, respectively, in the 8-OH-Gua repair activity. Hall *et al.* (29) have also reported oxidative DNA

damage repair enzyme (Fapy-DNA glycosylase) activity in peripheral blood leukocytes from 20 smokers and 17 nonsmokers. They observed that the Fapy-DNA glycosylase activity was two times higher in smokers than in nonsmokers, and that the standard deviation of its activity was about three times higher in smokers than in nonsmokers.

In Fig. 1, the 8-OH-Gua level of one individual in the smoking group was an extremely high value (1.04). It appears from Fig. 4 that this individual was in the middle level of repair activity, and from Fig. 6 that he has the highest Brinkman index. These data may suggest that some smokers have an unusually high risk of oxidative damage. We hope that the measurement of 8-OH-Gua level and its repair activity in human leukocytes, which are easily obtained from subjects, may be of significance in analyzing the molecular epidemiology of cancer.

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