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Chemical repair of base lesions, AP-sites, and strand breaks on plasmid DNA in dilute aqueous solution by ascorbic acid

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ABSTRACT

We quantified the damage yields produced in plasmid DNA by γ-irradiation in the presence of low concentrations (10–100 μM) of ascorbic acid, which is a major antioxidant in living systems, to clarify whether it chemically repairs radiation damage in DNA. The yield of DNA single strand breaks induced by irradiation was analyzed with agarose gel electrophoresis as conformational changes in closed circular plasmids. Base lesions and abasic sites were also observed as additional conformational changes by treating irradiated samples with glycosylase proteins.

By comparing the suppression efficiencies to the induction of each DNA lesion, in addition to scavenging of the OH radicals derived from water radiolysis, it was found that ascorbic acid promotes the chemical repair of precursors of AP-sites and base lesions more effectively than those of single strand breaks. We estimated the efficiency of the chemical repair of each lesion using a kinetic model. Approximately 50–60% of base lesions and AP-sites were repaired by 10 μM ascorbic acid, although strand breaks were largely unrepaired by ascorbic acid at low concentrations. The methods in this study will provide a route to understanding the mechanistic aspects of antioxidant activity in living systems.

1. Introduction

Ascorbic acid (AA), in addition to some types of catechins derived from plants or animal cells, are antioxidants used in medical and pharmaceutical fields as well as the health food industry. Many *in vitro* experiments have shown that antioxidants play an important role as scavengers of Reactive Oxygen Species (ROS), such as hydroxyl (OH) and other free radicals [1–5]. ROS are produced in cells by external physical or chemical stresses and are induced continuously by metabolic stresses, frequently causing irreversible molecular changes in the cell structure (i.e., damage). DNA damage includes strand breaks and base lesions. A double-strand break (DSB) consisting of two or more single-strand breaks (SSBs) in close proximity produced in genomic DNA is thought to be a harmful lesion that can cause serious biological effects, including cell lethality. On the other hand, base lesions can cause cell mutations that may contribute to the carcinogenic process. Some antioxidants are thought to protect our bodies from stresses, such as radiation, by scavenging ROS. Furthermore, it has been reported that injecting ocimum flavonoids or edaravone to mice exposed to ionizing radiation increases the survival rate [6,7].

The radioprotective effects of antioxidants are thought to be due to their reactivity with OH radicals. The rate constants of the reactions of antioxidants with OH radicals in aqueous solution have been established as 10^9–10^{10} M^{-1} s^{-1} [1,2], which are close to diffusion-controlled reaction rates. On the other hand, most biomolecules are also known to show high reactivity with OH radicals. Roots and Okada measured the average lifetime of OH radicals reacting with DNA in mammalian tissue. They determined the lifetime to be about 4×10^{-9} s by measuring the amount of SSBs produced by the reaction of DNA with OH radicals [8]. The scavenging capacity, given as the reciprocal of the lifetime, has been calculated to be 2.5×10^8 s^{-1} in the cell, suggesting that antioxidant concentrations of several tens or hundreds of millimolars are required for effective OH radical-scavenging in cell mimetic conditions. However, it is reported that concentrations of catechins in human plasma reaches only a few micromolars after ingestion of green tea [9].

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Thus, it is likely that, at such low concentrations of catechins, the health benefits of antioxidants arise from another mechanism additional to their OH-scavenging properties.

