remnant cancer cases were excluded because of their unique pathologic features. Furthermore, 18 cases (12 male and 6 female cases) with LELC-type tumors were also excluded from the present analysis. Among the remaining 1,918 cases, 1,693 cases ascertained during the period 1976-1992 were those used in the previous study reported by Tokunaga *et al* (3). The remaining 225 new Japanese gastric cancer cases were collected from Kagoshima City Hospital during the period 1993-1995.

Histology. Gastric carcinomas were classified according to the classification scheme of the Japanese Research Society for Gastric Cancer (12). Histological patterns were considered as follows: well-differentiated tubular adenocarcinoma (tub1), moderately-differentiated tubular adenocarcinoma (tub2), solid poorly-differentiated adenocarcinoma (por1), non-solid poorly-differentiated adenocarcinoma (por2), signet-ring cell carcinoma (sig) and mucinous carcinoma (muc). According to Lauren classification, intestinal-type tumors included carcinomas with types tub1, tub2 and muc, and diffuse-type tumors included carcinomas with types por1, por2 and sig.

The location of a tumor, defined as the predominant location of the tumor, was divided into the following three categories: cardia or upper third part, middle part, and antrum or lower third part according to the guidelines of the Japanese Research Society for Gastric Cancer (12).

ISH assay to detect EBER. The ISH assay of paraffin-embedded tissue samples obtained from the main tumor was conducted using a digoxigenin-labeled EBER-1 oligonucleotide probe as previously described (30). A case was considered to be EBER positive based on an intensive nuclear dark purple signal under microscopy. In every ISH assay, lymph node section from a patient with infectious mononucleosis and a sense probe for EBER-1 were used as positive and negative controls, respectively.

Statistical analysis. We examined the proportion of EBV-GCs using logistic regression analysis. Gender, age and tumor subsite were included in logistic models as covariates. Maximum likelihood estimates of odds ratios (OR) and 95% confidence interval (CI) were calculated. The P-value for trend of age was calculated using age as a continuous variable in a logistic model. All the P-values presented were two-sided.

Results

EBV-GCs accounted for 4.5% (n=49) and 6.1% (n=51) of 1,088 intestinal-type and 830 diffuse-type gastric carcinomas, respectively. When the gastric carcinomas were classified according to the classification scheme of the Japanese Research Society for Gastric Cancer, the percentage of EBV-GCs in tub1-, tub2-, por1-, por2-, sig- and muc-type tumors were 0.8%, 7.7%, 10.1%, 2.8%, 1.9% and 3.3%, respectively. EBV-GCs were most common in tub2- and por1-type carcinomas.

Table I shows the distribution of EBV-GCs according to gender, age, tumor location and histology. ORs and 95% CIs were obtained from logistic analysis using gender, age and tumor location as covariates. EBV-GC showed male predominance, and a relatively low frequency in the antrum,

Table I. Results of logistic analysis, both intestinal and diffuse types.

Variables	EBER+/N	(%)	OR*	95% CI[a]
Gender				
Female	17/706	(2.4)	1	reference
Male	83/1212	(6.8)	3.2	1.9-5.6
		P<0.001		
Age				
≤49	17/296	(5.7)	1	reference
50-69	59/1052	(5.6)	1.1	0.6-1.9
≥70	24/570	(4.2)	0.8	0.4-1.6
		P for trend = 0.233		
Location				
Upper third (cardia)	19/229	(8.3)	1	reference
Middle	58/759	(7.6)	1.0	0.6-1.7
Lower third (antrum)	23/930	(2.5)	0.3	0.2-0.6
		P for heterogeneity <0.001		
Histology				
Diffuse	51/830	(6.1)	1	reference
Intestinal	49/1088	(4.5)	0.7	0.4-1.0
		P=0.065		

[a]ORs and 95% CIs were obtained from logistic regression analysis using gender, age, and tumor location as covariates.

confirming clinicopathological features described by many previous studies. Diffuse-type tumors were slightly more common than intestinal-type carcinomas, but the difference between the 2 types was not statistically significant (P=0.065).

Table II summarizes the results obtained from logistic analyses conducted separately for the intestinal and diffuse types. Among intestinal-type tumors, EBV-GCs appeared to be predominant among the males but the gender difference was not statistically significant (P=0.143). Interestingly, age was inversely related to the ratio between EBER-positive and -negative tumors (P for trend = 0.002). The age-dependent decrease was observed regardless of tumor location (data not shown). Even when intestinal-type tumors were restricted to tub2-type tumors, which was most common among intestinal-type EBV-GCs, the age-dependent decrease of EBV-GC proportion was evident and statistically significant (P=0.004). Intestinal-type EBV-GCs were relatively less prevalent in the antrum. The proportion of intestinal-type EBV-GCs did not show evident dependence on calendar years (data not shown).

The results for diffuse-type carcinoma are also shown in Table II. In diffuse-type carcinomas, male predominance was evident and statistically significant (P<0.001). The magnitude of OR, comparing the ratio between EBER-positive and -negative tumors in 2 genders was larger in diffuse-type carcinomas (OR=5.1) than in intestinal-type carcinomas (OR=1.7). However, the 95% CIs of their ORs overlapped with each other; there was no statistically significant

Table II. Results of logistic analysis conducted separately for diffuse-type and intestinal-type gastric carcinomas.

	Intestinal type		Diffuse type	
Variables	EBER+/N (%)	OR (95% CI)[a]	EBER+/N (%)	OR (95% CI)[a]
Gender				
Female	9/316 (2.8)	1 (reference)	8/390 (2.1)	1(reference)
Male	40/772 (5.2)	1.7 (0.8-3.6)	43/440 (9.8)	5.1 (2.3-11.1)
	P=0.143		P<0.001	
Age				
≤49	10/95 (10.5)	1 (reference)	7/201 (3.5)	1 (reference)
50-69	30/611 (4.9)	0.4 (0.2-0.9)	29/441 (6.6)	2.1 (0.9-5.0)
≥70	9/382 (2.4)	0.2 (0.1-0.5)	15/188 (8.0)	2.3 (0.9-5.8)
	P for trend = 0.002		P for trend = 0.429	
Location				
Upper third	8/141 (5.7)	1 (reference)	11/88 (12.5)	1 (reference)
Middle	23/364 (6.3)	1.4 (0.6-3.3)	30/395 (7.6)	0.8 (0.4-1.6)
Lower third	13/583 (2.2)	0.4 (0.2-1.0)	10/347 (2.9)	0.2 (0.1-0.6)
	P for heterogeneity <0.001		P for heterogeneity = 0.001	

[a]ORs and 95% CIs were obtained from logistic regression analysis using gender, age and tumor location as covariates.

difference in the magnitude of the 2 ORs. The pattern of age-dependence in diffuse-type EBV-GCs was different from that of intestinal-type EBV-GCs. The proportion of EBV-GCs in diffuse-type carcinomas showed an increase in those ≥50 years of age. The trend of this increase was not statistically significant. The difference in the age-dependent patterns between the 2 histological types of Lauren classification was statistically significant in logistic analysis (P=0.002). As was the case with the intestinal-type tumors, the proportion of EBV-GCs among diffuse-type tumors was the lowest in cancer of the antrum. The proportion of diffuse-type EBV-GCs did not show an evident time trend (data not shown).

Discussion

The present study showed that the proportion of EBV-GCs in intestinal-type carcinomas decreased with age. On the other hand, age was not related to the proportion of EBV-GCs in diffuse-type tumors. The relatively low proportion of diffuse-type EBV-GCs in those aged <50 may be reflecting the presence of diffuse-type tumors with hereditary backgrounds in this relatively young age group (31). The results obtained here are similar to those reported by Hao *et al*, where only intestinal-type EBV-GCs showed an age-dependent decrease (6). Similar results were also obtained in a Chilean study [unpublished data, collected by the study conducted by Corvalan *et al* (11)]. Histology-specific age distribution of EBV-GCs was also examined by Hsieh *et al* (9). This Taiwanese study showed an age-dependent increase of intestinal-type EBV-GCs (statistical tests were not conducted). On the other hand, a study conducted in Colombia showed an age-dependent decrease both in intestinal and diffuse types (7).

Using the proportions of EBV-GCs specific for age and histology observed in the present study, as well as gastric cancer incidence in Japan in 1985 (32), we estimated age-specific incidence of intestinal- and diffuse-type EBV-GCs among men (Fig. 1). Incidence of EBV-GC among women was not estimated because the number of cases was too small to obtain reliable estimates. However, in statistical analysis, there was no evidence suggesting a significant gender difference in the EBV-GC incidence. The estimated incidence of intestinal-type EBV-GCs reaches its peak around 70 years of age (Fig. 1A). On the other hand, that of diffuse-type EBV-GCs does not have a peak in its age-specific incidence curve (Fig. 1B), suggesting a much older age peak, if any. Nasopharyngeal carcinoma (NPC), which is one of the most frequently observed carcinomas in southern China, and strongly suspected to be related to EBV, has its incidence peak at 50-60 years of age (33), which is much younger than that observed in intestinal-type EBV-GCs. The difference in the peak age between NPC and EBV-GC incidence curves suggests that age at exposure to etiologically important factors differs in NPC and EBV-GC patients. In most of the countries around the world, including southern China and Japan, the EBV infection takes place in early childhood (34). Therefore, it is unlikely for the ages at primary EBV infection to explain the different age distributions of these 2 cancers. Another possibility is the difference of ages at exposure to cofactors. In the case of NPC, salted fish intake in early childhood is an important cofactor in its etiology (35). Although no cofactors of EBV-GC are known, the male predominance of EBV-GCs strongly suggests the involvement of lifestyle-related factors in its etiology. The results obtained from the present study suggest that the age at exposure to those cofactors may be much older in the case of intestinal-type EBV-GCs, and even older in the case of diffuse-type EBV-GCs, when compared to NPC. As reviewed in the previous paragraph, the patterns of age-dependence observed in EBV-GCs are different from country to country. This fact may also explain the different age at exposure to cofactors from country to country.

