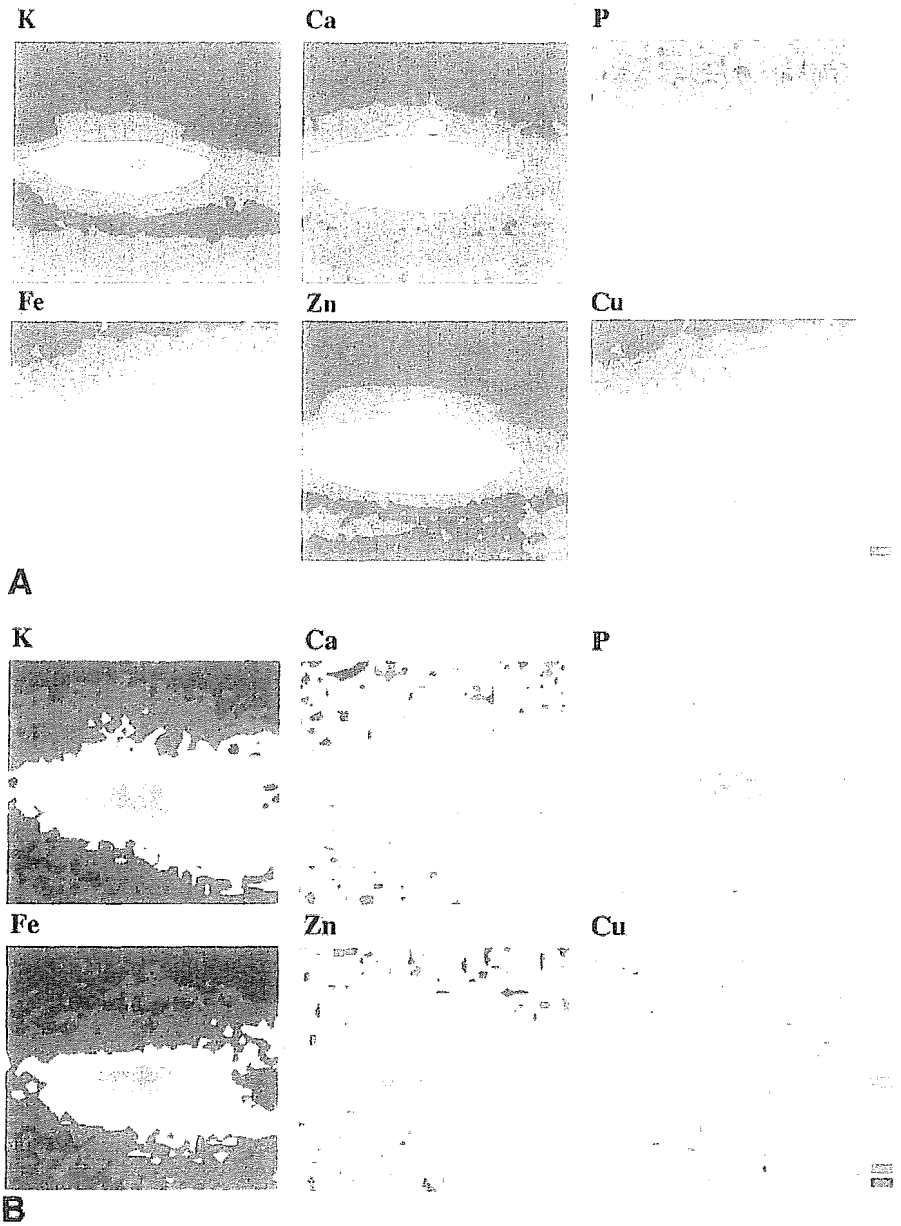

Fig. 1. Typical XRF imaging of LAD2. The figure represents elemental maps of Zn, Fe, K, Cu, Ca, and P. The scanning area was 40×40 μm^2. The image was obtained from LAD2 and cultured in medium for 30 min. **(A)** Control samples. The ranges of measured fluorescent intensities are from 0 to 1800 photons for Zn, from 20 to 260 photons for Fe, from 30 to 90 for Cu, from 0 to 2800 for K, from 0 to 700 for Ca, and from 20 to 100 for P. **(B)** IgE-mediated degranulation. The ranges of measured fluorescent intensities are from 50 to 160 photons for Zn, from 20 to 220 photons for Fe, from 20 to 70 for Cu, from 60 to 340 for K, from 30 to 130 for Ca, and from 10 to 60 for P. Each range is divided into eight levels. Each level has been assigned a shade of red, green, and blue, respectively.

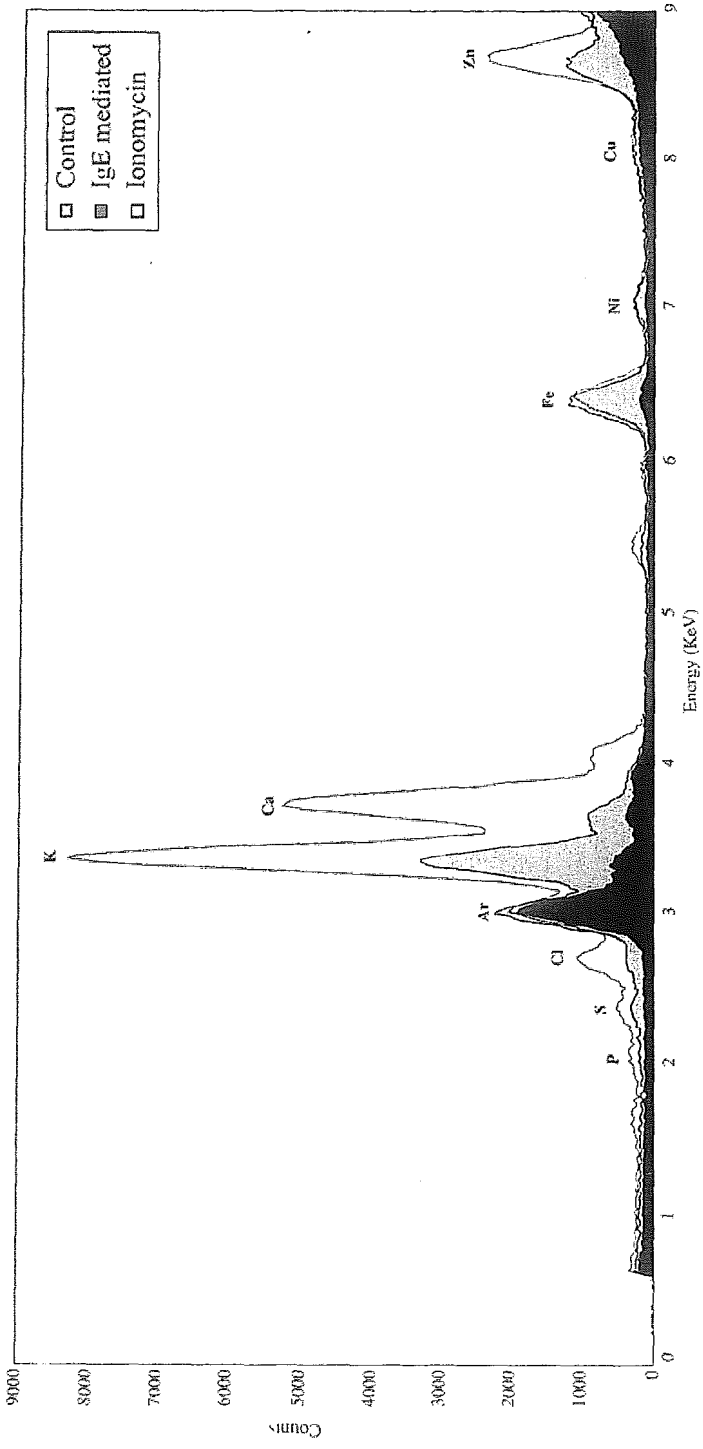

Fig. 2. Typical XRF spectra of LAD2: Control, sample, inducing IgE-mediated degranulation, and ionomycin stimulation. Measurement time was 200 s.