Some sulfhydryl compounds, such as glutathione, reduce DNA radicals by hydrogen donation [10]. Mediated by some reducing agents, this process is called "chemical repair" [11]. Antioxidants generally react with free radicals by donating hydrogen (or electron transfer reactions) from their hydroxyl groups. However, because of the similarity of the reductive reactions of antioxidants to the chemical repair reactions of sulfhydryl compounds, antioxidants are also presumed to reduce DNA radicals by chemical repair. It is reported that the yield of deoxyguanosine monophosphate (dGMP) radical is reduced by an electron transfer reaction of AA [12] and catechins [13]. Furthermore, the lifetime of some DNA radicals is longer than that of OH radicals (several seconds [14,15] compared with only a few nanoseconds in living cells [8], respectively). It is quite possible that antioxidants, even at low concentrations, react with DNA radicals, which have long lifetimes in irradiated cells, in addition to scavenging OH radicals. However, it is not known whether the DNA radicals undergo chemical repair by antioxidants, as in the case of dGMP radicals [12,13]. Anderson and co-workers concluded that DNA radicals, as precursors of SSBs and base lesions of DNA induced by γ-irradiation in aqueous solutions, undergo uniform chemical repair by catechins [16–18] but they did not observe the chemical repair properties of AA. Their method, however, was based on competitive reactions for OH radicals between antioxidants and Tris, which was added as a radical scavenger to maintain the scavenging capacity of the solution at a constant value. Thus, the results strongly depended on the accuracy of the rate constants of the reactions of catechins or Tris with OH radicals and the rate constant values differ by two- or three-fold between reports. The rate constant used by Anderson for (+)-catechin was $(1.5 \pm 0.2) \times 10^{10}\,M^{-1}\,s^{-1}$ but another report measuring the rate constant by Bors was $2.2 \times 10^{10}\,M^{-1}\,s^{-1}$ [1].

In terms of a reduction in the frequency of mutations, it is important to reduce base lesions, which are generally produced several times more frequently than SSBs in aqueous conditions [19]. If antioxidants do repair base lesions as effectively as they reduce dGMP radicals, they could be operating as potent radioprotectors in living systems. Thus, to further understand their antioxidative effects, it is important to reveal the chemical repair processes of antioxidants. In the present study, we investigated the chemical repair properties of antioxidants on DNA radicals from a different angle, which was independent of the competitive reactions of the scavengers. Qualitative verification of the chemical repair of base lesion precursors by antioxidants was achieved by applying an enzymatic method to detect the base lesions and apurinic/apyrimidinic sites (AP-sites), as well as SSBs. Comparing the suppression efficiency of this method on the induction of DNA lesions has highlighted the potential ability of antioxidant-mediated chemical repair on specific DNA radicals. Diluted DNA solutions in the presence of low concentrations of AA (10–100 μM) were irradiated by γ-rays, and the suppression efficiencies of induced DNA lesions were analyzed using biochemical assay and chemical kinetics analysis method.

2. Materials and methods

2.1. Chemicals and enzymes

AA was purchased from Sigma–Aldrich (St. Louis, USA). Sodium dihydrogen phosphate dehydrate ($NaH_2PO_4{\cdot}2H_2O$), disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4{\cdot}12H_2O$), Tris, boric acid, EDTA (ethylenediaminetetraacetic acid)·2Na·$2H_2O$, sodium hydrate (NaOH), 1% bromophenol blue, and Orange G were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Formamidopyrimidine-DNA glycosylase (Fpg), endonuclease III (Nth), and endouclease IV (Nfo), and reaction buffer solutions used for treatment of the enzymes were purchased from New England BioLabs Inc. (Ipswich, MA).

Stock solutions of the enzymes (concentrations were 10 units μL^{-1} for Nth and Nfo, and 8 units μL^{-1} for Fpg) and the reaction buffers were stored at −20 °C. Nth protein mainly excises ring-saturated pyrimidines (e.g., 5, 6-dihydrothymine [DHT]), thymine glycol, and AP-sites. Fpg protein excises mainly 2,6-diamino-4-hydroxy-5-*N*-methyl formamidopyrimidine, 7,8-dihydro-8-oxo-2′-deoxyguanine (8-oxoGua), and AP-sites. Nfo protein excises various AP-sites. [20,21].

Phosphate buffer solutions (pH 6.9) were prepared from $NaH_2PO_4{\cdot}2H_2O$ and $Na_2HPO_4{\cdot}12H_2O$. TBE buffer solutions consisted of 89 mM Tris, 89 mM boric acid, and 2 mM EDTA. The loading buffer solutions for electrophoresis consisted of 2% (w/v) g/L bromophenol blue, 2% (w/v) Orange G, 30% (w/v) glycerol, and 10 mM EDTA. The pH of the TBE buffer and the loading buffer were adjusted to 8.0 with 1N NaOH. All irradiation samples and buffer solutions were prepared with deionized water purified by Milli-Q system (Millipore, Billerica, MA). The deionized water and buffer solutions were sterilized at 120 °C for 20 min before sample preparation.

