

# Site-specific DNA damage at GGG sequence by oxidative stress may accelerate telomere shortening

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**Abstract** Telomere shortening during human aging has been reported to be accelerated by oxidative stress. We investigated the mechanism of telomere shortening by oxidative stress. H<sub>2</sub>O<sub>2</sub> plus Cu(II) caused predominant DNA damage at the 5' site of 5'-GGG-3' in the telomere sequence. Furthermore, H<sub>2</sub>O<sub>2</sub> plus Cu(II) induced 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) formation in telomere sequences more efficiently than that in non-telomere sequences. NO plus O<sub>2</sub><sup>-</sup> efficiently caused base alteration at the 5' site of 5'-GGG-3' in the telomere sequence. It is concluded that the site-specific DNA damage at the GGG sequence by oxidative stress may play an important role in increasing the rate of telomere shortening with aging.

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**Key words:** DNA damage; Telomere; Aging; Hydrogen peroxide; Nitric oxide

## 1. Introduction

In humans, a critical shortening of telomeres has been related to cell senescence [1–3]. Telomeres have essential roles in chromosomal structure and function, including stabilization of the chromosome during DNA replication and possible prevention of aberrant chromosomal recombination [4,5]. These functions are mediated by highly conserved repeats which consist of a characteristic hexameric telomere sequence (TTAGGG) in vertebrates [6,7]. Recently, von Zglinicki et al. reported an increase in the rate of telomere shortening by oxidative stress in human fibroblasts [8,9]. Furthermore, age-dependent telomere shortening was shown to be slowed down by enrichment of intracellular vitamin C via suppression of oxidative stress [10]. However, the mechanism for the increase in telomere shortening rate by oxidative stress remains to be clarified.

In recent years, evidence has been accumulating in favor of the free radical theory of aging [11–14], first proposed by Harman [15]. Mitochondria are well documented as a major intracellular source of O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> [16]. Aging is associated with increased rates of mitochondrial oxygen free radical production [17–19]. Oxygen free radical, O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> play im-

portant roles in DNA damage [20–26]. In addition, nitric oxide (NO) may be one of the contributors to age-related oxidative stress [27]. A recent report has suggested that aging of SJL mice correlated with increased NO production [28]. The propensity of NO to react rapidly with O<sub>2</sub><sup>-</sup> leads to formation of peroxynitrite (ONOO<sup>-</sup>) causing DNA damage [29–32]. In this study, we investigated the mechanism for telomere shortening induced by oxidative stress (H<sub>2</sub>O<sub>2</sub>, NO plus O<sub>2</sub><sup>-</sup>) using a <sup>32</sup>P 5' end-labeled DNA fragment including the telomeric sequence. Furthermore, we examined 8-hydroxylation of the guanine residue induced by oxidative stress.

## 2. Materials and methods

### 2.1. Materials

[ $\gamma$ -<sup>32</sup>P]ATP (222 TBq/mmol) was purchased from New England Nuclear. T<sub>4</sub> polynucleotide kinase was from New England Biolabs. Acrylamide, bisacrylamide, H<sub>2</sub>O<sub>2</sub> and piperidine were from Wako Chemical Industries, Ltd. Osaka, Japan. 1,4-Benzoquinone (BQ), NADH and CuCl<sub>2</sub> were from Nacalai Tesque, Inc., Kyoto, Japan. Diethylenetriaminepentaacetic acid (DTPA), 3-morpholinodisodium-N-ethylcarbamate (SIN-1) and 3-(2-hydroxy-1-methylethyl-2-nitrosohydrazino)-N-methyl-1-propanamine (NOC-7) were from Dojin Chemicals Co., Kumamoto, Japan. Nuclease P<sub>1</sub> (400 U/mg) was from Yamasa Shoyu Co., Chiba, Japan. Alkaline phosphatase from calf intestine was from Boehringer Mannheim GmbH. DNA fragments, (TAGTAG)<sub>4</sub>(TTAGGG)<sub>4</sub>, (CCCTAA)<sub>4</sub>(CTACTA)<sub>4</sub>, CGC(TTAGGG)<sub>7</sub>CGC, GCG(CCCTAA)<sub>7</sub>GCG, CGC(TGTGAG)<sub>7</sub>CGC and GCG(CTCACA)<sub>7</sub>GCG were synthesized and purified by HPLC at Sawady Technology Co., Ltd., Tokyo, Japan.

### 2.2. Analysis of damage to <sup>32</sup>P 5' end-labeled DNA by oxidative stress

The 48-base fragment 5'-(TAGTAG)<sub>4</sub>(TTAGGG)<sub>4</sub>-3' was phosphorylated with [ $\gamma$ -<sup>32</sup>P]ATP and T<sub>4</sub> polynucleotide kinase according to the method described previously [33]. The <sup>32</sup>P 5' end-labeled 48-base fragment and its complementary strand 5'-(CCCTAA)<sub>4</sub>(CTACTA)<sub>4</sub>-3' were annealed.

The standard reaction mixture in a microtube (1.5-ml Eppendorf) contained 1 mM SIN-1 or 250  $\mu$ M H<sub>2</sub>O<sub>2</sub> plus 50  $\mu$ M CuCl<sub>2</sub>, 20  $\mu$ M per base of sonicated calf thymus DNA and <sup>32</sup>P 5' end-labeled DNA fragment in 200  $\mu$ l of 10 mM sodium bicarbonate buffer (pH 7) containing 5  $\mu$ M DTPA. Where indicated, after incubation at 37°C, the DNA fragments were heated at 90°C in 1 M piperidine and treated as described previously [32]. The treated DNA fragments were electrophoresed on an 8% polyacrylamide/8 M urea gel and the autoradiogram was obtained by exposing X-ray film to the gel.