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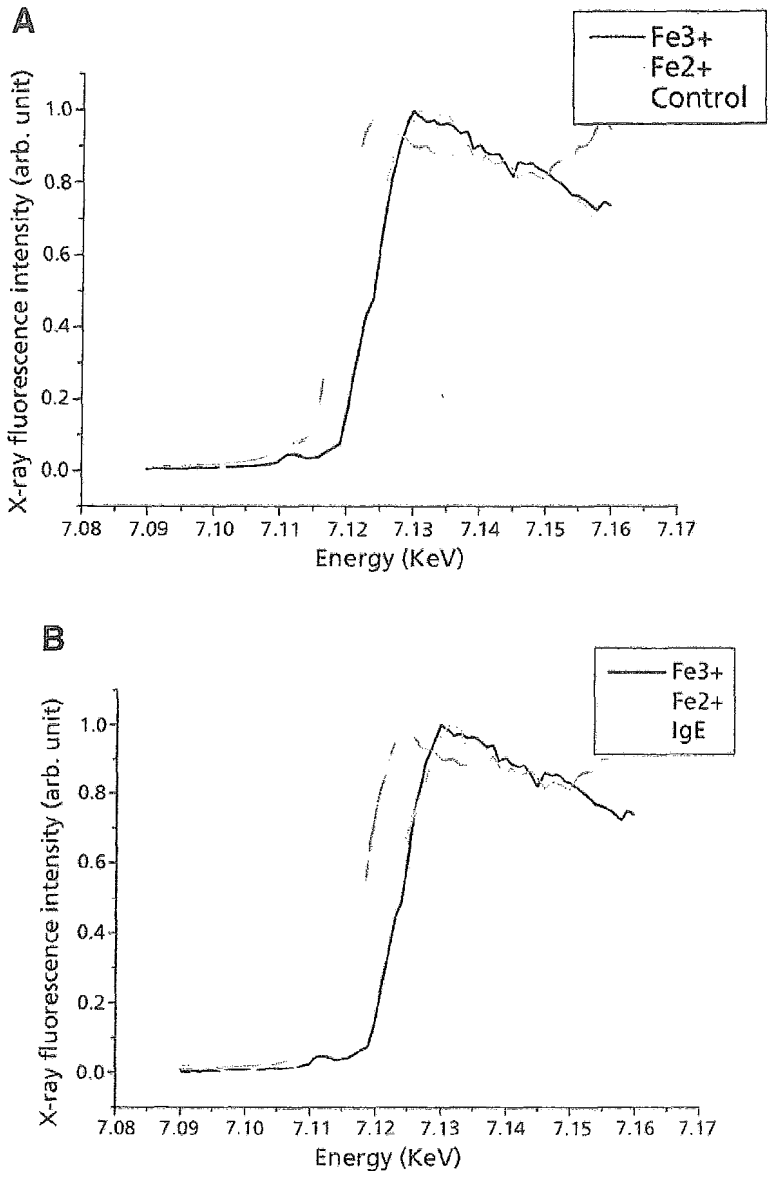

Fig. 3. Fe-K edge micro-XANES spectra: **(A)** control sample, **(B)** leading IgE-mediated degranulation, **(C)** sample under chemical stimulation. The spectra of Fe^{2+} [$FeSO_4$] and Fe^{3+} [$Fe_2(SO_4)_3$] are superimposed as reference. *(Figure continues)*

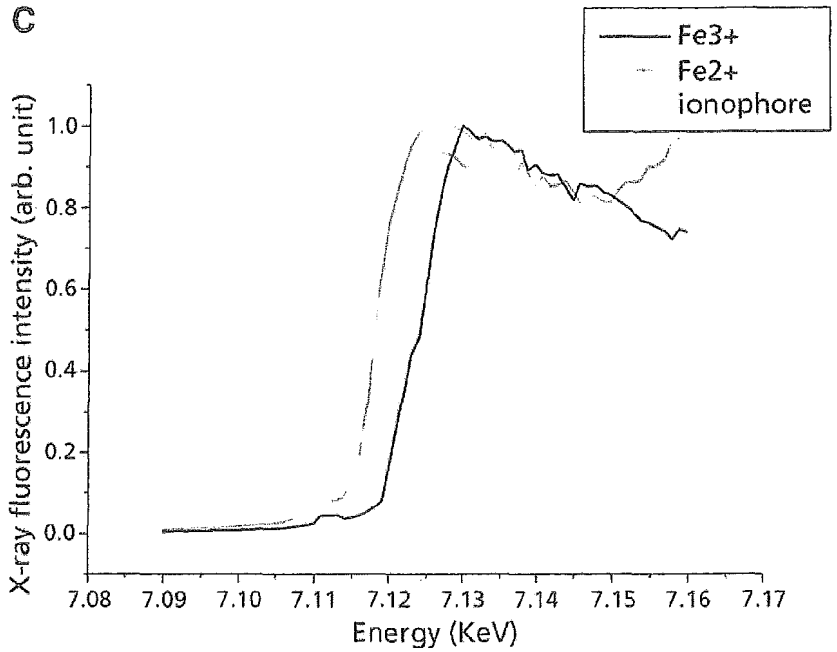

Fig. 3. *(Continued)*

in the intracellular region. The same tendency in the distribution of Fe and Cu was observed (Fig. 1A). After IgE-mediated degranulation, Zn was moved to the extracellular region (Fig. 1B).

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In Fig. 2, the SRXRF spectra is shown for each element. The counts show the dynamic change of several intracellular elements. After ionomycin stimulation, all of the elements are decreased in the cells. Although the phenomenon is the same for the six elements, it appears that there are different pathways involved in degranulation.

Chemical State of Iron

Figure 3 gives the micro-XANES spectra for the intracellular region of the samples and the Fe(II) and Fe(III) sulfates used as reference materials. The ordinate and abscissa represent the absorption coefficient and incident X-ray energy, respectively. The spectra were normalized by the absorption jump, which was defined as the difference between the highest and the lowest points in each spectrum. The spectra of three samples are identical to that of the reference materials: controls (Fig. 3A), IgE-mediated degranulation (Fig. 3B), and ionomycin stimulation (Fig. 3C). The chemical state of Fe by ionophore stimulation is about 2.5 eV from that of Fe^{2+} in $FeSO_4$, whereas the iron by IgE-mediated degranulation is closer to the chemical shift of Fe^{3+} in $Fe_2(SO_4)_3$

DISCUSSION

Intracellular trace element levels are increased by antigen stimulation. Ionomycin was used as a physical stimulant, leading to an increase in Ca and Na and a decrease of the other elements. The chemical state of Fe is also different as a result of antigen or chemical stimulation. Ionomycin seems to induce degranulation in mechanisms that are different than for antigen stimulation. The latter induces degranulation via crosslinking of IgE, whereas ionomycin physically stimulates the cell surface. The Haber–Weiss reaction is the main pathway when antigens crosslink the IgE on mast cells, resulting in Fe(III) accumulation in the intracellular region. Under physical stimulation by chemicals, both the Haber–Weiss and the Fenton reaction are at work; thus, the chemical state does not seem to shift. We previously reported the distribution of Fe and other trace elements in the HL60, which is human leukemia cell line *(12)*. Iron distribution seemed to correlate with superoxide anion formation. In the oxidation mechanism, Fe acts as the electron source, and several trace elements play an indispensable role as antioxidants. In immune cells, trace elements trap electrons by various mechanisms. In the present study, the valence state of Fe was determined in order to discover what role it plays in the oxidation process. In inflammation processes, Fe accumulation contributes to the generation of oxygen reactive species through the Fenton reaction. The XANES spectrum of Fe contained in mast cells shifted toward Fe(III), indicating that the Fe involved in the inflammation process might be in its higher oxidation state.

The IgE-dependent activation of mast cells is characterized by an influx of extracellular Ca, essential for the subsequent release of preformed (granule-derived) mediators and newly generated autacoids and cytokines. In addition, flow of ions such as K^+ and Cl^- is likely to play an important role in mast cell activation, proliferation, and chemotaxis through their effect on membrane potential and, thus, on Ca^{2+} influx. It is, therefore, important to identify these critical molecular effectors of mast cell function. The excessive accumulation of Fe and its oxidation state in LAD2 cells are related to the degranulation leading to NIP-BSA or ionomycin stimulation. XANES spectroscopy showed that the oxidation state of Fe in the cells changed from Fe(II) to Fe(III) as a result of degranulation. This finding might have implications for understanding the mechanisms involved in IgE-mediated cell responses such as those seen in allergic reactions. In addition, Fe chelation treatment by desferrioxiamine significantly suppresses IgE production and intracellular enzyme activity *(13,14)*. IgE-dependent activation had little effect on ion channel expression *(15)*. Trace elements work in inflammation as antioxidants and activators of several enzymes. In addition, their intracellular and extracellular balances seems to be relevant to this process.