2.2. Plasmid DNA preparation

Plasmid DNA pUC18 (2686 bp) was obtained from *Escherichia coli* JM109 by Qiagen HiSpeed Plasmid Kit (QIAGEN, Hilden, Germany) and purified by dialysis using a nitrocellulose membrane of pore size 0.025 μm (Millipore, Billerica, MA). After dialysis, we confirmed that over 90% of the plasmids were intact (closed circular form) using agarose electrophoresis (see below). Plasmids were stored at −20 °C at a concentration of 188 ng/μL dissolved in 20 mM phosphate buffer solution (pH 6.9).

DNA stock solutions were diluted with 20 mM phosphate buffer solutions (pH 6.9) to a concentration of 10 ng/μL for the irradiation samples. AA was added to the solutions just before irradiation. The concentrations of AA were adjusted to 0, 10, 100, and 1000 μM. As the number of electrons of the solutes, DNA and AA, is less than that of water in a unit volume, over 99% of the radiation energy is thought to be deposited onto water molecules under these conditions. Thus, DNA damage is predominantly induced by free radicals, such as OH radicals, derived from water radiolysis.

2.3. Irradiation

One hundred microliter samples were placed into 1.5 mL polypropylene tubes (Eppendorf, Hamburg, Germany) for irradiation. Each tube was placed into 20 mL of icy water maintained at 4 °C during irradiation and exposed to ^{60}Co γ-rays in aerobic conditions using a γ-ray apparatus (University of Tokyo, Tokyo, Japan). Because the thickness of the surrounding water is greater than that of the sample solution, secondary electron equilibrium is thought to be achieved in this setup. The dose rate, measured by a Fricke dosimeter, ranged from 0.05–3.2 Gy/min depending on the position of the sample. Samples were irradiated at least three times.

2.4. Electrophoresis

After irradiation, 10 μL of each irradiated DNA solution was mixed with 2 μL of the loading buffer. The mixture was placed into wells of a 1% agarose type 1-A (Sigma–Aldrich, St. Louis, MO) gel in TBE buffer. The leftover irradiated solutions were also placed into the same gel following enzyme treatment as described below. The samples were typically run at 3.0 V/cm for 270 min at 5.4 °C. After electrophoresis, closed-circular, open-circular, and linear forms of plasmid DNA were separated due to mobility differences

in the gel. DNA was stained by TBE buffer containing 0.02% ethidium bromide for 30 min, and gel images were obtained using Ettan Dige (GE Healthcare UK Ltd, England). The relative amount of DNA in closed-circular form (cc%) was then quantified, as described previously [22]. According to Milligan et al., the band intensities of the closed-circular form were multiplied by a factor of 1.4 because of the lower binding constant of ethidium bromide to the closed-circular forms than to the open-circular and linear forms.

The dose response of the residual closed-circular DNA was determined from the logarithmic loss. The D_{37} value was calculated from the slope of the response curve and the value of D_{37} represents the dose of radiation required to give on average one SSB per plasmid molecule. The chemical yield of the SSBs (G_{ssb}) is represented using the value of D_{37} as follows:

$$G_{ssb} = \frac{[\text{plasmid}]}{\rho D_{37}} \quad (1)$$

where [plasmid] is a molar concentration of plasmid DNA molecules in an irradiated solution, and ρ is the density of the solution (almost 1.0 kg l^{-1}).

2.5. Detection of enzyme effects

Prior to applying the base excision repair enzymes to the irradiated DNA solutions, the optimum concentrations of Nth, Fpg, and Nfo were determined by treating unirradiated and irradiated DNA solutions with 2 Gy of X-ray (150 kV, 6 mA) with an X-ray generator (SOFTEX, Kanagawa, Japan). The optimum concentrations of Nth, Fpg, and Nfo were determined as 10, 10, 40 unit per 1 pmol of plasmid DNA.

