8-Hydroxydeoxyguanosine Levels in White Blood Cell DNA and ex vivo Oxidation Resistance of Plasma in Smokers

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WAKO, Y., SATOH, M. and SUZUKI, K. *8-Hydroxydeoxyguanosine Levels in White Blood Cell DNA and ex vivo Oxidation Resistance of Plasma in Smokers.* Tohoku J. Exp. Med., 2001, **194**(2), 99–106 — Oxidative DNA damage in peripheral white blood cell of smokers were estimated in accordance with the levels of 8-Hydroxydeoxyguanosine (8-OHdG) in nuclear DNA and the antioxidant status of these smokers' plasma was investigated in terms of the ex vivo oxidation resistance of plasma. In a survey of 12 smokers (4 women) aged 22 to 48, the mean level of 8-OHdG was 3.79 ± 0.65 residue/ 10^6 dG (mean $\pm$ s.d.) with a range from 2.83 to 4.62 residue/ 10^6 dG. These measurements showed approximately 1.6-fold inter-individual variations of 8-OHdG level in smokers. A higher level of 8-OHdG was found for smokers whose ex vivo plasma oxidation resistance was weak. Significant association is seen between oxidized bases in white blood cells and plasma oxidation resistance, whereas signs of any association with plasma concentration of α -tocopherol, ascorbic acid, bilirubin, and uric acid are weak and sporadic. These findings indicate that apparent heterogeneity exists among smokers in some sort of resistance to the oxidative effects of smoking. — oxidative DNA damage; 8-hydroxydeoxyguanosine; antioxidant status; smoker
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Endogenous free radicals and other reactive oxygen species (ROS) are capable of damaging cellular biomolecules such as lipid and DNA (Halliwell and Aruoma 1991; Morrow et al. 1995). A frequent type of oxidative DNA damage is the 8-hydroxylation of the guanine base. Elevated levels of 8-hydroxydeoxy-

guanosine (8-OHdG) in human white cell DNA and in urine are found in subjects, such as patients treated with ionizing radiation and smokers, who are suspected of any enhanced oxidative stress (Shigenaga et al. 1989; Kiyosawa et al. 1990). 8-OHdG has been regarded as a useful biomarker for oxidative

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damage because of its potential importance and the relative ease with it can be measured (Shigenaga et al. 1994). Cellular biomolecules damage caused by ROS has been suggested as an important factor in aging as well as in a number of age-related degenerative diseases, including cancer, heart disease, and arthritis (Totter 1980; Ames and Shigenaga 1993; Steinberg and Lewis 1997). Therefore, antioxidant supplementation and dietary factors that reduce the impact of ROS attack are likely to protect against such diseases (Ames 1983; Byers and Perry 1992). But the findings of antioxidant activity obtained in vitro do not always correspond to the observations in vivo from intervention trials. While β -carotene was recognized as an effective antioxidant along with vitamins E and C, the ATBC study (alpha-tocopherol, beta-carotene study) suggested that the administration of β -carotene increases lung cancer incidence in smokers (The alpha-tocopherol, beta-carotene cancer prevention study group 1994). Podmore et al. (1998) reported that vitamin C administered as a dietary supplement to healthy humans exhibits a pro-oxidant effect at doses of 500 mg or more per day. Accordingly it is essential to establish that these dietary factors being evaluated really do decrease oxidative damage in vivo. And several biomarkers can be used in these intervention trials to study if dietary factors has any efficiency for antioxidative protection in vivo and the optimal intake of those dietary factors (Halliwell 1996). On the other hand, smokers are simply a good population in which to study the role of such nutrients in reducing oxidative damage because of the high rate at which their cellular biomolecules are oxidized. A number of intervention trials have been assembled with smoking volunteers from these standpoints.

In the food science field, the beneficial effects of food factors on health have recently attracted attention, and the authors themselves succeeded in the production of bioactive peptide fraction from a water-soluble fish protein using

a bioreactor system (Wako et al. 1999). This fraction contains antihypertensive peptides and antioxidative peptides. The authors have planned the study to determine with humans whether these peptides may really have an effect in vivo with smoking volunteers. A pilot study was carried out here to investigate the features of oxidative stress in smokers before conducting this trial. This paper will discuss the concentration of 8-OHdG in smokers' white blood cell nuclear DNA and also show a significant correlation between 8-OHdG levels and ex vivo oxidation resistance of plasma.

MATERIAL AND METHODS

Chemicals

Nuclease P1, alkaline phosphatase, 2-deoxyguanosine, 8-hydroxydeoxyguanosine, 2-propanol 2,2,5,7,8-pentamethyl-6-hydroxychroman and ascorbic acid were obtained from Wako Pure Chemical (Osaka). α -Tocopherol was purchased from Merck (Darmstadt, Germany). Metaphosphoric acid was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA).