In the present study, a new model for metal ion homoeostasis in mast cells is proposed. The natural resistance-associated macrophage protein 2,

Nramp2 (also called DMT1 and Slc11a2), localized in early endosomal membranes, brings extracellular bivalent cations into the cytosol. The related Nramp1, localized in late endosomal/lysosomal membranes, is in charge of delivering bivalent cations from the cytosol into an acidic compartment, where they can directly affect antimicrobial activity. Thus, we think that oxidative stress and trace elements kinetics are potential factors related to the pathogenesis of asthma and other allergies.

The use of a Synchrotron allows screening of trace elements. By using this technique, the distribution of several trace elements can be simultaneously measured and correlated. For confirmation of the present results, another highly sensitive measuring technique is being sought to do imaging and quantification of Zn by fluorescent reagents.

ACKNOWLEDGMENTS

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REFERENCES

1. P. Lydyard and C. Grossi, Development of the immune system, in *Immunology*, 5th ed. (I. Roitt, J. Brostoff, and D. Male, eds.), Mosby, London, pp. 156–170 (1998).
2. A. Walczak-Drzewiecka, J. Wyczolkowska, and J. Dastych, Environmentally relevant metal and transition metal ions enhance Fc epsilon RI-mediated mast cell activation, *Environ. Health Perspect.* **111**, 708–713 (2003).
3. S. J. Galli, New concepts about the mast cell, *N. Engl. J. Med.* **328**, 257–265 (1993).
4. Y. A. Mekori and D. D. Metcalfe, Mast cells in innate immunity, *Immunol. Rev.* **173**, 131–140 (2000).
5. M. Plaut, J. H. Pierce, C. J. Watson, et al., Mast cell lines produce lymphokines in response to cross-linkage Fc epsilon RI or to calcium ionophores, *Nature* **339**, 64–67 (1989).
6. A. Wodnar-Filipowicz, C. H. Heusser, and C. Moroni, Production of the haemopoietic growth factors GM-CSF and interleukin-3 by mast cells in response to IgE receptor-mediated activation, *Nature* **339**, 150–152 (1989).
7. P. Bradding, S. Feather, S. Wilson, et al., Immunjolocalization of cytokines in the nasal mucosa of normal and perennial rhinitic subjects. The mast cell as a source of IL-4, IL-5, and IL-6 in human allergic mucosal inflammation, *J. Immunol.* **151**, 3853–3865 (1993).
8. A. S. Kirshenbaum, C. Akin, Y. Wu, et al., Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcepsilonRI or FcgammaRI, *Leuk. Res.* **27**, 677–682 (2003).

9. S. Hayakawa, N. Ikuta, M. Suzuki, et al., Generation of an X-ray microbeam for spectromicroscopy at SPring-8 BL39XU, *J. Synchrotron Radiat.* **8,** 328–330 (2001).
10. S. Hayakawa, S. Tohno, K. Takegawa, et al., Ultra trace characterization using an X-ray microprobe at SPring-8 BL39XU, *Anal. Sci.* **17(Suppl.),** i115–i117 (2001).
11. S. Hayakawa, A. Yamaguchi, W. Hong, et al., A wave length dispersive X-ray spectrometer for small area X-ray fluorescence spectroscopy at SPring-8 BL39XU, *Spectrochim. Acta B* **54,** 171–177 (1999).
12. K. Nakashima, T. Shirakawa, and A. Ide-Ektessabi, Investigation of the relation of allergy and oxidative damage by metallic elements using SR micro beam, *AIP Conf. Proc.* **680,** 517–521 (2003).
13. H. Hasegawa, K. Oguro, Y. Naito, et al., Iron dependence of tryptophan hydroxylase activity in RBL2H2 cells and its manipulation by chelators, *Eur. J. Biochem.* **261,** 734–739 (1999).
14. Z. Wu, S. D. Holwill, and D. B. Oliveira. Desferrioxamine modulates chemically induced T helper 2-mediated autoimmunity in the rat, *Clin. Exp. Immunol.* **135,** 194–199 (2004).
15. P. Bradding, Y. Okayama, N. Kambe, et al., Ion channel gene expression in human lung, skin, and cord blood-derived mast cells, *J. Leukocyte Biol.* **73,** 614–620 (2003).

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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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Abstract

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: *8OHdG, 8-hydroxydeoxyguanosine; dG, deoxyguanosine; DPBS, Dulbecco's phosphate buffered saline; ESR, electron spin resonance; FDA, fluorescein diacetate;* H_2O_2*, hydrogen peroxide; HPLC, high-performance liquid chromatography; MDA, malondialdehyde;* NaN_3*, sodium azide; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive substance; TEP, 1,1,3,3-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,

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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 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).

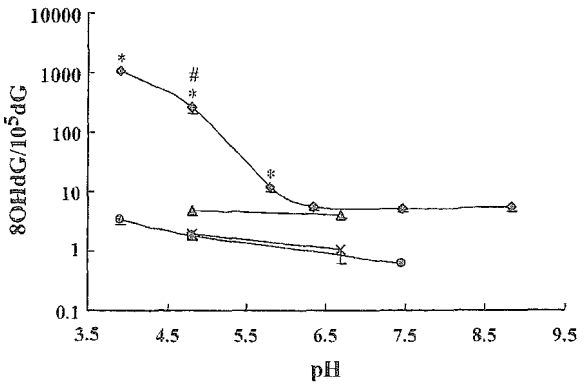

Figure 1. Correlation of pH with the effects of vitamin C on induction of 8OHdG by oxygen. Bacterial cells were exposed to oxygen under different pH conditions in the presence or absence of 10 mM vitamin C and then incubated at 37°C for 3 h. 8OHdG levels were determined as described in "Materials and 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).

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 Figure 1 and Table I experiments) or citrate-buffered saline (CBS, pH 5.0, used in

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.

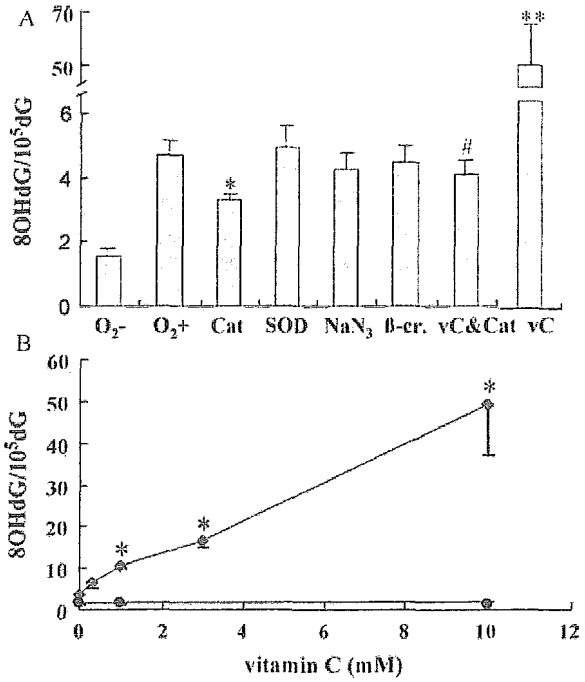

Figure 2. Effects of ROS scavengers (A) and dose-dependent effect of vitamin C (B) on induction of 8OHdG by oxygen. Bacterial cells were exposed to oxygen in the presence of vitamin C and/or ROS scavengers at pH 5.0 and then incubated at 37°C for 3 h. 8OHdG levels were determined as described in "Materials 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 exposed to oxygen (O_2+), 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.

Figures 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.

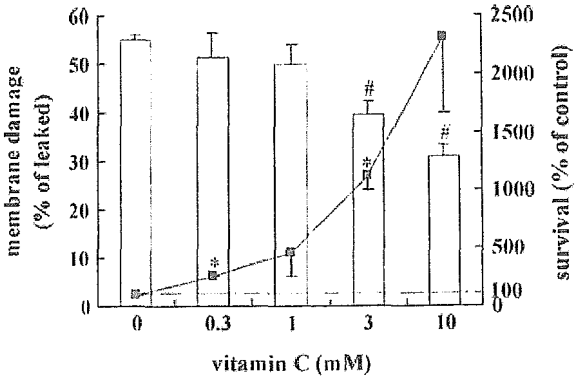

Figure 3. Effects of vitamin C on bacterial membrane damage (bar graph) and killing of bacterial cells (line graph) associated with oxygen exposure at pH 5.0. Membrane damage and bacterial cell mortality were investigated and analyzed as described in "Materials and Methods" section. Results from three 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.