To detect enzyme-sensitive lesions, enzymes were treated with the irradiated DNA solutions as below. Stock solutions of each enzyme were prepared for 10 samples just before use. Each stock solution contained 0.57 μL of Nth, 0.71 μL of Fpg, or 2.3 μL of Nfo with 20 μL of reaction buffer appropriate for each enzyme. For the Fpg stock solution, 20 μL of BSA was also added. These stock solutions were made up to 100 μL with sterilized water. Stock solutions without enzymes were also prepared. An aliquot of 10 μL of each stock solution was added to 10 μL of γ-irradiated DNA solution, and the mixed solutions were subsequently incubated at 37 °C for 1 h. After incubation, the solutions were analyzed using agarose gel electrophoresis, as described above. Base lesions excised by the enzymes, which resulted in strand breaks, were detected as a loss of closed circular plasmids. The base lesion yields revealed by the enzymatic treatments, $G_{\text{enzyme = (Nth, Fpg or Nfo)}}$, were determined by subtracting the G value of the prompt SSBs and heat-labile sites in the incubated samples without the enzyme, G_{enz-}, from that in the solution incubated with the enzyme, G_{enz+}.

$$G_{enzyme} = G_{enz+} - G_{enz-} \quad (2)$$

3. Results

Fig. 1 shows the semi-log plots of the dose response curves of fractions of closed-circular forms in γ-irradiated pUC18 plasmid DNA (10 ng/μL) aqueous solutions, with or without AA. The fractions of closed-circular forms of DNA in the solutions incubated at 37 °C for 1 h with or without Nth are also plotted. Nth-sensitive lesions and strand breaks, which are produced by irradiation, were suppressed by AA. Each fraction of the closed-circular forms decreased exponentially as the γ-ray dose increased. The closed circular forms decreased more dramatically on incubation with Nth rather than without, showing that Nth-sensitive lesions were produced by γ-ray irradiation in closed circular DNA molecules without strand breaks. The dose response curves of heat treatment

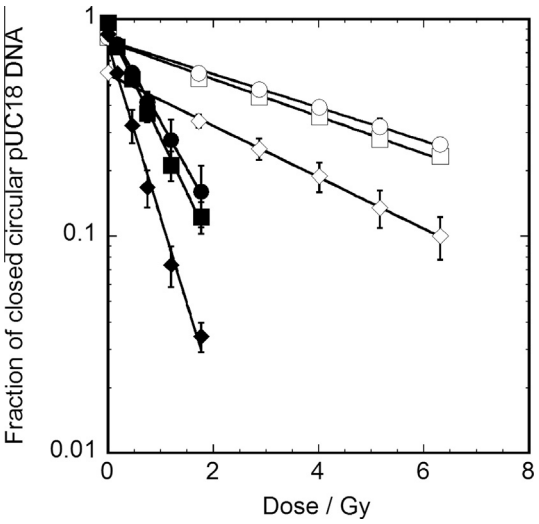

Fig. 1. Dose response curves of the remaining fraction of closed circular plasmid DNA in aqueous solution containing 0 (open symbol) or 100 μM (solid symbol) AA, 10 ng/μL pUC18 plasmid DNA, and 20 mM phosphate buffer (pH 7). The circles represent DNA directly analyzed without any chemical treatments after irradiation. The diamonds and squares represent DNA incubated with or without Nth treatment after irradiation, respectively.

showed slightly steeper slopes, regardless of whether AA was added (see the circle and square symbols in Fig. 1). This indicates that heat treatment induced damage in heat labile sites via OH radicals [23]. The production of heat labile sites was also observed in the treatment of Fpg and Nfo proteins. The G value of prompt SSBs, G_{SSB}, calculated from the dose-response curve shows the efficient production of SSBs through the reactions of DNA with ·OH following incubation. The G values of the Nth, Fpg, and Nfo-sensitive sites (G_{Nth}, G_{Fpg}, G_{Nfo}) were also determined (Table 1). Note that the data shown in the table are the values found after subtracting the contribution of the heat labile sites, G_{enz-}. The G value of prompt SSBs from pUC18 in γ-irradiated aqueous solution was previously reported as 3.5×10^{-2} μmol J^{-1} by Milligan [22], a value that is about five times greater than that determined in this study ($[6.4 \pm 2.2] \times 10^{-3}$ μmol J^{-1}). This discrepancy might be due to differences between the methods used by the two groups to purify plasmid DNA.