Almost all the studies so far showed male predominance of EBV-GC. Among the highest gender ratios were 7.0

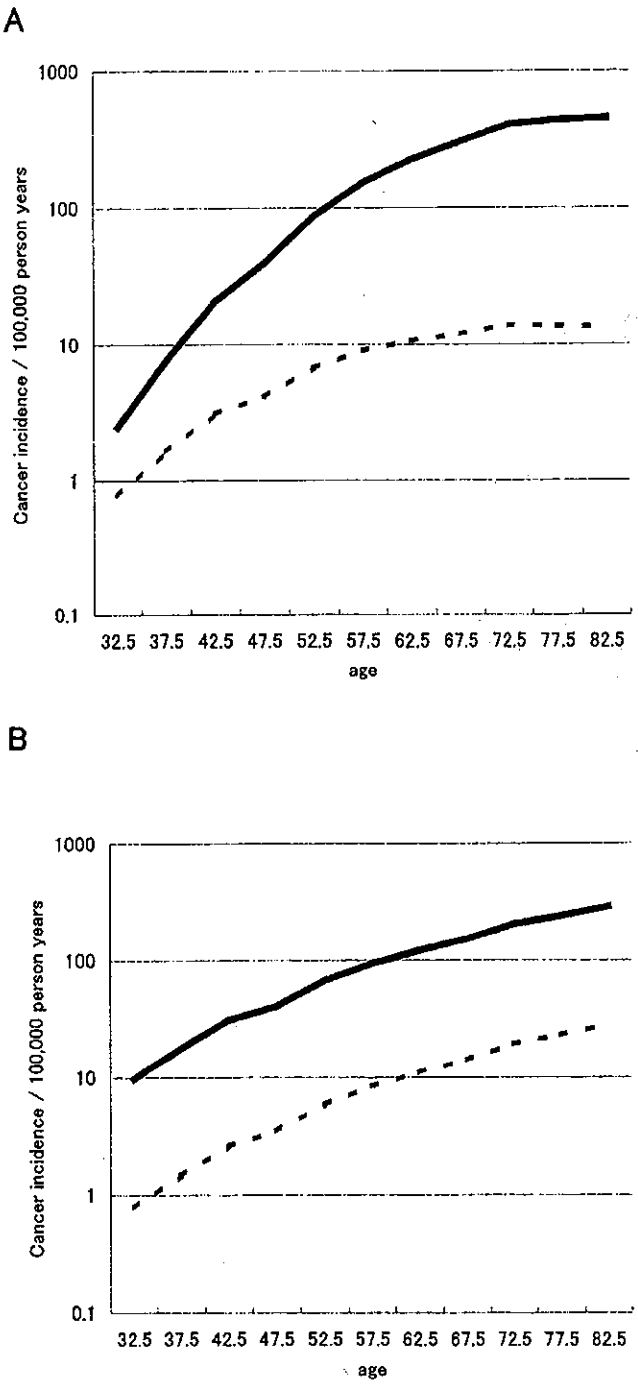

Figure 1. Estimated intestinal- and diffuse-type gastric cancer incidence in Japanese men. (A), Solid line, gastric cancer incidence of all intestinal-type tumors in Japanese men; dotted line, incidence of the intestinal-type EBV-GC in Japanese men. (B), Solid line, gastric cancer incidence of all diffuse-type tumors in Japanese men; dotted line, incidence of the diffuse-type EBV-GC in Japanese men.

observed in Caucasians living in Los Angeles (1), and 6.2 in Russians (9). Even among LELCs, which are known to be almost always EBER positive (22), the present study showed male predominance (LELC turned out to be EBER positive in all of the 12 male cases, and 4/6 female cases). To date, an exception is the Mexican study, reporting the gender ratio of only 1.2 (10). A study conducted in an area near Shanghai also reported a similar result (4). The present study showed the difference in magnitude of male predominance in intestinal- and diffuse-type tumors, suggesting that the varying gender ratio from country to country may be explained to some extent by the different distributions of various histological types. Although the underlying mechanism of male predominance in EBV-GC is not known, possible factors are the lifestyles more commonly observed in males than in females. Undifferentiated NPC, whose morphology is almost identical to that of gastric LELC, is known to have incidence about 2-fold higher in males than in females (33). The factors responsible for this male predominance have yet to be elucidated.

There are several studies reporting the predominance of diffuse-type tumors among EBV-GCs, as stated in the Introduction. This study did not confirm this notion. As shown in Table II, the ratio between intestinal- and diffuse-type EBV-GCs is affected by gender, age and tumor location. The conflicting results in the literature may be explained by the different distribution of those factors in various studies.

Another evident feature of EBV-GC observed in the present study is its low prevalence among carcinomas of the antrum regardless of histological type. Note here that fundic-gland mucosa exists in only a small part of the lower one-third of the stomach, whereas it covers the upper-third of the stomach except cardia. Thus, our observation suggests that EBV-GC tends to occur, but unlikely exclusively, in the fundic-gland epithelium, which is featured by oxyntic cells. Interestingly, as pointed out by Fukayama *et al*, EBV-GCs are more frequently found in the zone intermediate between fundic and pyloric gland mucosa (36). The intermediate zone moves in proximal direction as age advances (37). The age-dependent decrease of intestinal-type EBV-GCs observed in the present study may be explained by the shift of the zone, whose nature may be changed by the shift or the advancement of age (or both).

This study, examining by far the largest number of EBV-GCs in current literature, showed different patterns of age-dependence in intestinal- and diffuse-type EBV-GCs, suggesting that pathogenic pathways of EBV-GCs may be different in these 2 histological types.

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HTLV-1 provirus load in peripheral blood lymphocytes of HTLV-1 carriers is diminished by green tea drinking

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Human T-cell lymphotropic virus type 1 (HTLV-1) is causatively associated with adult T-cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Since a high level of HTLV-1 provirus load in circulating lymphocytes is thought to be a risk for ATL and HAM/TSP, diminution of HTLV-1 provirus load in the circulation may prevent these intractable diseases. Our previous study (*Jpn J Cancer Res* 2000; 91: 34–40) demonstrated that green tea polyphenols inhibit *in vitro* growth of ATL cells, as well as HTLV-1-infected T-cells. The present study aimed to investigate the *in vivo* effect of green tea polyphenols on HTLV-1 provirus load in peripheral blood lymphocytes on HTLV-1 carriers. We recruited 83 asymptomatic HTLV-1 carriers to examine HTLV-1 provirus DNA with or without administration of capsulated green tea extract powder. Thirty-seven subjects were followed up for 5 months by measuring HTLV-1 provirus load after daily intake of 9 capsules of green tea extract powder per day (equivalent to 10 cups of regular green tea), and 46 subjects lived *ad libitum* without intake of any green tea capsule. The real-time PCR quantification of HTLV-1 DNA revealed a wide range of variation of HTLV-1 provirus load among asymptomatic HTLV-1 carriers (0.2–200.2 copies of HTLV-1 provirus load per 1000 peripheral blood lymphocytes). Daily intake of the capsulated green tea for 5 months significantly diminished the HTLV-1 provirus load as compared with the controls (P=0.031). These results suggest that green tea drinking suppresses proliferation of HTLV-1-infected lymphocytes *in vivo*. (Cancer Sci 2004; 95: 596–601)

Human T-cell lymphotropic virus type 1 (HTLV-1) is a causative agent of adult T-cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP).[1–4] The major route of HTLV-1 infection is mother-to-child transmission via breast milk, and both bottle-feeding and short-term breast-feeding reduce the risk of neonatal infection with HTLV-1.[5,6] However, once HTLV-1 infects T lymphocytes and integrates its provirus into the host genome, the virus cannot be excluded from the body. A high level of HTLV-1 provirus load in circulating lymphocytes of HTLV-1 carriers is a risk factor for HTLV-1-related diseases.[7–10] It is thus likely that diminution of HTLV-1 provirus load in circulating lymphocytes may prevent HTLV-1 carriers from contracting ATL or HAM/TSP.

Green tea polyphenols have antioxidant and anti-mutagenic activities,[11–14] and induce apoptosis in a variety of tumor cells.[15,16] These anti-tumor activities of green tea have been supported by epidemiological findings that green tea drinking lowers the risk of stomach cancer.[17,18] Other Japanese studies showed that consumption of green tea amounting to more than 10 cups per day lowered the incidence of cancers of the stomach, lung and other sites.[19–21]

We previously reported that green tea polyphenols inhibit *in vitro* growth of ATL cells as well as HTLV-1-infected T-cells, by inducing apoptosis.[22] The present study was conducted to assess the *in vivo* effect of green tea to diminish HTLV-1 provirus load in peripheral blood lymphocytes of asymptomatic HTLV-1 carriers.

Materials and Methods

Study subjects and blood samples. Ninety-five subjects were recruited from asymptomatic HTLV-1 carriers living in Kagoshima prefecture, an HTLV-1 endemic area in southern Kyushu, Japan, all of whom had given informed consent to answer a questionnaire regarding their lifestyle (such as habitual green tea consumption, smoking and alcohol drinking habits), to make daily records of taking capsules of green tea extract powder, and to donate peripheral blood for monthly examination of HTLV-1 provirus load. The female subjects were recruited from those who had participated in the surveillance for mother-to-child transmission of HTLV-1,[6] and the male subjects were their husbands and relatives. The subjects were randomly assigned to two groups by the minimization method using age and gender as risk factors: 47 subjects who took the capsulated green tea extract powder, designated as the GT(+) group and 48 subjects who lived *ad libitum* without intake of any green tea capsules, designated as the GT(−) group. Both groups were followed up monthly for 5 months. We drew 5–7 ml of peripheral blood with citrate anticoagulant from each subject and collected the buffy coat to enrich peripheral blood lymphocytes by centrifugation at 2000g for 10 min at ambient temperature. The buffy coats were frozen at −30°C until used for examination of HTLV-1 provirus DNA. The study protocol was reviewed and approved by the Medical Ethical Committee of Kagoshima University.

Capsules of green tea extract powder. We used organic green tea leaves grown without the use of pesticides to prepare the capsules of green tea extract powder. In brief, the dry green tea leaves were infused into hot water (95°C) with ascorbic acid to stabilize green polyphenols, then the infusion was reduced to powder by a spray-dry method. The green tea extract powder was capsulated as "Nanchariki" (Satsuma Shuzo Co., Ltd., Kagoshima, Japan). Ingredients of the green tea extract powder were analyzed with a HPLC-UV detection system as depicted in Fig. 1. One capsule of the powder contained 27.3 mg of (−)-epigallocatechin-3-gallate (EGCg), which is the main constituent of green tea polyphenols. One cup of regular green tea, made by infusion of 2 g of dried leaves with 150 ml of hot wa-

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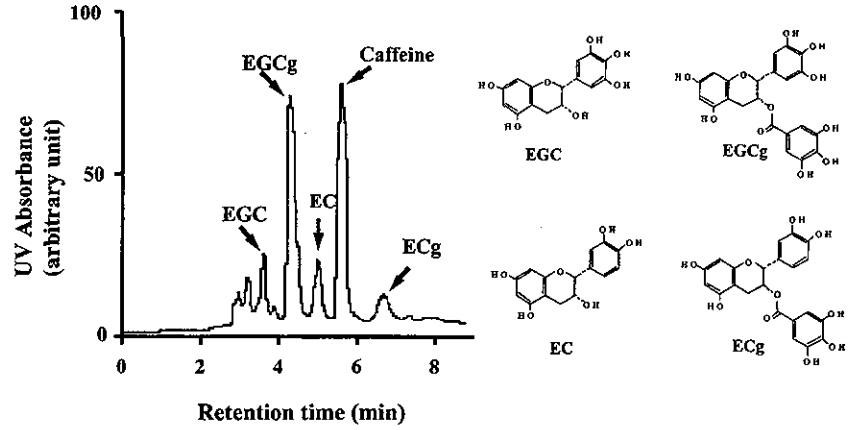

Fig. 1. HPLC profile of polyphenols and caffeine in green tea extract powder ("Nanchariki"). Ten milligrams of "Nanchariki" powder was dissolved in 10% methanol solution by sonication and vigorous vortexing. The mixture was passed through a 0.45 μm filter to get a sample solution of the green tea extract powder. An aliquot of the solution was subjected to HPLC with UV detection (mobile phase, methanol/water (35/65 containing 0.2% phosphoric acid); flow rate, 1.0 ml/min; column, C_{18} reversed-phase column (150×4.6 mm i.d., particle size 5 μm); column temperature, 40°C; UV detection, 280 nm). Polyphenol compounds were analyzed with reference to authentic (−)-epigallocatechin-3-gallate (EGCg), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECg), epicatechin (EC) and caffeine purchased from Mistui Norin Co., Ltd. (Shizuoka, Japan) and Nacalai Co., Ltd. (Tokyo), respectively. The chemical structures of EGC, EGCg, EC and ECg are shown.

ter, contains 24.0 mg of EGCg. Therefore, 9 capsules of "Nanchariki" contain 245.7 mg of EGCg, which is equivalent to 10 cups of regular green tea.