Study population

Twelve healthy men and women who are smokers (8 men and 4 women) and four non-smokers (3 men and a woman) aged 21 to 48 years were recruited with informed consent from the Hachinohe medical service and welfare group and Hachinohe institute of technology. Individuals on drug therapy were excluded. All subjects completed a questionnaire on the state of their health, smoking habits, tea and coffee consumption, and medical history (Table 1). Non-smokers were defined as individuals who had never smoked cigarettes.

The study was carried out by the observance of the Helsinki declaration.

Blood Sampling

The volunteers were asked not to smoke on the morning of the day when blood samples

TABLE 1. *Basic information, baseline 8-OHdG, ex vivo oxidation resistance of plasma, and plasma antioxidant concentrations in smokers and non-smokers*

Characteristic	Smokers (n = 12)	Non-smokers (n = 4)
Sex (M/F)	8/4	3/1
Age (years)	29.5 ± 8.8 (21~48)	29.5 ± 12.5 (22~48)
Body weight (kg)	63.8 ± 10.8 (46.2~81.5)	59.8 ± 11.6 (47.1~70.3)
Height (cm)	168 ± 2 (154~178)	169 ± 9.8 (159~180)
Cigarettes (per day)	19.6 ± 7.5 (10~40)	
Smoking years	10.8 ± 8.4 (4~30)	
8-OHdG (residue/10 ⁶ dG)	3.79 ± 0.65* (2.83~4.62)	3.13 ± 0.57 (2.61~3.89)
Antioxidation status of plasma (mmol/l; trolox equivalent)	1.07 ± 0.08 (0.95~1.18)	1.11 ± 0.06 (1.02~1.16)
α-Tocopherol (mg/100 ml)	1.00 ± 0.26 (0.62~1.43)	0.95 ± 0.19 (0.74~1.20)
Ascorbic acid (μg/100 ml)	632 ± 304 (126~1259)	947 ± 197 (715~1196)
Uric acid (mg/100 ml)	5.87 ± 1.34 (3.73~7.43)	6.16 ± 1.90 (4.16~8.24)
Bilirubin (μg/100 ml)	534 ± 141 (274~764)	565 ± 62 (507~647)

Values are means ± s.d. The range is shown in parentheses.

*Significantly different from non-smokers ($p < 0.05$).

were collected. Blood was drawn into two heparinized vacutainer tubes for each subject between 9:00 to 10:00 a.m. White blood cells and plasma were separated from blood samples immediately in each tube. Plasma was separated by centrifugation at $3000 \times g$ for 15 minutes at 4°C. An aliquot of plasma was then removed and stored at -80°C for measurement. Where samples were intended for ascorbic acid determination, an equal volume of 10% metaphosphoric acid was added to the plasma before freezing.

Isolation of white blood cell DNA

DNA isolation from whole blood was carried out by a modification of the method described by Miller et al. (1988) using a commercially available isolation kit (Puregene; Gentra Systems Inc., Minneapolis, MN, USA). Three volumes of RBC lysis solution were added to the fresh whole blood. They were mixed gently to lyse erythrocyte membranes, stored for 10 minutes at room temperature, and centrifuged at $2000 \times g$ for 10 minutes. Supernatant was removed, leaving behind the white cell pellets. Then, 10 ml of cell lysis solution was

added to the re-suspended cells and pipetted up and down to lyse the white cells. After RNase treatment and deproteinization of the sample, DNA was extracted from the sample mixture by addition of equal volumes of 100% 2-propanol. The sample was mixed gently until white threads of DNA were noted. The resultant DNA pellets were centrifuged at $2000 \times g$ for 3 minutes and rinsed with 70% ethanol. DNA isolation was carried out in a nitrogen atmosphere by operating under a nitrogen stream and filling tubes with nitrogen gas. The DNA concentration and purity of the DNA isolated was confirmed spectrophotometrically. The ratio of A_{260}/A_{280} was between 1.8 and 1.9.

Determination of 8-OHdG

Each sample of 25 μg DNA was suspended in 50 μl of 20 mM sodium acetate buffer (pH 4.1). The samples were digested to deoxynucleotides with 12 units of nuclease P1 at 37°C for 30 minutes. Then, 3 μl of 1 M Tris-HCl buffer (pH 7.4) was added before incubation with 1.3 units of alkaline phosphatase at 37°C for 1 hour. HPLC/ECD was used to measure the 8-OHdG in the DNA hydrolysate according

to the method described by Kaneko et al. (1997). The HPLC system (LC-10, Shimazu, Kyoto) was equipped with a TSKgel ODS-80Ts column (2.0 mm i.d. $\times$ 150 mm, Tosoh Co., Tokyo). The eluent consisted of 12.5 mM citric acid, 30 mM sodium hydroxide, 25 mM sodium acetate, 10 mM acetic acid, and 6% methanol at pH 5.1; the flow rate was 0.5 ml/minutes. ECD was used to detect 8-OHdG (ECD-300, Eicom Co., Kyoto) at 0.6 V. Oxidative damage to DNA was expressed as the molar ratio of 8-OHdG to 10^6 molecules of deoxyguanosine (dG) in the same sample. The amount of dG was calculated from the absorbance at 260 nm in the same sample measured with an ultraviolet detector (SPD-10A, Shimazu).