The G values of these four types of irradiated DNA lesions in the presence of 10, 100, and 1000 μM AA were obtained (Table 1). The yield of each lesion decreased with increasing AA concentration. The ratio of the G value of each lesion produced in the presence of AA to G_0, the G value representing the absence of AA, was calculated for each concentration (shown in Fig. 2 as "G/G_0"). The G/G_0 ratios decreased with increasing AA concentration, and differences were found among the lesions. In the presence of 10 μM AA, the G value for the Nfo-sensitive sites decreased to less than 20% of the G_0, but the G value of prompt SSBs decreased to 60%. The G/G_0 ratios of enzyme-sensitive sites were obviously lower than that of prompt SSBs in the presence of 10 μM of AA. This result shows that AA significantly reduced the base-lesions and AP-sites detected by Nth, Fpg, or Nfo more efficiently than the SSBs. At higher concentrations of AA, however, the differences were within the limits of experimental error (see Fig. 2.).

4. Discussion

In this study, solutes, plasmid DNA, and AA were prepared at low concentrations to confirm that more than 99% of the γ-ray energy is absorbed and deposited onto water molecules (see Section

Table 1
The chemical yields (G values) of strand breaks, Nth-, Fpg-, and Nfo-sensitive sites, and the fraction of repairable DNA radicals (p).

	G values/μmol J^{-1}				p
	[AA] = 0 μM	[AA] = 10 μM	[AA] = 100 μM	[AA] = 1000 μM	
G_{Nth}	$(4.7 \pm 1.2) \times 10^{-3}$	$(1.3 \pm 0.2) \times 10^{-3}$	$(4.5 \pm 1.3) \times 10^{-4}$	$(7.8 \pm 1.9) \times 10^{-5}$	0.55
G_{Fpg}	$(7.3 \pm 2.2) \times 10^{-3}$	$(2.4 \pm 0.4) \times 10^{-3}$	$(7.3 \pm 0.3) \times 10^{-4}$	$(1.8 \pm 0.4) \times 10^{-4}$	0.50
G_{Nfo}	$(2.9 \pm 0.5) \times 10^{-3}$	$(6.9 \pm 3.4) \times 10^{-4}$	$(2.4 \pm 0.6) \times 10^{-4}$	$(5.0 \pm 2.0) \times 10^{-5}$	0.63
G_{SSB}	$(6.4 \pm 2.2) \times 10^{-3}$	$(4.5 \pm 0.3) \times 10^{-3}$	$(1.0 \pm 0.0) \times 10^{-3}$	$(1.9 \pm 0.7) \times 10^{-4}$	−0.08

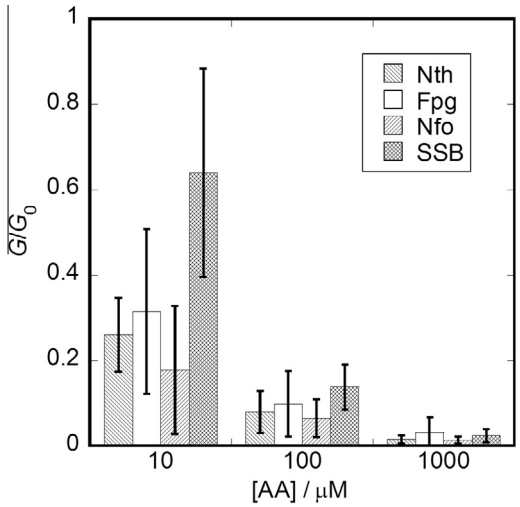

Fig. 2. Ratios of the chemical yields of prompt SSBs Nth-, Fpg- and Nfo-sensitive sites obtained in the presence of AA at several concentrations (G values) to the yields obtained without AA (G_0-values).