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

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 growing per dish from test samples by the number of colonies

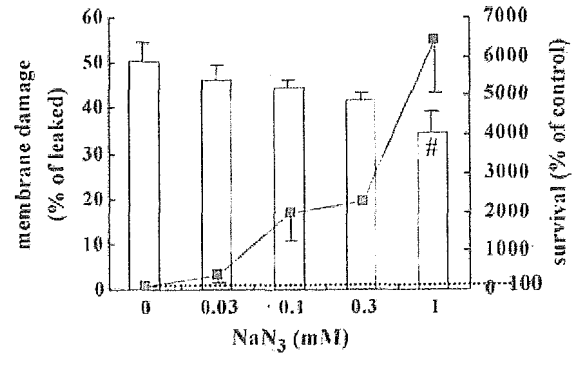

Figure 4. Effects of sodium azide on bacterial membrane damage (bar graph) and bacterial cell mortality (line graph) associated with oxygen exposure at pH 5.0 Bacterial cell mortality and membrane damage were investigated and analyzed as described in "Materials and Methods" section. For membrane damage, 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.

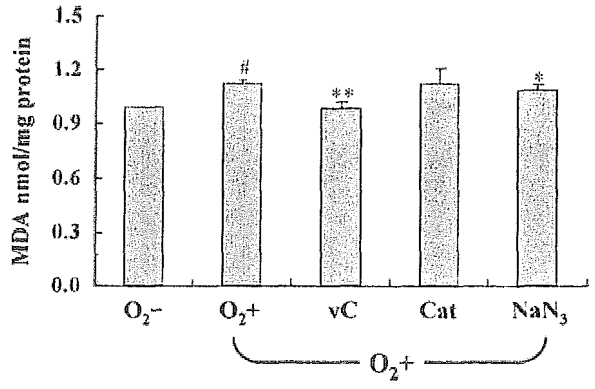

Figure 5. Effects of vitamin C and ROS scavengers on oxygen-induced lipid peroxidation. Bacteria were exposed to oxygen in the presence of vitamin C or ROS scavengers at pH 5.0 and subsequently incubated at 37°C for 1 h. Results are shown as mean + SE from four independent experiments. See Fig. 2A for 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.

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 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.

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.

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.

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 (Figure 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 Figure 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 (Figure 2B). Catalase almost completely inhibited this effect (Figure 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 (Figure 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 (Figure 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 (Figure 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 (Figure 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 (Figure 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 cell suspension. 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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References

[1] Gutteridge JM, Halliwell B. Free radicals and antioxidants in the year 2000. A historical look to the future. Ann N Y Acad Sci 2000;899:136–147.

[2] Halliwell B, Gutteridge JMC. Free Radicals in Biology and Medicine. Oxford: Oxford University Press; 1999.

[3] Harman D. The aging process. Proc Natl Acad Sci USA 1981;78:7124–7128.

[4] Ames BN. Dietary carcinogens and anticarcinogens. Oxygen radicals and degenerative diseases. Science 1983;221:1256–1264.

[5] Poli G, Parola M. Oxidative damage and fibrogenesis. Free Radic Biol Med 1997;22:287–305.

[6] Carney JM, Starke-Reed PE, Oliver CN, Landum RW, Cheng MS, Wu JF, Floyd RA. Reversal of age-related increase in brain protein oxidation, decrease in enzyme activity, and loss in temporal and spatial memory by chronic administration of the spin-trapping compound *N*-tert-butyl-alpha-phenylnitrone. Proc Natl Acad Sci USA 1991;88:3633–3636.

[7] Kang SW, Chae HZ, Seo MS, Kim K, Baines IC, Rhee SG. Mammalian peroxiredoxin isoforms can reduce hydrogen peroxide generated in response to growth factors and tumor necrosis factor-alpha. J Biol Chem 1998;273:6297–6302.

[8] Block G, Patterson B, Subar A. Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. Nutr Cancer 1992;18:1–29.

[9] Gaziano JM, Manson JE, Buring JE, Hennekens CH. Dietary antioxidants and cardiovascular disease. Ann N Y Acad Sci 1992;669:249–258.

[10] Block G. Vitamin C and cancer prevention: the epidemiologic evidence. Am J Clin Nutr 1991;53:270S–282S.

[11] Drake IM, Davies MJ, Mapstone NP, Dixon MF, Schorah CJ, White KL, Chalmers DM, Axon AT. Ascorbic acid may protect against human gastric cancer by scavenging mucosal oxygen radicals. Carcinogenesis 1996;17:559–562.

[12] Frei B, England L, Ames BN. Ascorbate is an outstanding antioxidant in human blood plasma. Proc Natl Acad Sci USA 1989;86:6377–6381.

[13] Frei B, Forte TM, Ames BN, Cross CE. Gas phase oxidants of cigarette smoke induce lipid peroxidation and changes in lipoprotein properties in human blood plasma. Protective effects of ascorbic acid. Biochem J 1991;277:133–138.

[14] Haliwell B. Vitamin C: antioxidant or pro-oxidant *in vivo*? Free Radic Res 1996;25:439–454.

[15] Sakagami H, Satoh K. Modulating factors of radical intensity and cytotoxic activity of ascorbate (review). Anticancer Res 1997;17:3513–3520.

[16] Shamberger RJ. Genetic toxicology of ascorbic acid. Mutat Res 1984;133:135–159.

[17] Podmore ID, Griffiths HR, Herbert KE, Mistry N, Mistry P, Lunec J. Vitamin C exhibits pro-oxidant properties. Nature 1998;392:559.

[18] Carr A, Frei B. Does vitamin C act as a pro-oxidant under physiological conditions? FASEB J 1999;13:1007–1024.

[19] Halliwell B. Vitamin C: poison, prophylactic or panacea? Trends Biochem Sci 1999;24:255–259.

[20] Takeuchi T, Nakaya Y, Kato N, Watanabe K, Morimoto K. Induction of oxidative DNA damage in anaerobes. FEBS Lett 1999;450:178–180.

[21] Kasai H. Chemistry-based studies on oxidative DNA damage: formation, repair, and mutagenesis. Free Radic Biol Med 2002;33:450–456.

[22] Dizdaroglu M. Oxidative damage to DNA in mammalian chromatin. Mutat Res 1992;275:331–342.

[23] Floyd RA. The role of 8-hydroxyguanine in carcinogenesis. Carcinogenesis 1990;11:1447–1450.

[24] Nakajima M, Takeuchi T, Morimoto K. Determination of 8-hydroxydeoxyguanosine in human cells under oxygen-free conditions. Carcinogenesis 1996;17:787–791.

[25] Takeuchi T, Nakajima M, Ohta Y, Mure K, Takeshita T, Morimoto K. Evaluation of 8-hydroxydeoxyguanosine, a typical oxidative DNA damage, in human leukocytes. Carcinogenesis 1994;15:1519–1523.

[26] Armitage WJ, Mazur P. Osmotic tolerance of human granulocytes. Am J Physiol 1984;247:C373–C381.

[27] Mishra KP. Cell membrane oxidative damage induced by gamma-radiation and apoptotic sensitivity. J Environ Pathol Toxicol Oncol 2004;23:61–66.

[28] Rotman B, Papermaster BW. Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. Proc Natl Acad Sci USA 1966; 55:134–141.

[29] Fukunaga K, Yoshida M, Nakazono N. A simple, rapid, highly sensitive and reproducible quantification method for plasma malondialdehyde by high-performance liquid chromatography. Biomed Chromatogr 1998;12:300–303.