Measurement of total antioxidant status of plasma

The antioxidant potential of plasma in a hydrogen peroxide-metmyoglobin induced oxidation system was estimated by the colorimetric method using a commercially available assay kit (Total Antioxidant Status, Randox Laboratories Ltd., Antrim, United Kingdom). Twenty μ l of plasma and 1 ml of chromogen containing 6.1 μ M metmyoglobin and 610 μ M 2, 2'-Azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) in phosphate buffered saline (pH 7.4) were mixed. The reaction was started by the addition of 200 μ l of hydrogen peroxide (250 μ M), and the mixture was left for 3 minutes at 37°C. Then, the production of radical cation ABTS was measured by the colorimetric method at 600 nm. Antioxidants in the plasma cause suppression of this color production to a degree that is proportional to their oxidation resistance ability. The antioxidant potential of plasma was calculated in relation to 1.0 mmol/liter 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox).

Measurement of plasma antioxidant levels

The plasma α -tocopherol concentration was measured by HPLC according to the

method described by Ueda and Igarashi (1987). The plasma ascorbic acid concentration was measured by HPLC according to the method described by Ross (1994). The plasma uric acid concentration was measured by the enzymatic method using a commercially available assay kit (Uric acid B-Test Wako; Wako Pure Chemical, Osaka). The plasma total bilirubin concentration was measured by the colorimetric method using a commercially available assay kit (Bilirubin BII-Test Wako; Wako Pure Chemical).

Statistics

Statistical comparisons between the two groups and significance of regression coefficients were determined using the *t*-test. *p*-Values of <0.05 were considered statistically significant. All values were expressed as mean $\pm$ s.d.

RESULTS AND DISCUSSION

8-OHdG level in total white blood cell DNA

In smokers, various critical biological substances are believed to be damaged by oxidants present in cigarette smoke and by the activation of phagocytic cells that generate oxygen species (Church and Pryor 1985). Thus, the levels of 8-OHdG in the white blood cell DNA of our volunteers were estimated. Smokers were found to possess 3.79 ± 0.65 8-OHdG/ 10^6 dG with a range from 2.83 to 4.62 8-OHdG/ 10^6 dG in blood cell DNA (Table 1). These results showed that the 8-OHdG levels varied widely (about 1.6-fold inter-individual variations). This finding is similar to that described earlier (Kiyosawa et al. 1990). Fig. 1 shows that there is no significant difference in 8-OHdG levels between groups with a short (4~6 years) and long (9~30 years) smoking period (3.56 ± 0.70 residue/ 10^6 dG vs. 4.02 ± 0.57 residue/ 10^6 dG). A distinct relationship has not been found for the 8-OHdG level with age nor with the number of cigarettes smoked/day (data not shown). Present data cannot explain the individual

Fig. 1. Levels of 8-OHdG in white blood cells from smokers (●) and non-smokers (○). The smoking subjects were divided into two classes according to the length of smoking history: a 4~6 year-class and a 9~30 yr-class. The thick vertical bar represents the mean and s.d. of determinations for each group.

variations in 8-OHdG levels by the length of smoking history or age. This scattering of 8-OHdG measurements could be attributed to various factors, for example difference in life-style, nutritional state and susceptibility to the oxidative effects of smoking. The levels of non-smokers were also estimated to the reference. Though it was a result of limiting 4 individuals (one woman) aged 22 to 48, the mean 8-OHdG levels from non-smokers were slightly lower than those from smoker (3.13 ± 0.57 residue/ 10^6 dG vs. 3.79 ± 0.65 residue/ 10^6 dG) (Table 1). Many investigators have reported that the oxidant injury of white blood cells are increased in smokers (Kiyosawa et al. 1990; Asami et al. 1996). In contrast, other studies have failed to show such an increase (Phillips et al. 1988; Takeuchi et al. 1994).