2). The direct ionization of DNA and AA by radiation is therefore negligible and almost all DNA radicals are thought to be formed by reactions with OH radicals (·OH) produced from water radiolysis. DNA reacts with OH radicals at several reaction sites (A, B, C,...), producing DNA radicals (A·, B·, C·,...). These DNA radicals eventually result in various DNA lesions (A′, B′, C′,...).

$$\mathrm{DNA} + {}^{\bullet}\mathrm{OH} \rightarrow \mathrm{A}^{\bullet} \rightarrow\rightarrow \mathrm{A}' \tag{3}$$

$$\mathrm{DNA} + {}^{\bullet}\mathrm{OH} \rightarrow \mathrm{B}^{\bullet} \rightarrow\rightarrow \mathrm{B}' \tag{4}$$

$$\mathrm{DNA} + {}^{\bullet}\mathrm{OH} \rightarrow \mathrm{C}^{\bullet} \rightarrow\rightarrow \mathrm{C}' \tag{5}$$

In this experiment, AA and phosphate buffer (PB) also reacted with OH radicals, generating products AA· and PB·.

$$\mathrm{AA} + {}^{\bullet}\mathrm{OH} \rightarrow \mathrm{AA}^{\bullet} \tag{6}$$

$$\mathrm{PB} + {}^{\bullet}\mathrm{OH} \rightarrow \mathrm{PB}^{\bullet} \tag{7}$$

Under the assumption that DNA and AA react competitively with OH radicals, and thereby AA does not chemically repair DNA radical damage, we applied competitive kinetics as below. The G value of lesion A′ ($G_{A'}$) was found to be

$$G_{A'} = \frac{k_{A'}[\mathrm{DNA}]}{k_{A'}[\mathrm{DNA}] + k_{B'}[\mathrm{DNA}] + k_{C'}[\mathrm{DNA}] + \cdots + k_{PB}[\mathrm{PB}] + k_{AA}[\mathrm{AA}]} \times G_{\bullet OH} \tag{8}$$

where k is the rate constant of the reaction of OH radical with each site (A, B, C,...), phosphate buffer, and AA. We assumed that recombination between OH radicals is negligible. The yield of A′ in the solutions in the absence of AA was also calculated.

$$G_{A'0} = \frac{k_{A'}[\mathrm{DNA}]}{k_{A'}[\mathrm{DNA}] + k_{B'}[\mathrm{DNA}] + k_{C'}[\mathrm{DNA}] + \cdots + k_{PB}[\mathrm{PB}]} \times G_{\bullet OH} \tag{9}$$

From Eq. (8) and (9), $G_{A'}/G_{A'0}$ was obtained as follows:

$$\frac{G_{A'}}{G_{A'0}} = \frac{k_{A'}[\mathrm{DNA}] + k_{B'}[\mathrm{DNA}] + k_{C'}[\mathrm{DNA}] + \cdots + k_{PB}[\mathrm{PB}]}{k_{A'}[\mathrm{DNA}] + k_{B'}[\mathrm{DNA}] + k_{C'}[\mathrm{DNA}] + \cdots + k_{PB}[\mathrm{PB}] + k_{AA}[\mathrm{AA}]} = \frac{K[\mathrm{DNA}] + k_{PB}[\mathrm{PB}]}{K[\mathrm{DNA}] + k_{PB}[\mathrm{PB}] + k_{AA}[\mathrm{AA}]} \tag{10}$$

The rate constants of the reactions of each DNA lesion are summed up and described as K. The right side of this equation shows that this value is independent of the damaged DNA sites. Therefore, G/G_0 of every DNA lesion is represented as the same equation under the assumption that there is no chemical repair process by AA:

$$\frac{G_{A'}}{G_{A'0}} = \frac{G_{B'}}{G_{B'0}} = \frac{G_{C'}}{G_{C'0}} = \cdots = \frac{S}{S + k_{AA}[\mathrm{AA}]} \tag{11}$$

The scavenging capacity on DNA and PB is summed up and described as S.

Fig. 2 shows the G/G_0 of SSBs and enzyme-sensitive sites. The Nth- and Nfo-sensitive sites clearly have lower ratios than those of prompt SSBs, indicating that the present data contradict Eq. (11). The yield of Fpg-sensitive sites also seems lower than that of SSBs, although the experimental error was higher than that for the Nth- and Nfo-sensitive sites. According to a previous report, AA reduced the amount of dGMP radical produced from the reaction of dGMP with OH radical [12]. Taking this report into account, the present data also show that DNA radicals, as precursors of base lesions or AP-sites, may undergo chemical repair by AA. In living organisms, AA might play an important role in protecting genomic DNA from oxidative damage through chemical repair, in addition to OH radical-scavenging activities.