[30] Liaudet L, Pacher P, Mabley JG, Virag L, Soriano FG, Hasko G, Szabo C. Activation of poly (ADP-ribose) polymerase-1 is

a central mechanism of lipopolysaccharide-induced acute lung inflammation. Am J Respir Crit Care Med 2002; 165:372–377.
[31] Takeuchi T, Kato N, Watanabe K, Morimoto K. Mechanism of oxidative DNA damage induction in a strict anaerobe *Prevotella melaninogenica*. FEMS Microbiol Lett 2000; 192:133–138.
[32] Halliwell B, Dizdaroglu M. The measurement of oxidative damage to DNA by HPLC and GC/MS techniques. Free Radic Res Commun 1992;16:75–87.
[33] Halliwell B, Aruoma OI. DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems. FEBS Lett 1991;281:9–19.
[34] Shigenaga MK, Ames BN. Assays for 8-hydroxy-2′-deoxyguanosine: a biomarker of *in vivo* oxidative DNA damage. Free Radic Biol Med 1991;10:211–216.
[35] Halliwell B, Gutteridge JM. Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol 1990;186:1–85.
[36] Halliwell B, Gutteridge JM. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J 1984;219:1–14.
[37] Lynch SR, Cook JD. Interaction of vitamin C and iron. Ann N Y Acad Sci 1980;355:32–44.
[38] Herbert V, Shaw S, Jayatilleke E. Vitamin C-driven free radical generation from iron. J Nutr 1996;126:1213S–1220S.
[39] Drouin R, Rodriguez H, Gao SW, Gebreyes Z, O'Connor TR, Holmquist GP, Akman SA. Cupric ion/ascorbate/hydrogen peroxide-induced DNA damage: DNA-bound copper ion primarily induces base modifications. Free Radic Biol Med 1996;21:261–273.
[40] Hu ML, Shih MK. Ascorbic acid inhibits lipid peroxidation but enhances DNA damage in rat liver nuclei incubated with iron ions. Free Radic Res 1997;26:585–592.
[41] Woodmansee AN, Imlay JA. Reduced flavins promote oxidative DNA damage in non-respiring *Escherichia coli* by delivering electrons to intracellular free iron. J Biol Chem 2002;277:34055–34066.
[42] Srinivasan C, Liba A, Imlay JA, Valentine JS, Gralla EB. Yeast lacking superoxide dismutase(s) show elevated levels of "free iron" as measured by whole cell electron paramagnetic resonance. J Biol Chem 2000;275:29187–29192.
[43] Schafer FQ, Buettner GR. Acidic pH amplifies iron-mediated lipid peroxidation in cells. Free Radic Biol Med 2000; 28:1175–1181.
[44] Wills ED. Mechanisms of lipid peroxide formation in animal tissues. Biochem J 1966;99:667–676.
[45] Burhans WC, Weinberger M, Marchetti MA, Ramachandran L, D'Urso G, Huberman JA. Apoptosis-like yeast cell death in response to DNA damage and replication defects. Mutat Res 2003;532:227–243.
[46] Norbury CJ, Zhivotovsky B. DNA damage-induced apoptosis. Oncogene 2004;23:2797–2808.
[47] Nissen SB, Tjonneland A, Stripp C, Olsen A, Christensen J, Overvad K, Dragsted LO, Thomsen B. Intake of vitamins A, C, and E from diet and supplements and breast cancer in postmenopausal women. Cancer Causes Control 2003;14:695–704.
[48] Feskanich D, Willett WC, Hunter DJ, Colditz GA. Dietary intakes of vitamins A, C, and E and risk of melanoma in two cohorts of women. Br J Cancer 2003;88:1381–1387.
[49] Epe B, Hegler J, Wild D. Singlet oxygen as an ultimately reactive species in *Salmonella typhimurium* DNA damage induced by methylene blue/visible light. Carcinogenesis 1989;10:2019–2024.
[50] Dahl TA, Midden WR, Hartman PE. Comparison of killing of gram-negative and gram-positive bacteria by pure singlet oxygen. J Bacteriol 1989;171:2188–2194.
[51] Dahl TA, Midden WR, Hartman PE. Pure exogenous singlet oxygen: nonmutagenicity in bacteria. Mutat Res 1988;201:127–136.
[52] Nakano M, Kambayashi Y, Tatsuzawa H, Komiyama T, Fujimori K. Useful 1O2 (1delta g) generator, 3-(4′-methyl-1′-naphthyl)-propionic acid, 1′,4′-endoperoxide (NEPO), for dioxygenation of squalence (a skin surface lipid) in an organic solvent and bacterial killing in aqueous medium. FEBS Lett 1998;432:9–12.
[53] Chamberlain J, Moss SH. Lipid peroxidation and other membrane damage produced in *Escherichia coli* K1060 by near-UV radiation and deuterium oxide. Photochem Photobiol 1987;45:625–630.
[54] Bartoli GM, Galeotti T. Growth-related lipid peroxidation in tumour microsomal membranes and mitochondria. Biochim Biophys Acta 1979;574:537–541.
[55] Bocking T, Barrow KD, Netting AG, Chilcott TC, Coster HG, Hofer M. Effects of singlet oxygen on membrane sterols in the yeast *Saccharomyces cerevisiae*. Eur J Biochem 2000;267:1607–1618.
[56] Chou PT, Khan AU. L-ascorbic acid quenching of singlet delta molecular oxygen in aqueous media: generalized antioxidant property of vitamin C. Biochem Biophys Res Commun 1983;115:932–937.
[57] Bodannes RS, Chan PC. Ascorbic acid as a scavenger of singlet oxygen. FEBS Lett 1979;105:195–196.
[58] Singh DP, Kshatriya K. NaCl-induced oxidative damage in the cyanobacterium *Anabaena doliolum*. Curr Microbiol 2002;44:411–417.
[59] Kawanishi S, Inoue S. Mechanism of DNA cleavage induced by sodium chromate (VI) in the presence of hydrogen peroxide. J Biol Chem 1986;261:5952–5958.
[60] Takayama F, Egashira T, Yamanaka Y. Singlet oxygen generation from phosphatidylcholine hydroperoxide in the presence of copper. Life Sci 2001;68:1807–1815.
[61] Tanaka K, Miura T, Umezawa N, Urano Y, Kikuchi K, Higuchi T, Nagano T. Rational design of fluorescein-based fluorescence probes. Mechanism-based design of a maximum fluorescence probe for singlet oxygen. J Am Chem Soc 2001;123:2530–2536.
[62] Hvorup R, Chang AB, Saier Jr, MH. Bioinformatic analyses of the bacterial L-ascorbate phosphotransferase system permease family. J Mol Microbiol Biotechnol 2003; 6:191–205.
[63] Buettner GR, Jurkiewicz BA. Ascorbate free radical as a marker of oxidative stress: an EPR study. Free Radic Biol Med 1993;14:49–55.
[64] O'Connor HJ, Schorah CJ, Habibzedah N, Axon AT, Cockel R. Vitamin C in the human stomach: relation to gastric pH, gastroduodenal disease, and possible sources. Gut 1989;30:436–442.
[65] Wike-Hooley JL, Haveman J, Reinhold HS. The relevance of tumour pH to the treatment of malignant disease. Radiother Oncol 1984;2:343–366.
[66] Arndt H, Palitzsch KD, Scholmerich J. Leucocyte endothelial cell adhesion in indomethacin induced intestinal inflammation is correlated with faecal pH. Gut 1998;42:380–386.

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.

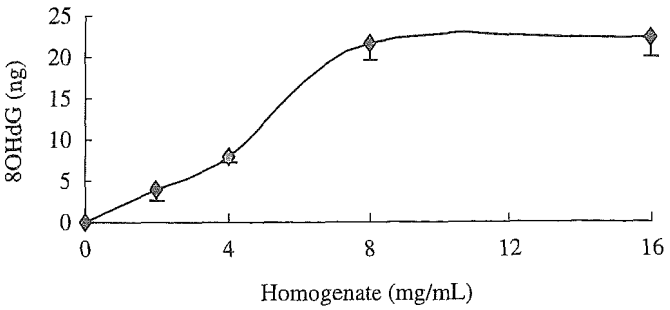

Fig. 1. Release of 8-hydroxydeoxyguanosine (8OHdG) from oxidatively damaged DNA. After ultraviolet irradiation in the presence of 50 μg/mL riboflavin, consequently damaged DNA was incubated with the indicated concentrations of rat liver homogenates at 37°C for 24 h. After incubation, the reaction mixtures 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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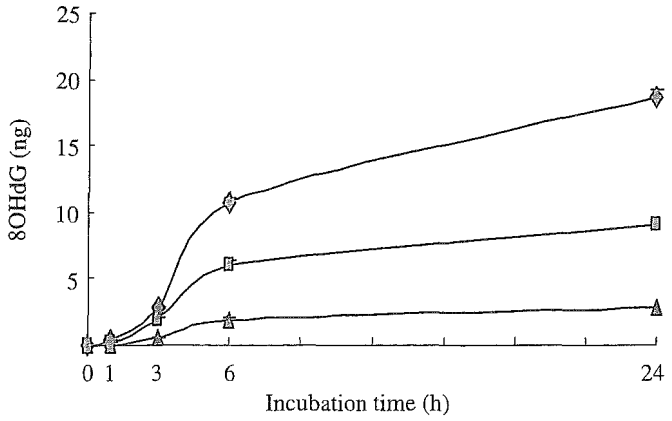

Fig. 2. Time course of 8-hydroxydeoxyguanosine (8OHdG) generation from DNA with different levels of 8OHdG. Ultraviolet irradiation with (◆) 50 μg/mL, (■) 10 μg/mL or (▲) 2 μg/mL riboflavin-damaged DNA samples were incubated with rat liver homogenates at 37°C for 1, 3, 6, or 24 h. Then the incubation mixtures were ultrafiltered through YM10 and the quantities of 8OHdG in the ultrafiltrates were determined as described in Materials and Methods. Results from a typical experiment conducted in triplicate is presented as mean ± SE.