Real-time PCR quantification of HTLV-1 provirus load in peripheral blood lymphocytes. High-molecular DNA was isolated from the frozen buffy coat of blood samples pretreated with RCLB solution (Genome Science Laboratories, Fukushima, Japan) to remove red blood cell components and with SMI TEST EX-R&D (Genome Science Laboratories) to extract DNA. The standard HTLV-1 DNA was prepared from MT-2 cells.[23] The real-time PCR quantification of HTLV-1 DNA was performed in a LightCycler System (Roche Diagnostics, Mannheim, Germany) by intra-assay using a series of duplicate measurements of 12 test samples with standard DNA of 4 different dilutions for each assay. The duplicate intra-assay for HTLV-1 provirus load in peripheral blood lymphocytes was run by simultaneous measurements of β-globin DNA and HTLV-1 DNA using the standard DNAs, β-globin DNA from Roche Diagnostics, and HTLV-1 provirus DNA from MT-2 cells. One peripheral blood lymphocyte has 2 copies of *β-globin* gene (equivalent to 6 pg of β-globin DNA) and one MT-2 cell has 8 copies of HTLV-1 provirus DNA (equivalent to 6 pg of HTLV-1 DNA). The β-globin PCR primer and probe sets were commercial kits (Roche Diagnostics). The HTLV-1 primer set corresponded to the highly conserved HTLV-1 pX region, SK43 (CGGATACCCAGTCTACGTGT, nucleotide positions 7358–7377) and SK44 (GAGCCGATAACGCGTCCATCG, nucleotide positions 7516–7496).[24] The HTLV-1 pX probe set was newly designed by ourselves for the two adjacent parts of the pX region which were labeled with different fluorophores (SONPX1: 5′-TACATCGTCACGCCCTACTGGCCAC-fluorescein-3′, nucleotide positions 7438–7462 and SONPX2: 5′-LC-red640-TGTCCAGAGCATCAGATCACCTGGG-phosphate-3′, nucleotide positions 7464–7488) according to the manufacturer's instructions.

The PCR amplification was initiated with pre-incubation at 95°C for 10 min using FastStart polymerase (Roche Diagnostics). For β-globin amplification, 40 cycles of PCR were carried out: 95°C for 10 s, 50°C for 10 s and 72°C for 10 s. For HTLV-1 pX amplification, we performed 45 cycles of PCR at 95°C for 10 s, 50°C for 10 s and 72°C for 10 s. The final volume (20 μl) of the PCR amplification mixture for HTLV-1 pX, 3 m*M* $MgCl_2$, 0.5 μ*M* each of the primer set, 0.2 μ*M* each of the oligonucleotide probes, 2 μl of LightCycler FastStart DNA Master Mix (Roche Diagnostics) and 2 μl of template DNA solution diluted to 25 ng/μl, while those for β-globin were from commercially available kits (Roche Diagnostics). The HTLV-1 provirus load was expressed as number of copies per 1000 cells using the following formula: HTLV-1 provirus load=[(HTLV-1 pX copy number)/(β-globin copy number/2)]×1000. The detection limit of this method was 0.2 copies of HTLV-1 provirus/1000 cells.

Statistical analysis of difference in HTLV-1 provirus load. To examine the significance of the diminution of HTLV-1 provirus load after taking the green tea capsules, we calculated the difference of HTLV-1 provirus load between the baseline (0 month) and each month, and compared the difference between the GT(−) and GT(+) groups by time after taking the capsules, using a *t* test. We also performed subgroup analysis of subjects whose values of HTLV-1 provirus load at baseline were either below or above median value (lower or higher provirus load group). The trend of change in HTLV-1 provirus load values during the intervention period was compared between the GT(−) and GT(+) groups using a linear regression model with a group interaction term (GT(−)=0 and GT(+)=1) and time (month), after adjusting for the contribution of habitual green tea drinking (0, 1–3, 4–9, 10–14, ≥15 cups of regular green tea per day). Differences of demographic distribution and lifestyles at baseline between the GT(−) and GT(+) groups were examined by using the *t* test and χ^2 test. Statistical analysis was carried out using STATA ver.7 (Stata Corp., TX).

Results

Profile of study subjects. We recruited 95 subjects at the beginning of this study; however, 12 dropped out due to difficulty in monthly donation of blood samples and low compliance with daily intake of the capsules of green tea extract powder. Thus, we followed up 83 subjects for 5 months, 37 subjects in the GT(+) group and 46 subjects in the GT(−) group. The age distribution in the GT(+) group was slightly shifted to the younger side as compared with the GT(−) group, but the difference was not statistically significant. Lifestyle factors (green tea consumption, smoking and alcohol drinking habits) in the GT(+)

and GT(−) groups were similar at baseline, and there was no significant difference between the groups (Table 1). Most subjects tolerated the daily intake of "Nanchariki" without any complaint of gastrointestinal symptoms, except for one subject who developed an indisposition in stomach and bowel movement after 2 months of daily intake of "Nanchariki" capsules.

Properties of green tea extract powder and content of EGCg. Green tea contains antioxidants and other bioactive components.[12, 14, 22] HPLC-UV analysis revealed that the "Nanchariki" powder contained 4 polyphenols (EGC, EGCg, EC, ECg) and caffeine as major ingredients (Fig. 1). Amounts of EGC, EGCg, EC and ECg in the "Nanchariki" powder were estimated to be 84.7, 68.8, 32.5 and 10.7 mg/g, respectively. The amount of caffeine was 32.7 mg/g. Freshly prepared regular green tea showed the same profile of polyphenols and caffeine as that of "Nanchariki" powder (data not shown). Nine capsules of "Nanchariki" contained 245.7 mg of EGCg (equivalent to 10 cups of regular green tea). This is estimated to be as much as one-third of the maximum tolerated dose of green tea extract in humans, 4.2 g green tea extract (34 cups of regular green tea)/m^2/day.[25]

Real-time PCR quantification of HTLV-1 provirus load among HTLV-1 carriers. We used the real-time PCR system in LightCycler for quantification of HTLV-1 provirus load in peripheral blood lymphocyte DNA. Our duplicate intra-assay system reduced the error in PCR measurement, providing a coefficient of variation of less than 8% for test samples containing 18.0–200.2 copies of HTLV-1 provirus/1000 cells, and enabled accurate quantitative measurements of HTLV-1 provirus load in peripheral blood lymphocytes (Fig. 2). We found a wide range of individual variation of HTLV-1 provirus load, from 0.2–200.2 copies of HTLV-1/1000 cells, and the median value was 18.0 copies/1000 cells, giving a skewed distribution of HTLV-1 provirus load among HTLV-1 carriers (Fig. 3).

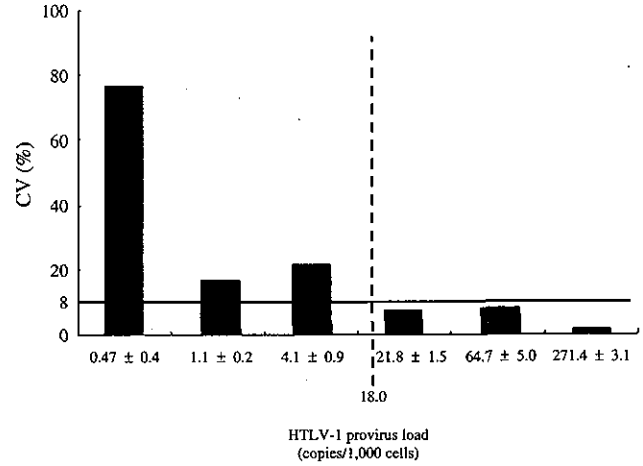

Fig. 2. Relationship between HTLV-1 provirus load and coefficient of variation. Coefficient of variation (CV) was determined by quadruplicate measurements of HTLV-1 provirus load using serially diluted DNA samples of HTLV-1 carrier's lymphocytes. CV (%) was calculated by means of the following formula: CV (%)=(SD of HTLV-1 provirus load in quadruplicate measurement/mean of HTLV-1 provirus load in quadruplicate measurement)×100. The axis shows the amount of HTLV-1 provirus load in each dilution of HTLV-1 DNA (copies/1000 cells). The ordinate shows CV (%). Subjects with more 18.0 copies of HTLV-1 provirus load/1000 cells had a CV of less than 8%.

Table 1. Characteristics of study subjects

	Number (%)		*P* value[3), 4)]
	GT(−)[1)]	GT(+)[2)]	
Age			
25–39	23 (50)	22 (59)	0.692
40–59	22 (48)	14 (38)	
60–77	1 (2)	1 (3)	
Total	46 (100)	37 (100)	
Sex			
Male	2 (4)	1 (3)	0.690
Female	44 (96)	36 (97)	
Habitual consumption of green tea at baseline			
0 cup/day	12 (26)	14 (37)	0.188
1–3 cups/day	15 (32)	11 (30)	
4–9 cups/day	12 (26)	10 (27)	
10–14 cups/day	1 (2)	1 (3)	
15 or more cups/day	3 (7)	0 0	
Unknown	3 (7)	1 (3)	
Smoking habit at baseline			
Never or former	35 (76)	31 (84)	0.573
Current	8 (17)	5 (14)	
Unknown	3 (7)	1 (3)	
Alcohol drinking habit at baseline			
None	24 (52)	19 (51)	0.117
<1 day/week	7 (15)	9 (24)	
1–4 days/week	3 (7)	6 (16)	
5> day/week	7 (15)	1 (3)	
Unknown	5 (11)	2 (6)	

1) GT(−): Without administration of green tea capsules.
2) GT(+): With administration of green tea capsules (9 capsules per day).
3) Distributions of age between GT(−) and GT(+) were compared by *t* test.
4) Differences of sex and lifestyle between GT(−) and GT(+) were examined by χ^2 test.