To evaluate the chemical repair activity of AA on each type of DNA lesion, a simple chemical kinetic model was considered. First, DNA radicals resulting in a specific DNA lesion can be categorized into two groups: the chemically-repairable and unrepairable groups. The ratio of the former group to the latter group is p: $(1 - p)$. Here p is the fraction of DNA radicals repairable by AA. DNA radicals in the unrepairable group inevitably result in the stable DNA lesions detected in the experiment. The ratio of the G values of the lesions in the unrepairable group to G_0 is represented by a revision of Eq. (11).

$$\frac{G}{G_0} = (1 - p)\frac{S}{S + k_{AA}[\mathrm{AA}]} \tag{12}$$

The value of k_{AA} was measured previously by the authors as $4.5 \times 10^9\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$ by pulse radiolysis (unpublished data). We fitted Eq. (11) to the G/G_0 values obtained in the experiment using reported values of K and k_{PB} ($2.7 \times 10^9\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$ for K and $1.5 \times 10^5\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$ for k_{PB}) and the molarity of the nucleotides (30.8 μM) for the DNA concentration [22]. The estimated optimal values of p are shown in Table 1 (each R^2 of p was greater than 0.99). The values of p of the enzyme-sensitive sites were 0.50–0.63, showing that about half of the precursors of base lesions

or AP-sites could be chemically repaired by AA. The value of p of SSBs, on the other hand, was almost zero within the limits of experimental error. These results indicate that SSBs are seldom repaired by AA. Anderson and co-workers [16] also investigated chemical repair by AA and concluded that AA does not repair SSBs. The present study reveals that even a low concentration (10 μM) of AA chemically repairs certain types of precursors of base lesions or AP-sites in DNA (30.8 μM of nucleotides in the present study) but not SSB precursors. Furthermore, according to the chemical repair model, half of the base lesions and AP-sites could be repaired even under low concentration of AA.

In conclusion, our study indicates that AA is a potent chemical repair agent for DNA damage. The enzymatic method revealed that AA works preferentially on precursors of base lesions and AP-sites, which are possible inducers of mutations. The method used in this study could be applied to other antioxidants and offers a new research field to explore radioprotection mechanisms. Further study is in progress to investigate the chemical repair abilities of some flavonoids and edaravone, which is used as a therapeutic medication for brain infarction.