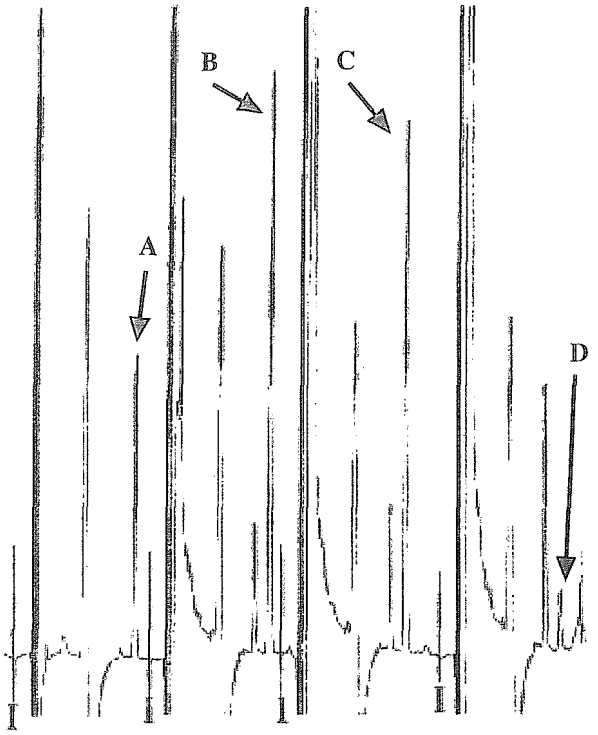

Fig. 3. High-performance liquid chromatography–electrochemical detector chromatogram of the final ultrafiltrates incubated with anti-8-hydroxydeoxyguanosine (8OHdG) antibody or control IgG1. After incubation of DNA (damaged by ultraviolet irradiation with 50 μg/mL riboflavin) with rat liver homogenates at 37°C for 18 h, the incubation mixture was ultrafiltered. The ultrafiltrate was incubated with Dulbecco's phosphate-buffered saline (B), control IgG1 (C), or anti-8OHdG antibody (D), then the mixtures were ultrafiltered again with YM10. Quantities of 8OHdG in the final ultrafiltrates were determined as described in Materials and Methods. Peak A indicates authentic 8OHdG, and 'I' indicates the injection points of samples.

time as authentic 8OHdG. Control IgG1 did not absorb the peak in the ultrafiltrate; however, anti-8OHdG antibody at the same concentration as control IgG1 absorbed the peak almost completely.

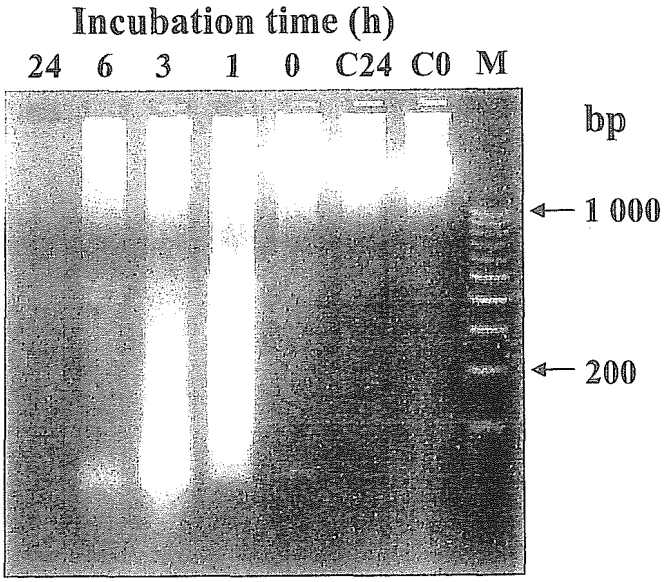

Fig. 4. Electrophoretic determination of DNA degradation by rat liver homogenates. DNA (damaged by ultraviolet irradiation with 50 μg/mL riboflavin) was incubated with rat liver homogenates at 37°C for the indicated durations. Then incubation mixtures containing 1.0 μg DNA were subjected to electrophoresis as described in Materials and Methods. C0, C24: samples of damaged DNA were incubated with homogenization buffer at 37°C for 0 h (C0) or 24 h (C24). M, marker DNA fragments, sizes of marker fragments are indicated on the right.

Degradation of DNA by rat liver homogenates. As Figure 4 shows, rat liver homogenates degraded DNA that had been damaged by UV irradiation in the presence of 50 μg/mL riboflavin. Oxidatively damaged DNA was considerably degraded even after 1 h of incubation, and the DNA degradation by rat liver homogenates was dependent on the incubation time. In contrast, when damaged DNA was incubated for 24 h without rat liver homogenates, it was not degraded (C24 *vs* C0). Although the DNA was extensively damaged by UV irradiation in the presence of riboflavin, its size was larger than 1000 bp (C0).

Changes in the amount of dG, 8OHG or 8OHdG in the presence of rat liver homogenates. As Figure 5A shows, when dG was incubated with rat liver homogenates, a small amount of 8OHdG was detected at 0 h of incubation, and this amount increased slightly after 6 h of incubation. At 6 h, however, the amount of 8OHdG generated from dG was <1/10 000 of dG (Fig. 5A). In the presence of rat liver homogenates, dG disappeared rapidly. More than 70% of dG had disappeared even after 0.5 h of incubation: after 3 h none could be detected. By contrast, in the presence of rat liver homogenates, 8OHG and 8OHdG did not breakdown significantly after 6 h, and more than 75% of 8OHG and 8OHdG were detected unchanged even after 24 h of incubation (Fig. 5B,C). 8OHdG was not generated from 8OHG by rat liver homogenates.

Discussion

Urinary 8OHdG is widely used as a biological marker with which to evaluate oxidative stress in the body.[22,34,35] Its usefulness, however, has so far been limited because we do not know enough about how 8OHdG comes to be present in urine.[23,31] No evidence has been presented that 8OHdG is released from DNA by tissues, cells, or by their extracts. Here, the authors clearly show that 8OHdG is generated from DNA by rat liver homogenates (Fig. 1). Because other compounds that are present in rat liver homogenates might have, in HPLC analysis, produced peaks at the same position as 8OHdG, the authors tested with an anti-8OHdG antibody. The antibody absorbed the peak almost