Diminution of HTLV-1 provirus load by intake of encapsulated green tea extract powder. The HTLV-1 provirus loads in the GT(−) group and GT(+) group at the beginning of the follow-up study were 0.2–200.2 and 0.2–192.0 copies/1000 cells, and the mean and standard deviation were 43.2±52.3 and 33.4±45.9, respectively (Fig. 4, A and B, 0 month). The HTLV-1 provirus load in both groups showed no statistically significant difference (P=0.369). The follow-up observation revealed that GT(−) group subjects above the median value had a wide range of fluctuation in HTLV-1 provirus load during the study period, whereas those below the median value maintained consistently low levels of HTLV-1 provirus load (Fig. 4A, 0–5 months). The GT(+) group showed a lesser fluctuation of HTLV-1 provirus load above the median value after intake of the green tea capsules (Fig. 4B, 0–5 months).

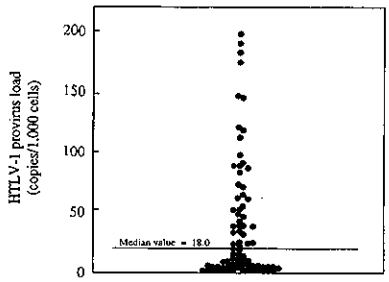

Fig. 3. Individual variation of HTLV-1 provirus load among HTLV-1 carriers. The median value of HTLV-1 provirus load among 83 study subjects was 18.0 copies/1000 cells. Note the skewed distribution, with a wide range of variation and clustering at levels below the median value of HTLV-1 provirus load.

The difference in HTLV-1 provirus load from baseline to each follow-up month between the GT(−) and GT(+) groups was not great, but reached statistical significance at 5 months in the subgroup of higher provirus load (Table 2, P=0.031). No significant difference was observed in the lower provirus load group.

Changing trend of HTLV-1 provirus load over time among HTLV-1 carriers. Among the subgroup of higher provirus load group, a decreasing trend of the HTLV-1 provirus load values was observed in the GT(+) group (regression coefficient (RC)= −0.072, SE=0.430), but not in the GT(−) group (RC=+0.012, SE=0.043), and the difference showed a marginal significance (P=0.077). This trend did not change after adjustment for age (RC of GT(+) and GT(−)=−0.072 and +0.012, respectively), smoking (RC=−0.071, +0.013), and alcohol drinking (RC= −0.072, +0.012).

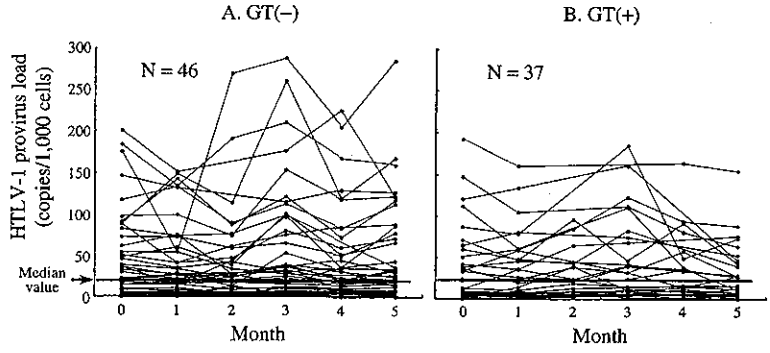

Fig. 4. Change in HTLV-1 provirus load during 5 months follow-up of HTLV-1 carriers with or without administration of green tea powder capsules ("Nanchariki"). (A) GT(−); 46 subjects lived without restriction of their lifestyle as a control group. They were followed up for 5 months by measuring HTLV-1 provirus DNA by duplicate intra-assay using 6 blood samples obtained from each subject. The median value of 18.0 copies/1000 cells was the same as that in Fig. 3. (B) GT(+); 37 subjects received 9 capsules of "Nanchariki" and the HTLV-1 provirus loads were measured by duplicate intra-assay as described above.

Table 2. Comparison of HTLV-1 provirus load difference from baseline to each follow-up month between GT(−) and GT(+) groups

	Number	Values of provirus load					
		Baseline	Difference between baseline and each month				
		0 month	1 month	2 months	3 months	4 months	5 months
		Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Total							
GT(−)	46	42.1±52.4	−4.1±25.6	−2.5±20.2	11.6±39.0	−2.1±14.4	1.5±27.4
GT(+)	37	32.8±46.3	−2.6±15.4	5.5±14.1	8.7±24.9	−4.9±26.5	−7.8±27.4
P values		—	0.616	0.946	0.364	0.301	0.063
Lower provirus load group[1]							
GT(−)	20	4.6±5.2	−0.2±1.7	0.4±6.2	0.8±7.0	−0.1±4.1	−0.1±3.0
GT(+)	21	3.8±3.4	1.6±6.5	4.8±10.6	2.0±3.2	2.4±3.5	1.6±3.6
P values		—	0.873	0.892	0.722	0.936	0.936
Higher provirus load group[2]							
GT(−)	26	72.6±53.2	−7.2±34.0	−4.1±25.0	17.2±47.2	−3.2±17.8	2.7±36.6
GT(+)	16	66.6±49.1	−8.1±21.3	6.6±18.8	15.4±34.3	−12.2±36.7	−20.2±38.7
P values		—	0.46	0.874	0.45	0.169	0.031

Note the statistical significance of the difference of HTLV-1 provirus load at 5 months in the higher load group.
1) The subjects with an HTLV-1 provirus load lower than 18.0 copies/1000 cells at baseline.
2) The subjects with an HTLV-1 provirus load higher than 18.0 copies/1000 cells at baseline.

Discussion

The present study was designed to investigate the *in vivo* effect of green tea on HTLV-1 provirus load by administering green tea powder capsules ("Nanchariki") to HTLV-1 carriers. The real-time PCR quantification of HTLV-1 provirus DNA has an inherent error of 25% in terms of coefficient of variation (CV), as was seen in different inter-assay runs for HTLV-1 provirus load.[26] However, our real-time PCR quantification with duplicate intra-assay reduced the CV to less than 8% (Fig. 2). By virtue of this method, we could perform accurate measurements of HTLV-1 provirus load in peripheral blood lymphocytes of asymptomatic HTLV-1 carriers, revealing a wide range of variation and fluctuation of HTLV-1 provirus load among HTLV-1 carriers (Fig. 3, Fig. 4). Similar findings were documented by the Miyazaki cohort study.[10]

Daily intake of 9 capsules of "Nanchariki" for 5 months diminished the HTLV-1 provirus load in the higher provirus load group. The extent of diminution was not great but the HTLV-1 provirus load showed a consistent diminution over time in the GT(+) group (RC=−0.072). This marginal extent of HTLV-1 diminution can be explained by three possibilities. The first possibility is that the dose of the green tea extract powder used in this study (9 capsules of "Nanchariki") is insufficient for suppression of HTLV-1 carriers' lymphocytes *in vivo*, since the concentration of EGCg in plasma is estimated to be about 0.3 μg/ml,[27] which is far less than the range of 3–27 μg/ml required to inhibit the growth of HTLV-1-infected T-cells *in vitro*.[22] The second is that the majority of the HTLV-1 carriers are females, who may have fewer abnormal lymphocytes in the circulation than males[28,29] and their HTLV-1-infected lymphocytes may be less sensitive to apoptosis induction by green tea components.[22] The third is that individual variation in absorption and metabolism of tea polyphenols[30] may produce heterogeneous responses in diminution of HTLV-1 provirus load, so that it is difficult to obtain a consistent result with statistical significance.

Work on the molecular mechanisms of HTLV-1 suppression by green tea polyphenols has been focused on the transcriptional factors, I-κB/NF-κB.[31] HTLV-1 Tax protein degrades I-κB and activates NF-κB, and then enhances IL-2/IL-2 receptor expression in HTLV-1-infected T-cells.[32] EGCg of green tea polyphenols stabilizes I-κB and abrogates NF-κB activation in keratinocytes.[31] Stabilization of I-κB and abrogation of NF-κB activation may occur in HTLV-1 carrier lymphocytes after intake of green tea capsules. Individuals with a higher provirus load, who are at risk for ATL, may derive particular benefit from green tea drinking because they have increased number of abnormal lymphocytes that are prone to apoptosis.[22,29,33]

"Nanchariki" is made of whole extract of green tea, which contains caffeine and polyphenol compounds. Decaffeinated capsules of green tea extract powder may minimize in the effects on the stomach and bowel movements encountered in the drop-out cases in this study. We have already developed decaffeinated capsules, and a further study is planned.

In conclusion, the present study has demonstrated a diminution of HTLV-1 provirus load in peripheral blood lymphocytes of HTLV-1 carriers taking "Nanchariki" capsules. Further study on the mechanism of suppression of transcriptional factors and induction of apoptosis of HTLV-1-infected lymphocytes by green tea may provide an insight into chemoprevention of other virus-related cancers.

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Dual Role of Vitamin C in an Oxygen-sensitive System: Discrepancy between DNA Damage and Cell Death

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Although vitamin C is considered to act both as pro-oxidant and antioxidant, the mechanisms underlying these actions are still unclear. Using the oxygen-sensitive system of a strict anaerobe, *Prevotella melaninogenica*, we investigated both the pro-oxidant and antioxidant mechanisms of vitamin C. In the presence of vitamin C, the 8-hydroxydeoxyguanosine (8OHdG) formation induced by oxygen exposure was enhanced, probably due to the action of vitamin C on hydrogen peroxide generated during oxygen exposure: while catalase almost completely suppressed the enhancing effect of vitamin C, 8OHdG formation induced by hydrogen peroxide was enhanced by vitamin C. By contrast, the presence of vitamin C inhibited bacterial cell death, membrane damage, and lipid peroxidation induced by oxygen exposure. Sodium azide showed similar effects to vitamin C, thus the antioxidant action of vitamin C may be due to its quenching of the singlet oxygen generated in this system. Both the pro-oxidant and antioxidant effects of vitamin C were observed only in acidic conditions.