Ex vivo oxidation resistance of plasma

The method used here to assess intrinsic antioxidant status of subjects was an ex vivo test for antioxidant protection that depends on challenging plasma in vitro with H_2O_2 . If the

antioxidant potential of plasma is high, the expected result would be strong resistance to oxidative damage. The trolox equivalent antioxidant status of plasma ranged from 0.95 to 1.18 mmol/liter in the study subjects consisting of smokers and non-smokers (Table 1). There was no significant difference between the mean values of antioxidant status for smokers and non-smokers (1.07 ± 0.08 mmol/liter vs. 1.11 ± 0.06 mmol/liter). However, a significant negative correlation was noted between the antioxidant status of plasma and 8-OHdG levels in white blood cells in smokers ($r = -0.63$, $p = 0.029$) and in subjects as a whole ($r = -0.69$, $p = 0.003$) (Fig. 2A). This finding is of interest that a higher level of 8-OHdG was found for smokers whose ex vivo plasma oxidation resistance was weak.

Plasma antioxidant levels

The antioxidant system has many components. One component traps radicals to prevent chain reactions; examples include tocopherol, ascorbic acid, β -carotene, uric acid, bilirubin, and albumin. A deficiency in any of these components can cause a reduction in the overall antioxidant status of an individual. Table 1 gives the mean plasma concentrations of α -tocopherol, ascorbic acid, uric acid, and bilirubin for subjects. Smokers had lower levels of ascorbic acid than non-smokers (632 ± 304 μ g/100 ml vs. 947 ± 197 μ g/100 ml), supporting many researchers' observations (Cross and Halliwell 1993), but this difference was not statistically significant in the current study. No significant differences between the plasma from smokers and non-smokers were observed in the concentration of other antioxidants. DNA damage in white cells and ex vivo oxidation resistance of plasma were examined for subjects as a whole for any correlation with the individual plasma antioxidant levels (Table 2). However, almost all of these correlation coefficients are not significant. The only significant associations are seen between oxidized bases and

Fig. 2. The levels of 8-OHdG in white blood cell according to plasma antioxidant status (A) and plasma ascorbic acid concentration (B) in smokers (●) and nonsmokers (○). There was an inverse relationship between the levels of 8-OHdG and plasma antioxidant status ($r = -0.6$, $p < 0.01$) and between the levels of 8-OHdG and plasma ascorbic acid concentration ($r = -0.58$, $p < 0.05$).

TABLE 2. The regression analyses between plasma antioxidant levels and white blood cell DNA damage, and *ex vivo* oxidation resistance of plasma in subjects as a whole (smokers and non-smokers)

	Correlation coefficient (r)	
	DNA damage	Oxidation resistance
α -Tocopherol	0.40	-0.15
Ascorbic acid	-0.57*	0.46
Uric acid	-0.20	0.52*
Bilirubin	-0.31	0.21

*Significant correlation is indicated as $p < 0.05$.

ascorbic acid concentrations ($r = -0.57$, $p = 0.020$) (Fig. 2B), and between *ex vivo* plasma oxidation resistance and uric acid concentrations ($r = 0.52$, $p = 0.039$). Negative correlations are seen between oxidized bases and ascorbic acid, uric acid, and bilirubin concentrations. But the correlation between oxidized bases and α -tocopherol is positive. Reverse correlations with oxidized bases are seen between oxidation resistance and antioxidant concentrations. We could not find distinct correlation between assessed antioxidants statuses and DNA dam-

age, since there should be many other variables that could affect DNA damage, including intrinsic antioxidant defenses and DNA repair.

Assessment of oxidative stress

The present paper attempted to assess the oxidant injury of smokers from the determination of 8-OHdG levels in white blood cell DNA. The measurements of 8-OHdG varied widely. However, this research has indicated a significant correlation between the 8-OHdG levels in white blood cells and the *ex vivo* plasma oxidation resistance (Fig. 2A). The ability to demonstrate the level depending on the antioxidant status will confirm the measurement of white blood cell 8-OHdG as a useful tool in assessment of the status of oxidative stress. Moreover damaged DNA is repaired by exonucleases and is excreted into the urine. The level of 8-OHdG in urine is thought to be unaffected by diet and this compound is not thought to be metabolized in humans (Shigenaga et al. 1989). Thus, the urinary excretion of 8-OHdG will reflect the whole body DNA damage and repair. Urinary 8-OHdG was found to correlate with metabolic rate and smoking (Loft et al. 1992, 1993, 1994). There are many uncer-

tainties in the application of biomarkers of oxidative DNA damage to human nutritional studies (Halliwell 1998; Takeuchi et al. 1994). However, it may be possible that urinary 8-OHdG excretion rate can be combined with the measurement of 8-OHdG in white blood cell DNA to study the assessment for oxidative stress in humans more reliably.

This study should be continued to identify the diets that decrease DNA damage and to attempt to isolate protective factors from this damage. This supplementation will help to protect against age-related degenerative disease. An essential part of these studies is to establish that these compounds being evaluated really do decrease oxidative damage in vivo in humans. When selecting smokers for participation in these studies, the individual heterogeneity in some sort of resistance to the oxidative effects of smoking must be taken into account.

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