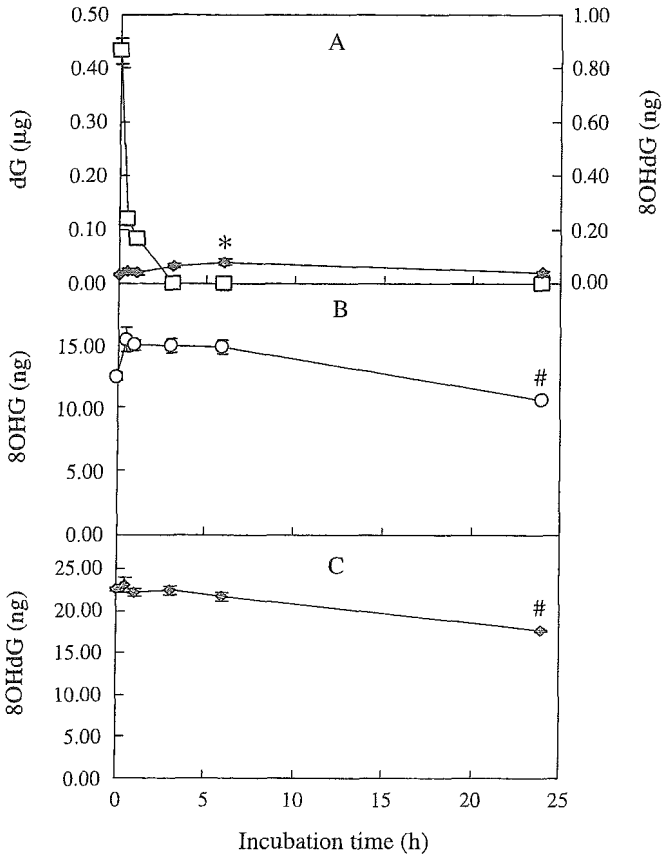

Fig. 5. Effect of incubation time on (□) quantity of deoxyguanosine (dG), (○) 8-hydroxyguanine (8OHG), or (◈) 8-hydroxydeoxyguanosine (8OHdG) in the presence of rat liver homogenates. (A) dG (0.5 μg). (B) 8OHG (16 ng). (C) 8OHdG (25 ng). Amounts were calculated from samples of 10 μg of DNA that were damaged by ultraviolet irradiation with 50 μg/mL riboflavin. These were incubated at 37°C with rat liver homogenates for 0, 0.5, 1, 3, 6 or 24 h. After incubation, the mixtures were ultrafiltered and the quantities of dG, 8OHG, or 8OHdG in the ultrafiltrates were determined as described in Materials and Methods. Data from a typical experiment conducted in triplicate are presented as mean ± SE. *$P < 0.05$, significantly increased when compared with 0 h; #$P < 0.05$, significantly decreased when compared with 0 h.

completely, indicating that 8OHdG was generated from DNA (Fig. 3). Our data also show that the quantity of 8OHdG generated from the DNA corresponds with the level of oxidative damage in the DNA (Fig. 2). These findings indicate that, *in vivo*, 8OHdG is generated from DNA, and that the amounts of generated 8OHdG are useful for evaluating oxidative damage in DNA. However, attention should be paid when determining the 8OHdG quantity (see Fig. 2).

A semiquantitative assay of 8OHG using the 8OHdG detection system showed that the quantities of 8OHG produced from DNA were approximately 1/25 of the quantities of 8OHdG after incubation of DNA with rat liver homogenates, and that the proportion did not vary with the 8OHdG levels in DNA (data not shown). It might be thought that just 70% of 8OHdG was released from DNA after a 24-h incubation period (Table 1). However, Figure 5C shows that 75% of 8OHdG could be recovered after the same incubation time in the presence of rat liver homogenates. Thus, in this system, the authors considered that most of the 8OHdG in damaged DNA was released as 8OHdG.

The authors were surprised that most of the 8OHdG was released from DNA by the rat liver homogenates. At first, it was considered that 8OHdG was generated during the DNA repair process, because dG, a DNA degradation product,[1] was barely detectable in the ultrafiltrates. The result of electrophoresis (Fig. 4), however, indicated that the generation of 8OHdG co-occurred during DNA degradation. In the presence of rat liver homogenates, it is possible that dG rapidly disappeared, which was confirmed as shown in Figure 5A. When DNA was incubated with rat liver homogenates for 1 h, the electrophoretic mobility of oxidatively damaged DNA was decreased, probably due to the interaction between the DNA and the proteins in the homogenate.

It is also possible that 8OHdG is generated from dG or 8OHG. In particular, generation from dG has been reported in the co-presence of oxidants.[9,36,37] Commercially available dG preparations usually contain 1–5 molecules of 8OHdG per 100 000 of dG (data not shown). When dG was incubated with rat liver homogenates, however, <1/10 000 of the dG was converted to 8OHdG during 6 h of incubation (Fig. 5A). Meanwhile, 8OHdG was not generated when 8OHG was incubated with rat liver homogenates (Fig. 5B). These findings indicate that, during DNA degradation, 8OHdG was generated directly from DNA. The authors suggest that 8OHdG is generated when oxidative stress causes tissue or cell destruction: in such conditions, both oxidative DNA damage and tissue or cell homogenates could be produced. Because oxidative stress induces apoptosis,[38,39] and DNA is extensively degraded during apoptosis,[40] apoptotic cells might also be sources of 8OHdG. The authors are now investigating whether living cells could also generate 8OHdG from DNA using a cell culture system.

Liver contains many types of nuclease[41–44] that degrade DNA to nucleotides. In turn, these can be dephosphorylated to nucleosides by the phosphatases that are also present in the liver.[45,46] Some nucleases in the liver are reported to be sensitive to NaCl,[47,48] and when NaCl was added to the incubation mixtures, NaCl at concentrations of more than 150 mmol/L inhibited the generation of 8OHdG (data not shown). The finding further supports our conclusion that 8OHdG generation is coupled with DNA degradation. Thus it seems plausible that, in the present experiment, the nucleases and phosphatases present in the liver were responsible for the generation of 8OHdG from DNA. Additionally, in support of this conclusion, the technique for determining 8OHdG in DNA uses nuclease P1, an exonuclease, and alkaline phosphatase.[11,33,49] Further study, however, is required to identify which enzyme or enzymes are responsible for the generation of 8OHdG from DNA. Furthermore, investigation as to which organ most efficiently generates 8OHdG may eventually make it possible to use urinary 8OHdG to evaluate organ-specific oxidative stress. In contrast to rat liver homogenates, Fpg protein, a bacterial homolog of oxoguanine glycosylase that acts as a DNA BER enzyme,[50,51] generated 8OHG from DNA, but not 8OHdG (data not shown).

It is interesting that, while dG rapidly disappeared under the same conditions, in the presence of rat liver homogenates more than 75% of 8OHG and 8OHdG remained unchanged up to 24 h of incubation (Fig. 5). These findings suggest that 8OHdG and 8OHG are stable in the body and in the circulation, and so may be excreted into urine unchanged, whereas most of dG undergoes breakdown and may not be detectable in urine as intact dG. This hypothesis is supported by the finding that the quantities of 8OHdG and 8OHG in urine are greatly disproportionate to the quantity of dG in urine.[18,22,52] It is also interesting that 8OHdG and 8OHG seem not to be metabolized or reused, suggesting the presence of mechanisms that do not allow the naturally occurring damaged base to be incorporated into nucleic acids. Our discovery of the stability of 8OHdG in the presence of rat liver homogenates suggests a useful substrate that could be used to study nucleases. Because 8OHdG is a stable product of nuclease reaction and can be determined with high sensitivity, DNA with 8OHdG seems to be a better substrate than DNA without 8OHdG.

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In conclusion, 8OHdG is released from DNA by rat liver homogenates in quantities that correspond with the levels of oxidative damage in the DNA. Because 8OHdG is stable in the presence of rat liver homogenates, it is likely that 8OHdG is stable enough in circulation to be excreted into urine. Thus, urinary 8OHdG, if determined at appropriate times or with 24-h urine testing, is a useful marker of oxidative DNA damage that is induced by oxidative stress, particularly oxidative stress that leads to the organ or cell destruction, or apoptosis. Although the present results do not show the *in vivo* generation of 8OHdG from DNA directly, they show that 8OHdG is generated from DNA by a biological material, rat liver homogenate.