Keywords: Vitamin C; Oxidative DNA damage; Lipid peroxidation; Bacterial cell killing; Membrane damage; *Prevotella melaninogenica*

Abbreviations: 8OHdG8-hydroxydeoxyguanosine; dGdeoxyguanosine; DPBSDulbecco's phosphate buffered saline; ESRelectron spin resonance; FDAfluorescein diacetate; H_2O_2hydrogen peroxide; HPLChigh-performance liquid chromatography; MDAmalondialdehyde; NaN_3sodium azide; ROSreactive oxygen species; SDSsodium dodecyl sulfate; SODsuperoxide dismutase; TBAthiobarbituric acid; TBARSthiobarbituric acid-reactive substance; TEP1,1,3,3-tetraethoxypropane

INTRODUCTION

Numerous exogenous agents and endogenous processes are capable of inducing free radicals *in vivo*.[1] In particular, reactive oxygen species (ROS) are produced continuously during normal cellular metabolism, exposure to radiation, or metabolic activation by certain chemicals.[2] These endogenously generated ROS are held by some to cause such damage as to be a major contributing factor in aging and numerous degenerative processes including cancer, heart disease, cataracts, and cognitive dysfunction.[3–6] Two defense mechanisms that limit the levels of ROS and thus prevent oxidative damage, have evolved in organisms. One is mediated by antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase,[2,7] the other by dietary antioxidant chemicals contained in fruits and vegetables.[8,9]

Of the small-molecule antioxidants, vitamin C has received the most attention. Experimental and epidemiological studies have reported that by scavenging physiologically relevant ROS and reactive nitrogen species, vitamin C has anticarcinogenic and chemopreventive actions.[10,11] Moreover, vitamin C is an effective water-soluble antioxidant that, in plasma, can prevent lipid peroxidation induced by peroxyl radicals or the gas-phase of cigarette smoke, and possibly protect against cardiovascular disease.[12,13] On the other hand, substantial evidence has shown that vitamin C may also act as an pro-oxidant, depending upon the environment in which it is present.[14] For example, it can induce apoptotic cell death in human myelogenous leukemic cell lines[15] and shows genotoxic effects in some test systems.[16,17] More recently, the pro-oxidative effect of vitamin C on biomacromolecules has been receiving increasingly great attention but much remains to be understood. Further research is

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essential to elucidate the specific local effects of vitamin C in biological systems.[18,19].

In a previous study, using a strict anaerobe, we established a highly oxygen-sensitive biological system.[20] Now we have used this system to investigate the role of vitamin C. In particular, we investigated the effect of vitamin C on the generation of 8-hydroxydeoxyguanosine (8OHdG), typically present when there is oxidative DNA damage,[21–23] and on lipid peroxidation, membrane damage, oxidative protein damage, and the killing of bacterial cells exposed to oxygen.

MATERIALS AND METHODS

Materials

L-Ascorbic acid (vitamin C) was obtained from Katayama Chemicals, Inc. (Osaka, Japan). Superoxide dismutase (SOD) was purchased from Wako Pure Chemicals, Inc. (Osaka, Japan). Sodium azide (NaN_3), sodium dodecyl sulfate (SDS) and HPLC grade acetonitrile were supplied by Nacalai Tesque, Inc. (Kyoto, Japan). Polyoxyethylenesorbitan monolaurate (Tween 20) and β-carotene came from Sigma-Aldrich Chemie Gmbh. (Steinheim, Germany) and catalase from Boehringer-Mannheim (Mannheim, Germany). Hydrogen peroxide was supplied by Santoku Chemicals, Inc. (Tokyo, Japan), while fluorescein diacetate (FDA), thiobarbituric acid (TBA), 1,1,3,3-tetraethoxypropane (TEP), and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO). S7150 Oxyblot™ Protein Oxidation Detection Kits were obtained from Chemicon International (Serologicals Corporation, GA).

Growth and Collection of Bacteria

Prevotella melaninogenica (*P. melaninogenica*) (GAI5490, strict anaerobe) cultures were grown on Brucella HK agar (Kyokuto Seiyaku Kogyo, Tokyo, Japan) in an anaerobic incubator (model 1024, Forma Scientific Inc., Marietta, OH) at 37°C as previously described.[20] After incubation for 3 days, bacteria were harvested and suspended in Dulbecco's phosphate buffered saline (DPBS, pH 7.0, Nikken Seibutsu, Kyoto, Japan, used in Fig. 1 and Table I experiments) or citrate-buffered saline (CBS, pH 5.0, used in Figs. 2–5 and Table II experiments) under oxygen-free conditions. Bacterial cell density was spectrophotometrically measured with 660 nm light and adjusted to density of 2.6 during all experiments.

Exposure of Bacteria to Oxygen and Hydrogen Peroxide

Bacteria were exposed to oxygen (O_2) by bubbling gaseous O_2 at 100 ml/min for 30 s through 1 ml samples of bacterial cell suspensions in 15 ml centrifuge tubes. Then tubes were tightly sealed and samples were incubated at 37°C for 1 or 3 h.

Exposure to hydrogen peroxide (H_2O_2) was carried out by incubating similar bacterial cell suspensions with various concentrations of H_2O_2 at 37°C for 3 h.

All enzymes or other reagents were added to suspensions of *P. melaninogenica* just before exposure to O_2.

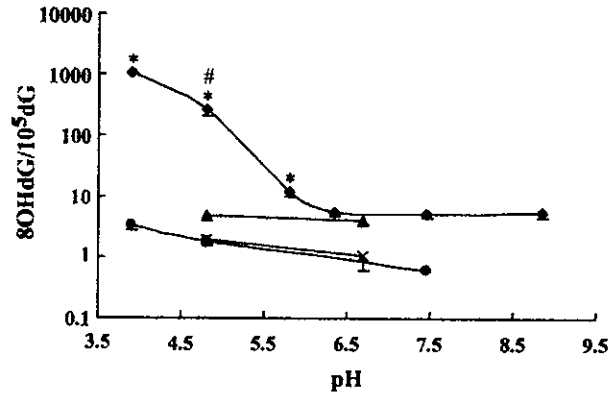

FIGURE 1 Correlation of pH with the effects of vitamin C on induction of 8OHdG by oxygen. Bacterial cells were exposed to oxygen under different pH conditions in the presence or absence of 10 mM vitamin C and then incubated at 37°C for 3 h. 8OHdG levels were determined as described in "Materials and Methods" section. ×, without oxygen exposure and without vitamin C; ▲, oxygen exposure without vitamin C; ●, without oxygen exposure but with vitamin C; ◆, oxygen exposure with vitamin C. Data are expressed as mean − SE. $^*P < 0.05$, indicates significantly enhanced presence of 8OHdG, compared with samples at pH 7.46. $^\#P < 0.05$, indicates significantly enhanced presence of 8OHdG, compared with ▲ samples (at the same pH exposed to oxygen without vitamin C).

Evaluation of Bacterial Cell Killing

After being exposed to O_2 and incubated for 3 h, bacterial cell suspensions were appropriately diluted with DPBS and spread on Brucella HK agar plates. Colonies were counted after 3–4 days incubation under anaerobic conditions at 37°C. Survival was calculated by dividing the number of colonies of

TABLE I Correlation of pH and effect of vitamin C on bacterial cell survival

pH	O_2^-	O_2^+	$vC(O_2^-)$	$vC(O_2^+)$
7.0	100	0	108.4 ± 29.2	0
5.0	100	0.0036 ± 0.0016	83.7 ± 3.4	0.1040 ± 0.029*

Bacterial cells suspended in neutral (pH 7.0) or acidic (pH 5.0) DPBS buffer were exposed or left unexposed to oxygen and then incubated at 37°C for 3 h in the presence or absence of 10 mM vitamin C. Bacterial cell survival was determined as described in "Material and Methods" section. Samples without oxygen exposure and without vitamin C (O_2^-) served as control (100%). The cell survival of other samples was calculated as percent of control. O_2^-, without oxygen exposure without vitamin C; O_2^+ oxygen exposure without vitamin C; $vC(O_2^-)$, without oxygen exposure with vitamin C; $vC(O_2^+)$, oxygen exposure with vitamin C. Results from three separate experiments are presented as mean ± SE. $^*P < 0.05$ compared with O_2^+ sample.

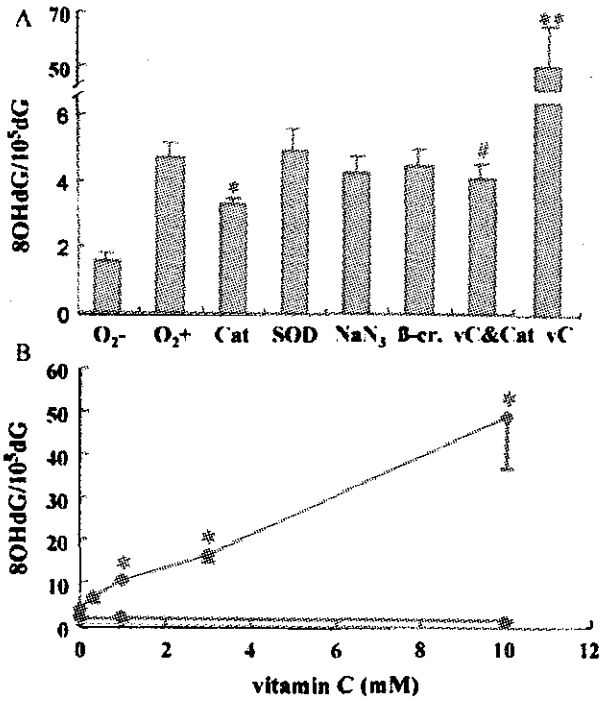

FIGURE 2 Effects of ROS scavengers (A) and dose-dependent effect of vitamin C (B) on induction of 8OHdG by oxygen. Bacterial cells were exposed to oxygen in the presence of vitamin C and/or ROS scavengers at pH 5.0 and then incubated at 37°C for 3 h. 8OHdG levels were determined as described in "Materials and Methods" section. (A) O_2^-, without oxygen exposure and without vitamin C or scavengers; O_2^+, oxygen exposure but without vitamin C or scavengers; the following samples were exposed to oxygen in the presence of various reagents. Cat, catalase (1000 units/ml); SOD, superoxide dismutase (300 units/ml); NaN_3, sodium azide (1 mM); β-cr., β-carotene (10 μM); vC and Cat, co-addition of vitamin C (10 mM) and catalase (1000 units/ml); vC, vitamin C (10 mM). $^*P < 0.05$, compared with samples (O_2^+) exposed to oxygen, indicates significant suppression of, and $^{**}P < 0.05$ significant increase in, induction of 8OHdG; $^*P < 0.05$, compared with samples with vC, indicates significantly suppressed induction of 8OHdG. (B) ●, without oxygen exposure but with vitamin C; ◆, oxygen exposure with vitamin C. $^*P < 0.05$ compared with samples exposed to oxygen without vitamin C. Results from three independent experiments are presented as mean ± SE.