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References

[1] W. Bors, C. Michel, Antioxidant capacity of flavanols and gallate esters: pulse radiolysis studies, Free Radic. Bio. Med. 27 (1999) 1413–1426.
[2] M. Bonifacic, I. Ljubenkov, M. Eckertmaksic, One-electron oxidation and reduction reactions of vitamin-C derivatives – 6-bromo and 6-chloro-6-deoxy-ascorbic acid, Int. J. Radiat. Biol. 66 (1994) 123–131.
[3] M. Erbenruss, W. Bors, M. Saran, Reactions of linoleic-acid peroxyl radicals with phenolic antioxidants – a pulse-radiolysis study, Int. J. Radiat. Biol. 52 (1987) 393–412.
[4] S.V. Jovanovic, Y. Hara, S. Steenken, M.G. Simic, Antioxidant potential of gallocatechins – a pulse-radiolysis and laser photolysis study, J. Am. Chem. Soc. 117 (1995) 9881–9888.
[5] S.V. Jovanovic, S. Steenken, M. Tosic, B. Marjanovic, M.G. Simic, Flavonoids as antioxidants, J. Am. Chem. Soc. 116 (1994) 4846–4851.
[6] P.U. Devi, A. Ganasoundari, B.S.S. Rao, K.K. Srinivasan, In vivo radioprotection by ocimum flavonoids, survival of mice, Radiat. Res. 151 (1999) 74–78.
[7] K. Anzai, M. Furuse, A. Yoshida, A. Matsuyama, T. Moritake, K. Tsuboi, N. Ikota, *In vivo* radioprotection of mice by 3-methyl-1-phenyl-2-pyrazolin-5-one (Edaravone; Radicut®), a clinical drug, J. Radiat. Res. 45 (2004) 319–323.
[8] R. Roots, S. Okada, Protection of DNA molecules of cultured mammalian cells from radiation-induced single-strand scissions by various alchols and SH sompounds, Int. J. Radiat. Biol. 21 (1972) 329–342.
[9] M.J. Lee, Z.Y. Wang, H. Li, L.S. Chen, Y. Sun, S. Gobbo, D.A. Balentine, C.S. Yang, Analysis of plasma and urinary tea polyphenols in human-subjects, Cancer Epidemiol. Biomarkers Prev. 4 (1995) 393–399.
[10] R.L. Willson, P. Wardman, K.-D. Asmus, Interaction of dGMP radical with cysteamine and promethazine as possible model of DNA repair, Nature 252 (1974) 323–324.
[11] C.V. Sonntag, Free-Radical-Induced DNA Damage and Its Repair A Chemical Rerspective, Springer, Berlin, 2006.
[12] P. O'Neill, Pulse radiolytic study of the interaction of thiols and ascorbate with OH adducts of dGMP and dG - implications for DNA-repair processes, Radiat. Res. 96 (1983) 198–210.
[13] H.Y. Fu, Y. Katsumura, M.Z. Lin, K. Hata, Y. Muroya, Y. Hatano, Fast repair activities towards dGMP hydroxyl radical adducts by silybin and its analogues, J. Radiat. Res. 49 (2008) 609–614.
[14] D. Schultefrohlinde, G. Behrens, A. Onal, Lifetime of peroxyl radicals of poly(U), poly(A) and single-stranded and double-stranded DNA and the rate of their reaction with thiols, Int. J. Radiat. Biol. 50 (1986) 103–110.
[15] K. Hildenbrand, D. SchulteFrohlinde, Time-resolved EPR studies on the reaction rates of peroxyl radicals of poly(acrylic acid) and of calf thymus DNA with glutathione. Re-examination of a rate constant for DNA, Int. J. Radiat. Biol. 71 (1997) 377–385.
[16] R.F. Anderson, C. Amarasinghe, L.J. Fisher, W.B. Mak, J.E. Packer, Reduction in free-radical-induced DNA strand breaks and base damage through fast chemical repair by flavonoids, Free Radical Res. 33 (2000) 91–103.
[17] R.F. Anderson, L.J. Fisher, Y. Hara, T. Harris, W.B. Mak, L.D. Melton, J.E. Packer, Green tea catechins partially protect DNA from ·OH radical-induced strand breaks and base damage through fast chemical repair of DNA radicals, Carcinogenesis 22 (2001) 1189–1193.
[18] R.F. Anderson, L.J. Fisher, T. Harris, The 'pivotal antioxidant' hypothesis for the role of flavonoids in their reduction of HO· radical-induced damage on DNA, Redox Rep. 6 (2001) 197–199.
[19] T. Shiina, R. Watanabe, I. Shiraishi, M. Suzuki, Y. Sugaya, K. Fujii, A. Yokoya, Induction of DNA damage, including abasic sites, in plasmid DNA by carbon ion and X-ray irradiation, Radiat. Environ. Biophys. 52 (2013) 99–112.
[20] S.S. David, S.D. Wiliams, Chemistry of glycosylases and endonucleases involved in base-excision repair, Chem. Rev. 98 (1998) 1221–1261.
[21] M. Haring, H. Rudiger, B. Demple, S. Boiteux, B. Epe, Recognition of oxidized abasic sites by repair endonucleases, Nucleic Acids Res. 22 (1994) 2010–2015.
[22] J.R. Milligan, J.A. Aguilera, J.F. Ward, Variation of single-strand break yield with scavenger concentration for plasmid DNA irradiated in aqueous-solution, Radiat. Res. 133 (1993) 151–157.
[23] G.D.D. Jones, T.V. Boswell, J.F. Ward, Effects of postirradiation temperature on the yields of radiation-induced single-strand and double-strand breakage in SV40 DNA, Radiat. Res. 138 (1994) 291–296.