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References

1 Lindahl T. Instability and decay of the primary structure of DNA. *Nature* 1993; **362**: 709–15.
2 Beckman KB, Ames BN. Oxidative decay of DNA. *J Biol Chem* 1997; **272**: 19633–6.
3 Halliwell B, Gutteridge JMC. *Free Radicals in Biology and Medicine*. Oxford: Oxford University Press, 1999.
4 Halliwell B, Aruoma OI. DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems. *FEBS Lett* 1991; **281**: 9–19.
5 Ames BN. Dietary carcinogens and anticarcinogens. Oxygen radicals and degenerative diseases. *Science* 1983; **221**: 1256–64.
6 Zhang J, Perry G, Smith MA, Robertson D, Olson SJ, Graham DG, Montine TJ. Parkinson's disease is associated with oxidative damage to cytoplasmic DNA and RNA in substantia nigra neurons. *Am J Pathol* 1999; **154**: 1423–9.
7 Shibutani S, Takeshita M, Grollman AP. Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. *Nature* 1991; **349**: 431–4.
8 Cheng KC, Cahill DS, Kasai H, Nishimura S, Loeb LA. 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G—T and A—C substitutions. *J Biol Chem* 1992; **267**: 166–72.
9 Floyd RA, Watson JJ, Wong PK, Altmiller DH, Rickard RC. Hydroxyl free radical adduct of deoxyguanosine: sensitive detection and mechanisms of formation. *Free Radic Res Commun* 1986; **1**: 163–72.
10 Kasai H. Analysis of a form of oxidative DNA damage, 8-hydroxy-2′-deoxyguanosine, as a marker of cellular oxidative stress during carcinogenesis. *Mutat Res* 1997; **387**: 147–63.
11 Foksinski M, Kotzbach R, Szymanski W, Olinski R. The level of typical biomarker of oxidative stress 8-hydroxy-2′-deoxyguanosine is higher in uterine myomas than in control tissues and correlates with the size of the tumor. *Free Radic Biol Med* 2000; **29**: 597–601.
12 Floyd RA. The role of 8-hydroxyguanine in carcinogenesis. *Carcinogenesis* 1990; **11**: 1447–50.
13 Kasai H, Crain PF, Kuchino Y, Nishimura S, Ootsuyama A, Tanooka H. Formation of 8-hydroxyguanine moiety in cellular DNA by agents producing oxygen radicals and evidence for its repair. *Carcinogenesis* 1986; **7**: 1849–51.
14 Collins AR, Gedik CM, Olmedilla B, Southon S, Bellizzi M. Oxidative DNA damage measured in human lymphocytes: large differences between sexes and between countries, and correlations with heart disease mortality rates. *FASEB J* 1998; **12**: 1397–400.
15 Yoshida R, Ogawa Y, Kasai H. Urinary 8-oxo-7,8-dihydro-2′-deoxyguanosine values measured by an ELISA correlated well with measurements by high-performance liquid chromatography with electrochemical detection. *Cancer Epidemiol Biomarkers Prev* 2002; **11**: 1076–81.
16 Tagesson C, Kallberg M, Klintenberg C, Starkhammar H. Determination of urinary 8-hydroxydeoxyguanosine by automated coupled-column high performance liquid chromatography: a powerful technique for assaying in vivo oxidative DNA damage in cancer patients. *Eur J Cancer* 1995; **31**: 934–40.
17 Lin HS, Jenner AM, Ong CN, Huang SH, Whiteman M, Halliwell B. A high-throughput and sensitive methodology for the quantification of urinary 8-hydroxy-2′-deoxyguanosine: measurement with gas chromatography-mass spectrometry after single solid-phase extraction. *Biochem J* 2004; **380**: 541–8.
18 Weimann A, Belling D, Poulsen HE. Quantification of 8-oxo-guanine and guanine as the nucleobase, nucleoside and deoxynucleoside forms in human urine by high-performance liquid chromatography-electrospray tandem mass spectrometry. *Nucl Acids Res* 2002; **30**: E7.
19 Wu LL, Chiou CC, Chang PY, Wu JT. Urinary 8-OHdG: a marker of oxidative stress to DNA and a risk factor for cancer, atherosclerosis and diabetics. *Clin Chim Acta* 2004; **339**: 1–9.
20 Loft S, Vistisen K, Ewertz M, Tjonneland A, Overvad K, Poulsen HE. Oxidative DNA damage estimated by 8-hydroxydeoxyguanosine excretion in humans: influence of smoking, gender and body mass index. *Carcinogenesis* 1992; **13**: 2241–7.
21 Gackowski D, Speina E, Zielinska M *et al.* Products of oxidative DNA damage and repair as possible biomarkers of susceptibility to lung cancer. *Cancer Res* 2003; **63**: 4899–902.
22 Shigenaga MK, Gimeno CJ, Ames BN. Urinary 8-hydroxy-2′-deoxyguanosine as a biological marker of in vivo oxidative DNA damage. *Proc Natl Acad Sci USA* 1989; **86**: 9697–701.
23 Gackowski D, Rozalski R, Roszkowski K, Jawien A, Foksinski M, Olinski R. 8-Oxo-7,8-dihydroguanine and 8-oxo-7,8-dihydro-2′-deoxyguanosine levels in human urine do not depend on diet. *Free Radic Res* 2001; **35**: 825–32.
24 Lunec J, Holloway KA, Cooke MS, Faux S, Griffiths HR, Evans MD. Urinary 8-oxo-2′-deoxyguanosine. redox regulation of DNA repair in vivo? *Free Radic Biol Med* 2002; **33**: 875–85.
25 Demple B, Harrison L. Repair of oxidative damage to DNA, enzymology and biology. *Annu Rev Biochem* 1994; **63**: 915–48.
26 Hazra TK, Izumi T, Maidt L, Floyd RA, Mitra S. The presence of two distinct 8-oxoguanine repair enzymes in human cells: their potential complementary roles in preventing mutation. *Nucl Acids Res* 1998; **26**: 5116–22.
27 Bessho T, Tano K, Kasai H, Ohtsuka E, Nishimura S. Evidence for two DNA repair enzymes for 8-hydroxyguanine (7,8-dihydro-8-oxoguanine) in human cells. *J Biol Chem* 1993; **268**: 19416–21.
28 Dianov G, Bischoff C, Piotrowski J, Bohr VA. Repair pathways for processing of 8-oxoguanine in DNA by mammalian cell extracts. *J Biol Chem* 1998; **273**: 33811–6.
29 Hayakawa H, Taketomi A, Sakumi K, Kuwano M, Sekiguchi M. Generation and elimination of 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-triphosphate, a mutagenic substrate for DNA synthesis, in human cells. *Biochemistry* 1995; **34**: 89–95.
30 Maki H, Sekiguchi M. MutT protein specifically hydrolyses a potent mutagenic substrate for DNA synthesis. *Nature* 1992; **355**: 273–5.
31 Cooke MS, Evans MD, Herbert KE, Lunec J. Urinary 8-oxo-2′-deoxyguanosine – source, significance and supplements. *Free Radic Res* 2000; **32**: 381–97.
32 Takeuchi T, Nakajima M, Morimoto K. Establishment of a human system that generates O2- and induces 8-hydroxydeoxyguanosine, typical of oxidative DNA damage, by a tumor promoter. *Cancer Res* 1994; **54**: 5837–40.
33 Takeuchi T, Nakajima M, Ohta Y, Mure K, Takeshita T, Morimoto K. Evaluation of 8-hydroxydeoxyguanosine, a typical oxidative DNA damage, in human leukocytes. *Carcinogenesis* 1994; **15**: 1519–23.
34 Loft S, Poulsen HE. Estimation of oxidative DNA damage in man from urinary excretion of repair products. *Acta Biochim Pol* 1998; **45**: 133–44.
35 Bergtold DS, Simic MG, Alessio H, Cutler RG. Urine biomarkers for oxidative DNA damage. In: Simic MG, Taylor KA, Ward JF, von Sontag C, eds. *Oxygen Radicals in Biology and Medicine*. New York: Plenum Press, 1988; 483–90.
36 Kasai H, Nishimura S. Hydroxylation of deoxyguanosine at the C-8 position by ascorbic acid and other reducing agents. *Nucl Acids Res* 1984; **12**: 2137–45.
37 Greenberg MM. In vitro and in vivo effects of oxidative damage to deoxyguanosine. *Biochem Soc Trans* 2004; **32**: 46–50.
38 Higuchi Y. Chromosomal DNA fragmentation in apoptosis and necrosis induced by oxidative stress. *Biochem Pharmacol* 2003; **66**: 1527–35.
39 Simon HU, Haj-Yehia A, Levi-Schaffer F. Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis* 2000; **5**: 415–18.
40 Hengartner MO, Apoptosis. DNA, destroyers. *Nature* 2001; **412**: 27–9.
41 Davies AM, Hershman S, Stabley GJ, Hoek JB, Peterson J, Cahill A. A Ca^{2+}-induced mitochondrial permeability transition causes complete release of rat liver endonuclease G activity from its exclusive location within the mitochondrial intermembrane space. Identification of a novel endo-exonuclease activity residing within the mitochondrial matrix. *Nucl Acids Res* 2003; **31**: 1364–73.
42 Barry ME, Pinto-Gonzalez D, Orson FM, McKenzie GJ, Petry GR, Barry MA. Role of endogenous endonucleases and tissue site in transfection and CpG-mediated immune activation after naked DNA injection. *Hum Gene Ther* 1999; **10**: 2461–80.
43 Hibino Y, Iwakami N, Sugano N. A nuclease from rat-liver nuclei with endo- and exonucleolytic activity. *Biochim Biophys Acta* 1991; **1088**: 305–7.
44 Cordis GA, Goldblatt PJ, Deutscher MP. Purification and characterization of a major endonuclease from rat liver nuclei. *Biochemistry* 1975; **14**: 2596–603.