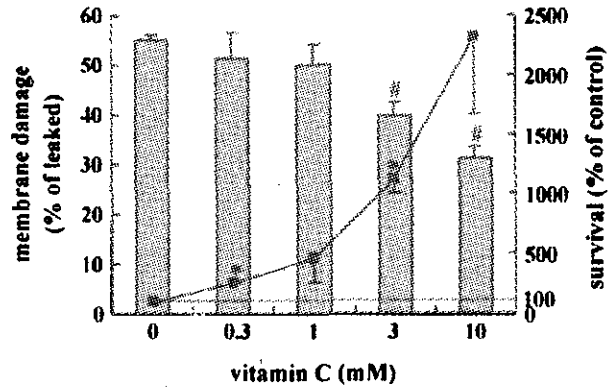

FIGURE 3 Effects of vitamin C on bacterial membrane damage (bar graph) and killing of bacterial cells (line graph) associated with oxygen exposure at pH 5.0. Membrane damage and bacterial cell mortality were investigated and analyzed as described in "Materials and Methods" section. Results from three independent experiments are presented as mean ± SE, except for mortality at 10 mM, for which two independent experiments were performed. $^*P < 0.05$ compared to the survival of samples exposed to oxygen without vitamin C. $^{\#}P < 0.01$, compared to the membrane damage of samples exposed to oxygen without vitamin C.

growing per dish from test samples by the number of colonies growing per dish from control samples (without O_2 exposure and without presence of vitamin C or other agents, such as enzymes).

Detection of 8OHdG

The DNA of the bacteria was extracted and digested under anaerobic conditions.[24] Quantities of 8OHdG and deoxyguanosine (dG) were determined by HPLC with electrochemical detection and UV absorption as previously described.[25] Oxidative DNA damage (8OHdG levels) was expressed as the molar ratio of 8OHdG per 10^5 dG.

Determination of Membrane Damage

Fluorescein diacetate (FDA) assay has been employed to assess cell membrane integrity.[26,27] FDA permeates the cells and is hydrolyzed by cellular esterases to form fluorescein, which is fluorescent and remains trapped within the cell.[28] Fluorescent cells are assumed, therefore, to have intact cell membranes. Bacterial membrane damage was evaluated by using flow cytometry to measure the leakage of fluorescent dye (FDA) from labelled bacteria.

Bacterial cells were incubated with FDA (4.8 μM) at 37°C for 15 min under oxygen-free conditions to allow the permeation of FDA, then these labelled cells were exposed to O_2 as described above. Exposed bacterial samples were incubated at 37°C for 1 h, then diluted 1000 times with DPBS and

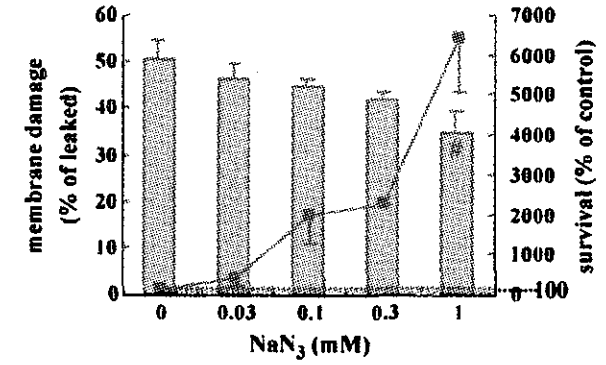

FIGURE 4 Effects of sodium azide on bacterial membrane damage (bar graph) and bacterial cell mortality (line graph) associated with oxygen exposure at pH 5.0 Bacterial cell mortality and membrane damage were investigated and analyzed as described in "Materials and Methods" section. For membrane damage, results from three independent experiments are presented as mean + SE and, for mortality, from two independent experiments (mean − range). $^*P < 0.05$, compared to samples exposed to oxygen in the absence of sodium azide, indicates significantly decreased membrane damage.

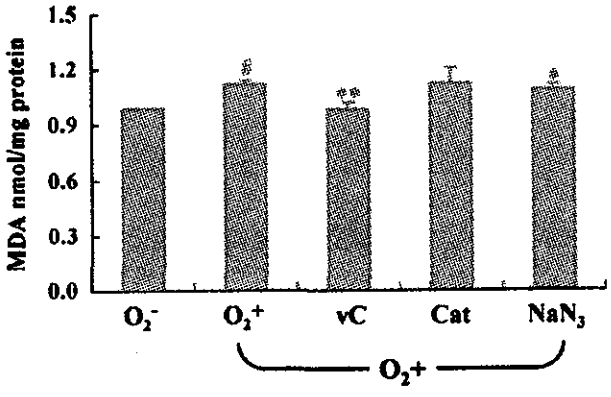

FIGURE 5 Effects of vitamin C and ROS scavengers on oxygen-induced lipid peroxidation. Bacteria were exposed to oxygen in the presence of vitamin C or ROS scavengers at pH 5.0 and subsequently incubated at 37°C for 1 h. Results are shown as mean + SE from four independent experiments. See Fig. 2A for concentrations of vC (vitamin C) and scavengers and explanation of O_2- and O_2^+. $^{\#}P < 0.05$, compared with O_2^- samples, indicates significantly greater induction of lipid peroxidation. $^{*}P < 0.05$ and $^{**}P < 0.01$,compared with O_2^+ samples, *indicate* significantly suppressed lipid peroxidation.

evaluated using flow cytometry and CellQuest software (FACScan, Becton Dickinson, Franklin Lakes, NJ). Membrane damage was expressed as percentage of leaked fluorescence compared with fluorescence of unexposed cells.

Detection of Lipid Peroxidation

Malondialdehyde (MDA) was used as an index of lipid peroxidation and measured using a previously described TBA method.[29,30] After oxygen exposure, bacterial samples (1 ml) were incubated at 37°C for 1 h, after which 100 µl 0.69% KCl and 100 µl 4.86% SDS were added. Then the mixture was centrifuged at 12,000 rpm. The supernatant was incubated at 95°C for 1 h in TBA working solution (0.2% TBA solution in 0.1 M sodium acetate buffer, pH 2.5). After cooling to room temperature, the samples were centrifuged at 10,000*g* for 10 min. Supernatant was used for the HPLC analysis. The TBA–MDA adduct was separated on an octadecylsilane column (TSK gel ODS 80Ts, TOSOH Corporation, Tokyo, Japan) and monitored with a fluorescence detector at 515 nm excitation and 553 nm emission. TEP was used as a standard. Lipid peroxidation was calculated as MDA equivalents and expressed as nmol MDA per milligram protein measured by the Bradford assay.

TABLE II Effect of vitamin C on induction of 8OHdG by Hydrogen peroxide

	Vitamin C (mM)			
H_2O_2 (mM)	0.0	0.3	1.0	3.0
0.2	18.2 ± 0.3	32.2 ± 1.4*	61.2 ± 5.1*	97.5 ± 11.2*
0.5	25.5 ± 1.4	46.3 ± 1.0*	85.2 ± 0.6*	156.5 ± 18.3*
1.0	34.5 ± 2.5	NT	103.0 ± 14.3*	182.8 ± 23.7*

With the indicated concentrations of H_2O_2, bacterial cells were incubated at pH 5.0, in the absence or presence of vitamin C, at 37°C for 3 h. 8OHdG levels were determined as described in "Materials and Methods" section. Data from three separate experiments are presented as mean ± SE. NT, not tested. $^{*}P < 0.05$ compared with samples which incubated with the same concentration of Hydrogen peroxide in the absence of vitamin C.

Statistical Analysis

Results are presented as mean ± SEM (standard error of the mean). Statistical analysis was performed using Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Effects of Vitamin C on Oxygen-induced Bacterial Cell Killing and the Relationship of pH to Oxidative DNA Damage

After bacterial cells were exposed to oxygen in neutral or acidic conditions, oxidative DNA damage and bacterial cell killing were evaluated. Oxygen significantly induced DNA damage under both neutral and acidic conditions. The resultant amount of 8OHdG did not significantly vary depending on pH (Fig. 1). When vitamin C was added to bacterial cells, however, the oxidative DNA damage due to oxygen exposure significantly increased, but only in samples with acidic conditions (pH < 6; $P < 0.05$). Table I shows the effect of vitamin C on bacterial cell survival in different pH conditions. If bacteria were not exposed to O_2, vitamin C made no difference to survival in either neutral or acidic conditions. Both in the presence and absence of vitamin C, at pH 7.0, oxygen exposure killed all bacteria. On the other hand, at pH 5.0, vitamin C suppressed the cell killing due to O_2 exposure.

Effects of Scavengers on Oxygen-induced 8OHdG

Exposure to O_2 resulted in 8OHdG formation in bacterial cells. We examined the effects of ROS scavengers on oxygen-induced 8OHdG at pH 5.0. As shown in Fig. 2A, superoxide scavenger SOD, and singlet oxygen scavengers sodium azide and β-carotene did not suppress the formation of 8OHdG in the presence of oxygen. By contrast, catalase significantly inhibited oxygen-induced 8OHdG formation. As described above, vitamin C on its own did not increase 8OHdG, however, it did dose-dependently enhance the production of 8OHdG in the presence of oxygen (Fig. 2B). Catalase almost completely inhibited this effect (Fig. 2A).

Effect of Vitamin C on H_2O_2-induced 8OHdG

Table II shows the effect of vitamin C on oxidative DNA damage induced by H_2O_2: 8OHdG increased depending on the amount of H_2O_2 present, and vitamin C also dose-dependently enhanced this production of 8OHdG in the presence of H_2O_2.

Effects of Vitamin C on Oxygen-induced Bacterial Cell Killing and Membrane Damage

Incubation of vitamin C with bacterial samples while they were exposed to oxygen dose-dependently increased survival of bacterial cells (Fig. 3). At lower concentrations, vitamin C provided less effective protection, but at 0.3 and 3 mM, the number of surviving cells significantly increased compared to bacteria exposed to O_2 without vitamin C.

Vitamin C dose-dependently suppressed membrane damage induced by oxygen exposure (Fig. 3). As with the effect of vitamin C on cell survival, significant protection was observed at 3 and 10 mM. In this experimental system using *P. melaninogenica*, no membrane damage was induced by H_2O_2.

Effects of ROS Scavengers on Oxygen-induced Cell Killing

Vitamin C suppressed oxygen-induced bacterial cell killing (Fig. 3). To gain insight into the mechanism of this suppressive effect, we examined the effects of ROS scavengers on oxygen-induced bacterial cell killing at pH 5.0. In line with results obtained in our previous report,[20] catalase suppressed the cell killing. Bacterial colony numbers per milliliter of bacterial suspension ($n = 3$–5 per category) were 6500 ± 3000 when vitamin C alone was added, 4300 ± 1600 with catalase alone, and $57{,}400 \pm 12{,}600$ in the presence of both vitamin C and catalase. Compared with vitamin C or catalase alone, the co-addition of catalase and vitamin C significantly suppressed the cell killing ($P < 0.05$). In a way similar to the effects of vitamin C, sodium azide, a typical singlet oxygen scavenger, concentration-dependently suppressed both oxygen-induced cell killing and membrane damage (Fig. 4). Another typical singlet oxygen scavenger β-carotene had similar effects to vitamin C and sodium azide. Superoxide scavenger SOD, however, had no effect (data not shown).

Effects of Scavengers and Vitamin C on Lipid Peroxidation Induced by Oxygen

Testing for lipid peroxidation, we found increased MDA (evaluated as a TBA-reactive substance, TBARS) after exposure to oxygen (Fig. 5). While both vitamin C and sodium azide significantly suppressed the production of MDA, no suppression was found with catalase or SOD (data not shown).

DISCUSSION

Although vitamin C is one of the most important nutrients and antioxidants in the human body, pro-oxidative effects have also been reported. To elucidate the working of this nutrient, we investigated some of the actions of vitamin C in a simple biological system. We have already established a highly oxygen-sensitive biological system using a strict anaerobe,[20] which has proved useful in investigating the biological effects of ROS and the activities of antioxidants and pro-oxidants. In the current study, we investigated both the pro-oxidative and antioxidative effects of vitamin C on the system. When there was exposure to O_2, the presence of vitamin C enhanced oxidative DNA damage even while suppressing bacterial cell killing, membrane damage, and lipid peroxidation. These actions are likely to result from different mechanisms and such findings further demonstrate the usefulness of this bacterial system for investigating the effects of ROS and antioxidants.

We have already reported that, after *P. melaninogenica* is exposed to O_2, hydrogen peroxide and superoxide are generated and 8OHdG is induced.[31] The suppressive effect of catalase on 8OHdG induction indicates that hydrogen peroxide induces oxidative DNA damage in the system. When vitamin C was added to the system, by itself it had no effect on 8OHdG induction, however, under acidic conditions, when there was exposure to O_2, the presence of vitamin C did significantly enhance oxygen-induced 8OHdG formation. Meanwhile, catalase almost completely suppressed this vitamin C enhancement of 8OHdG formation. Such findings indicate that this pro-oxidative action of vitamin C is most likely due to its capacity for enhancing the toxicity of hydrogen peroxide in the system. Using the same system, under the same pH conditions as during oxygen exposure, vitamin C enhanced H_2O_2-induced 8OHdG formation (Table II). Although membrane permeable hydrogen peroxide did induce oxidative DNA damage (Table II), it is unlikely to have a direct effect on DNA.[32] Rather, its effect is most probably mediated by the generation of a hydroxyl radical by the Fenton reaction, which involves iron-dependent reduction of hydrogen peroxide.[33–35] Vitamin C is a cellular reducing agent that is likely to be able to replace superoxide in reducing Fe^{3+} to Fe^{2+} and, consequently, promote the formation of hydroxyl radicals when hydrogen peroxide is present.[14,36–38] Several studies have

reported the genotoxicity of vitamin C and the interaction with iron was suggested to be the likely mechanism.[39,40] Moreover, in the Fenton reaction, free iron readily acts as a catalyst. Studies with *E. coli* and yeast have shown that free iron exists in growing cells.[41,42] In our system, when we added phenanthroline to *P. melaninogenica*, the bacterial cells turned red, indicating the presence of free iron. Spectrophotometric analysis confirmed that 5 μM of phenanthlorine chelatable iron was present in the cells. Since iron is more soluble in acidic conditions, it becomes more available for redox reactions.[43,44] Our findings indicate that vitamin C enhances oxygen-induced 8OHdG formation only in acidic conditions. Thus, when local conditions are acidic, we speculate that vitamin C acts as a pro-oxidant by reducing free iron, which accelerates the Fenton reaction.

Serious DNA damage is considered to induce cell death, including necrosis and apoptosis.[2,45,46] In the present study, however, with vitamin C present in acidic conditions, an inverse relation was observed between 8OHdG levels and cell survival. This suggests that vitamin C enhances the survival of cells with DNA damage, and this may help to explain the reported carcinogenicity of vitamin C.[47,48]

Vitamin C suppressed the oxygen-induced death of bacterial cells. It also suppressed membrane damage, which is a likely factor in cell death, and suppressed lipid peroxidation, which may be a cause of membrane damage. We found that while catalase also reduced cell mortality, it did not suppress membrane damage (data not shown) or lipid peroxidation. Meanwhile, at 1.0 mM, although H_2O_2 killed the bacteria completely, it did not induce membrane damage. These findings suggest that something other than H_2O_2 itself induces cell death. Several reports have described the important role of singlet oxygen in killing bacterial cells.[49–52] At the same time, singlet oxygen-induced cell death has been associated with the reactivity of singlet oxygen with lipids and/or proteins.[53–55] Consequently, we investigated the effects of singlet oxygen scavengers on our system. Both sodium azide and β-carotene suppressed cell death. Because sodium azide is so easy to handle, we further investigated its effects on membrane damage and lipid peroxidation and found that the presence of sodium azide had effects similar to the presence of vitamin C. The inhibiting effect of sodium azide on lipid peroxidation and membrane damage indicates that singlet oxygen induces lipid peroxidation and bacterial cell membrane damage. Without oxygen exposure, sodium azide in concentrations up to 1 mM did not affect the survival of *P. melaninogenica*. It seems reasonable to conclude that the cell death that we observed in this study was at least partly due to the presence of singlet oxygen, which altered lipids and damaged the membrane. Reports have shown that, after incubating vitamin C with chemically generated singlet oxygen, vitamin C can scavenge singlet oxygen.[56,57] Although the generation of singlet oxygen has been reported in some kinds of bacterium,[58] we are not able to find evidence of its generation in our system. Although we were unable to detect the presence of singlet oxygen, and in spite of the efforts of others with ESR[59,60] and HPLC with fluorescent detection,[61] we still find the most plausible explanation for the antioxidative effect of vitamin C is its action on singlet oxygen. Compared with the effect of vitamin C or catalase on their own, the co-presence of catalase and vitamin C significantly and greatly suppressed cell killing, and this also supports such a conclusion. When we studied protein oxidation, however, we found no evidence, evaluated by the subsequent amounts of protein carbonyls present, that exposure to oxygen induced protein oxidation (data not shown).

The increased damage caused by vitamin C to the DNA of bacterial cells suggests that vitamin C was present in the bacterial cells. Indicating that the transport of vitamin C into bacterial cells is possible, the presence of L-ascorbate permease has been reported for *E. coli* and has been identified in association with a wide variety of bacteria.[62] Vitamin C is a diacid with pK1 = 4.2 and pK2 = 11.6. Studies on the stability of vitamin C show that the rate of vitamin C oxidation increases as pH rises.[63] *In vitro*, vitamin C concentrations remained stable in acidic, but fell significantly during 24 h in neutral (pH 7.2), gastric aspirate.[64] The stability of vitamin C in acidic condition may explain why, in our system, both the pro-oxidant and antioxidant effects of vitamin C were observed only in acidic conditions.

In conclusion, in our *P. melaninogenica* model, oxygen exposure induced significant oxidative stress. In particular, there is evidence that the presence of oxygen resulted in the formation of H_2O_2 and singlet oxygen, which, in turn, induced oxidative DNA damage and cell death. Vitamin C showed both pro-oxidative activity by enhancing the oxidative DNA damage and, antioxidative activity by decreasing cell death, membrane damage, and lipid peroxidation subsequent to oxygen exposure. The dual role of vitamin C is probably due to its contrary action on two kinds of oxidative stress. In our model, the pro-oxidative effect of vitamin C probably results from its enhancement of hydrogen peroxide toxicity. At the same time, the antioxidative effect is likely due to its quenching of singlet oxygen. Numerous pathologies have been associated both with oxidative stress and with low tissue-pH values.[65,66] Therefore, before recommending the use of vitamin C as a dietary

antioxidant supplement, it would be prudent to consider the local physiological environments in which, after consumption, vitamin will be present.

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Generation of 8-hydroxydeoxyguanosine from DNA using rat liver homogenates

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In relation to carcinogenesis, aging and other pathologic conditions, urinary 8-hydroxydeoxyguanosine (8OHdG) is widely used as a marker for evaluating the effect of oxidative stress on DNA. Because no reports have described how 8OHdG is generated from DNA *in vivo* or by biological materials, and how it is excreted into urine, the authors investigated the generation of 8OHdG from DNA, using rat liver homogenate. Oxidatively damaged DNA samples containing different levels of 8OHdG were prepared using ultraviolet irradiation with three different concentrations of riboflavin. Following incubation of damaged DNA samples with rat liver homogenates, the generation of 8OHdG from the DNA was determined using high-performance liquid chromatography with electrochemical detection after ultrafiltration of the incubation mixtures. The generation of 8OHdG was also tested with an anti-8OHdG antibody. The quantity of 8OHdG generated from the DNA by rat liver homogenates was dependent on the 8OHdG levels in the DNA: almost all 8OHdG in the DNA was released as 8OHdG by rat liver homogenates. Generation of 8OHdG correlated with the degradation of DNA. Interestingly, the generated 8OHdG was stable in the presence of rat liver homogenates, whereas deoxyguanosine (dG) rapidly disappeared in the same conditions. Less than 1/10 000 of dG was converted to 8OHdG when dG was incubated with rat liver homogenate. Incubation of 8-hydroxyguanine with rat liver homogenates did not generate 8OHdG. These findings suggest that most of the 8OHdG in DNA is released as 8OHdG during DNA degradation and that, because of its stability, 8OHdG is excreted into urine, thus providing a convenient measure of oxidative damage to DNA. (*Cancer Sci* 2005; 96: 13–18)

Despite the presence of antioxidant defenses and DNA repair systems, oxidative damage to DNA is an inevitable consequence of metabolic activities, of ionizing radiation, and of environmental mutagens.[1–3] Such DNA damage is thought to play an important role in carcinogenesis, in aging and in a number of other pathological conditions.[4–6] Among the many types of oxidative base damage, 8-hydroxydeoxyguanosine (8OHdG) is the most extensively studied, both because of its mutagenicity,[7,8] and because its presence can be determined with high sensitivity.[9,10] In reactive oxygen species-related carcinogenesis, the level of 8OHdG in target tissues appears to play a critical role,[11,12] and this has led to 8OHdG being widely used as a marker of oxidative DNA damage.[13,14] However, because of the scantness of 8OHdG in DNA, and because of secondary formation during the analysis of 8OHdG in cellular DNA, urinary 8OHdG has been used to evaluate the level of 8OHdG in DNA, and a number of analytical methods have been developed with which to reliably measure 8OHdG in urine.[15–18] Furthermore, findings show that levels of urinary 8OHdG correlate well with many pathological conditions, particularly with carcinogenesis.[19–21]

Even so, although urinary excretion of 8OHdG has been proposed as a candidate biomarker of oxidative stress to DNA,[22] the ultimate source of urinary 8OHdG has not been clarified. In humans, urinary excretion of 8-hydroxyguanine (8OHG) and 8OHdG is reported to not depend on diet,[23] and may reflect the involvement of different repair mechanisms, namely base excision repair (BER) and nucleotide excision repair (NER).[24] BER is largely responsible for the removal of non-bulky base adducts, and involves specialized enzymes that recognize a specific repertoire of lesions. In this process, a number of glycosylases have been identified.[25,26] These enzymes, however, excise damaged bases, resulting in the excretion of damaged bases, rather than damaged nucleosides, into urine. Another set of human 8OHdG repair enzymes, endonucleases,[27] along with the NER process, which probably acts simply as a back-up system,[28] are likely to generate 8OHdG from DNA and thus contribute to the presence of 8OHdG in urine. No experimental evidence, however, has been provided to support this conjecture. Findings for several processes other than DNA repair indicate that other channels contribute to the background levels of 8OHdG that are excreted in urine. For example, even though proof of a defined role is still not forthcoming,[18] 8OHdG may derive from sanitation of the nucleotide pool by the action of human MutT homolog (MTH),[29,30] or from dead cells.[1] Potential sources of urinary 8OHdG have been collated in a comprehensive review.[31] Thus far, however, there have been neither reports that have described the generation of 8OHdG from DNA through incubation with tissue or cell extracts, nor have any researchers shown any correlation between the amount of 8OHdG generated and the 8OHdG levels in DNA.

In the present report, to more clearly elucidate the source of urinary 8OHdG, the authors investigated whether 8OHdG is generated from DNA by rat liver homogenate, and whether the amounts of generated 8OHdG correspond with the levels of oxidative damage in DNA.

Materials and Methods

Materials. 8OHdG and 8OHG were obtained from Cayman Chemical (Ann Arbor, MI, USA). Calf thymus DNA, bovine serum albumin, alkaline phosphatase, control mouse IgG1, protease inhibitor cocktail, deoxyguanosine (dG) and ethidium bromide were obtained from Sigma Chemical (St Louis, MO, USA). Nuclease P1 came from Seikagaku Corporation (Tokyo, Japan). IgG1 class mouse monoclonal anti-8OHdG antibody (Clone N45.1) was purchased from the Japanese Aging Control Institute (Shizuoka, Japan). DNA marker and loading buffer were from BEXEL Biotechnology (Union City, CA, USA). All other reagents were reagent grade and purchased from Nacalai Tesque (Kyoto, Japan).

Preparation of rat liver homogenate. Male Wistar rats aged 11 weeks were killed under deep ether anesthesia and the livers were promptly removed, frozen in liquid nitrogen, and stored at −80°C until needed. Using a Teflon-glass homogenizer, livers were homogenized in five volumes of ice-cold homogenization buffer (20 mmol/L Tris-HCl pH 7.4, containing 0.25 mol/L sucrose and 1% v/v protease inhibitor cocktail). The homogenates were filtered through nylon mesh to remove clumps of connective tissue attached to unbroken cells and then were stored at −80°C. The contaminated DNA concentration in the homogenates was 0.04 mg/mL.

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Preparation of oxidatively damaged DNA. Calf thymus DNA was dissolved with Dulbecco's phosphate-buffered saline (DPBS) at 2.0 mg/mL and incubated with three different concentrations of riboflavin (50, 10, 2 μg/mL). The mixtures were then irradiated with ultraviolet (UV) at 365 nm (UVGL-58; UVP, Upland, CA, USA) for 10 min at room temperature. The dose of irradiated UV was calculated to be 0.6 J/cm^2. After irradiation, DNA was precipitated and washed with ethanol. The DNA was then dissolved with DPBS and precipitated again with ethanol to remove residual riboflavin. Finally the DNA was dissolved in DPBS at 1.0 mg/mL. The levels of 8OHdG in the damaged DNA were determined as described below.

8OHdG release from oxidatively damaged DNA. For the indicated durations, 10-μL samples of oxidatively damaged DNA were incubated with 15 μL of rat liver homogenate at 37°C. After this, the incubation mixtures were diluted with 75 μL of double distilled water and ultrafiltered with YM10 (Millipore; Billerica, MA, USA) at 13 400 *g* for 30 min. The quantities of 8OHdG in the ultrafiltrates were determined as described below. In the degradation experiments, dG, 8OHG, or 8OHdG were incubated with the rat liver homogenates, and the mixtures were ultrafiltered as described above.

Determination of 8OHdG and dG. The level of 8OHdG in the damaged DNA was determined as described previously.[32] Briefly, DNA was heat denatured and then digested sequentially with nuclease P1 and alkaline phosphatase. The generated 8OHdG was determined using an electrochemical detector (ECD, Coulochem II; ESA, Chelmsford, MA, USA) and dG with a UV detector: both methods were combined with previously described[33] high-performance liquid chromatography (HPLC). As described above, the dG levels in the dG-degradation experiment and the quantities of 8OHdG that were generated from damaged DNA after incubation with rat liver homogenates were determined. The authors also detected some 8OHG under the same conditions as for the 8OHdG determination; however, because of the close proximity of other peaks close to the 8OHG peak, an accurate determination of the small amounts of 8OHG was not possible.

Absorption of 8OHdG with anti-8OHdG antibody. After incubation of oxidatively damaged DNA with rat liver homogenates at 37°C for 18 h, the incubation mixture was ultrafiltered through YM10, then the ultrafiltrate was incubated with either anti-8OHdG antibody, control IgG1 or DPBS at 37°C for 60 min. The molar ratio of 8OHdG to antibody or control IgG1 was 1:4. The mixtures were then ultrafiltered again with YM10, and the ultrafiltrates (final ultrafiltrates) were applied to the HPLC–ECD system to determine the quantity of 8OHdG.

DNA degradation determined using electrophoresis. After DNA was incubated with rat liver homogenates as described above, the incubation mixtures were loaded onto 2% agarose gels containing 0.5 × TBE (45 m*M* Tris-boric acid with 1 m*M* ethylenediamine tetra-acetic acid, pH 8.0) and ethidium bromide, and then electrophoresed with 0.5 × TBE buffer. The separated fragments were made visible on the agarose gel using a UV transilluminator and DNA profiles were taken using a camera.

Protein assay. The protein concentrations of rat liver homogenates were determined using a Bio-Rad protein assay solution (Bio-Rad, Hercules, CA, USA). Bovine serum albumin was used as a standard.

Statistical analysis. Data are presented as means ± standard error. Statistical analyses were carried out using one-way ANOVA; *P*-values of <0.05 were considered to be statistically significant.

Results

Generation of 8OHdG from oxidatively damaged DNA by rat liver homogenate. After subjecting the DNA to UV irradiation with different concentrations of riboflavin, 8OHdG in DNA was concentration-dependently induced. For 10 μg of DNA, the amount of induced 8OHdG was: 26.7 ng with riboflavin 50 μg/mL, 12.5 ng with 10 μg/mL, or 3.7 ng with 2 μg/mL (Table 1).

Table 1. Different levels of 8-hydroxydeoxyguanosine (8OHdG) in damaged DNA and generation by rat liver homogenates of 8OHdG from the DNA

	Quantity of 8OHdG in 10 μg DNA (ng)	Quantity of 8OHdG released (ng)	Quantity of 8OHdG released(%)
A	26.7 ± 0.59	18.58 ± 0.55	69.7 ± 3.5
B	12.5 ± 0.10	8.99 ± 0.39	72.1 ± 3.2
C	3.7 ± 0.02	2.78 ± 0.08	75.1 ± 2.6

Ultraviolet irradiation with (A) 50 μg/mL, (B) 10 μg/mL, or (C) 2 μg/mL riboflavin induced different levels of 8OHdG. The percentage released indicates the ratio of the amount of released 8OHdG to the total amount of 8OHdG in the DNA. Data from a typical experiment conducted in triplicate are presented as mean ± SE.

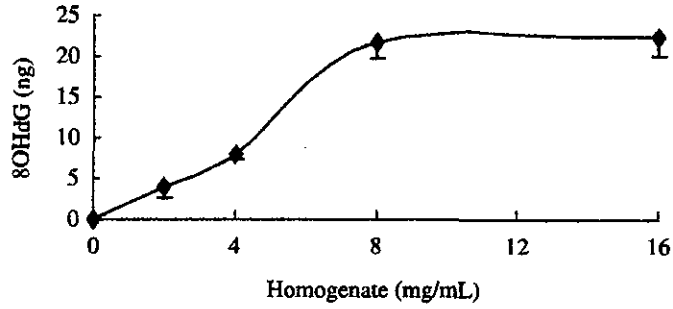

Fig. 1. Release of 8-hydroxydeoxyguanosine (8OHdG) from oxidatively damaged DNA. After ultraviolet irradiation in the presence of 50 μg/mL riboflavin, consequently damaged DNA was incubated with the indicated concentrations of rat liver homogenates at 37°C for 24 h. After incubation, the reaction mixtures were ultrafiltered and the quantity of 8OHdG in the ultrafiltrate was determined as described in Materials and Methods. Results from two independent experiments conducted in duplicate are presented as mean ± SE.

When oxidatively damaged DNA (prepared using UV irradiation with 50 μg/mL riboflavin) was incubated with varying concentrations of rat liver homogenates, concentration-dependent generation of 8OHdG was observed up to an 8 mg/mL concentration (Fig. 1). The authors found no further increase in 8OHdG generation from DNA at homogenate concentrations of >8 mg/mL. In the following experiments, to maximally generate 8OHdG, 12 mg/mL of rat liver homogenate was thus used. In contrast, no 8OHdG was generated when, for 24 h, rat liver homogenates were incubated alone without damaged DNA, or when the damaged DNA was incubated with homogenization buffer but without homogenate.

As shown in Figure 2, 8OHdG was time-dependently generated from oxidatively damaged DNA after incubation with rat liver homogenates. When DNA samples with different degrees of damage were treated for the same incubation time, the quantities of 8OHdG that were generated correlated with the 8OHdG levels in DNA. The greater the presence of 8OHdG in DNA, the greater its release by rat liver homogenates. After a 24-h incubation period, approximately 70% of the 8OHdG in DNA was released as 8OHdG (Table 1), and no significant differences in the percentage of 8OHdG released from the original levels of 8OHdG in the different samples were found.

Absorption of 8OHdG with anti-8OHdG antibody. To confirm the generation of 8OHdG from DNA by rat liver homogenates, the absorption of 8OHdG by anti-8OHdG antibody was tested. As Figure 3 shows, the final ultrafiltrate of the reaction mixture of rat liver homogenates and DNA peaked at the same elution

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