The preferred cleavage sites were determined by direct comparison of the positions of the oligonucleotides with those produced by the procedure of Maxam and Gilbert [34] using a DNA-sequencing system (LKB 2010 MacroPhor). A laser densitometer (LKB 2222 UltraScan XL) was used for the measurement of the relative amounts of oligonucleotides from treated DNA fragments.

### 2.3. Analysis of 8-oxodG formation in telomeric or non-telomeric DNA fragments by oxidative stress

A single-stranded DNA fragment containing the telomere sequence (5'-CGC(TTAGGG)<sub>7</sub>CGC-3') was annealed to its complementary strand 5'-GCG(CCCTAA)<sub>7</sub>GCG-3'. Another single-stranded DNA fragment containing no telomere sequence (5'-CGC(TGTGAG)<sub>7</sub>-

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**Abbreviations:** NOS, nitric oxide synthase; 8-oxodG, 8-oxo-7,8-dihydro-2'-deoxyguanosine (also known as 8-hydroxy-2'-deoxyguanosine); SIN-1, 3-morpholinodisodium-N-ethylcarbamate; DTPA, diethylenetriaminepentaacetic acid; SAM, senescence-accelerated mouse; HPLC-ECD, electrochemical detector coupled to an HPLC; NOC-7, 3-(2-hydroxy-1-methylethyl-2-nitrosohydrazino)-N-methyl-1-propanamine; BQ, 1,4-benzoquinone

CGC-3') was also annealed to the complementary strand 5'-GC-G(CTCACA)<sub>7</sub>GCG-3'. Telomeric or non-telomeric DNA fragments (10  $\mu$ M per base) were incubated with H<sub>2</sub>O<sub>2</sub> and 25  $\mu$ M CuCl<sub>2</sub> in 50  $\mu$ l of 10 mM sodium bicarbonate buffer (pH 7) containing 5  $\mu$ M DTPA at 37°C for 30 min. After ethanol precipitation, DNA was digested to nucleosides by incubation with nuclease P<sub>1</sub> and alkaline phosphatase and analyzed by an HPLC-ECD as described previously [35].

### 3. Results

#### 3.1. Site-specific damage of telomeric DNA fragments by oxidative stress

H<sub>2</sub>O<sub>2</sub> plus Cu(II) ion efficiently caused DNA damage at the

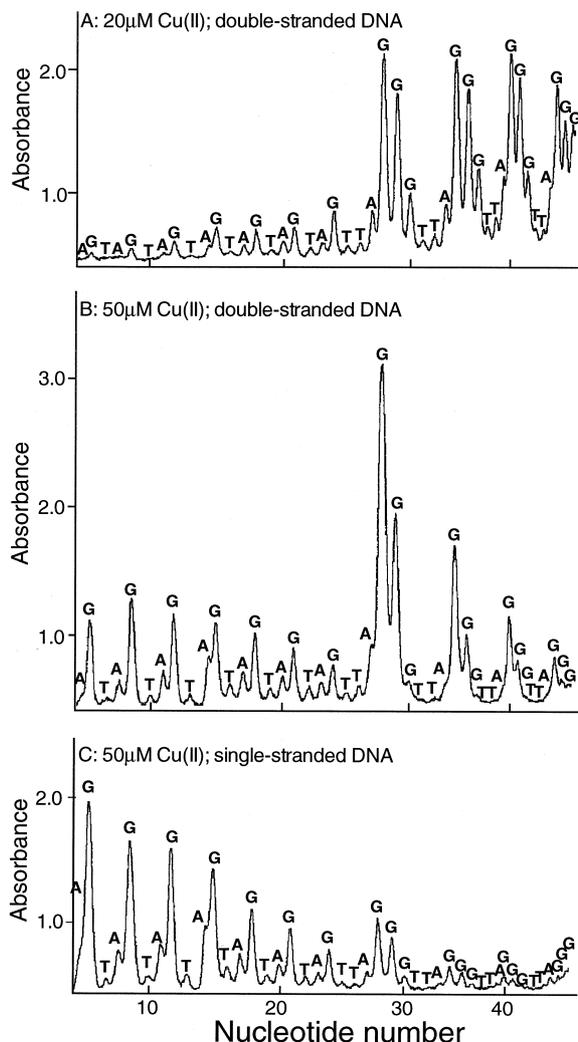


Fig. 1. Site specificity of DNA cleavage by H<sub>2</sub>O<sub>2</sub> in the presence of Cu(II). The <sup>32</sup>P 5' end-labeled 48-bp fragment (5'-(TAGTAG)<sub>4</sub>-(TTAGGG)<sub>4</sub>-3') in 200  $\mu$ l of 10 mM sodium bicarbonate buffer at pH 7 containing 5  $\mu$ M DTPA and 20  $\mu$ M per base of sonicated calf thymus DNA was incubated with 250  $\mu$ M H<sub>2</sub>O<sub>2</sub> in the presence of 20  $\mu$ M Cu(II) (A) or 50  $\mu$ M Cu(II) (B and C) at 37°C for 30 min. After piperidine treatment, DNA fragments were electrophoresed on an 8% polyacrylamide/8 M urea gel using a DNA-sequencing system and the autoradiogram was obtained by exposing X-ray film to the gel. The relative amounts of oligonucleotides produced were measured using a laser densitometer (LKB 2222 UltroScan XL). The piperidine-labile sites of the treated DNA were determined by direct comparison with the same DNA fragment after undergoing DNA sequencing reactions according to the Maxam-Gilbert procedure [34]. The horizontal axis shows the nucleotide number.

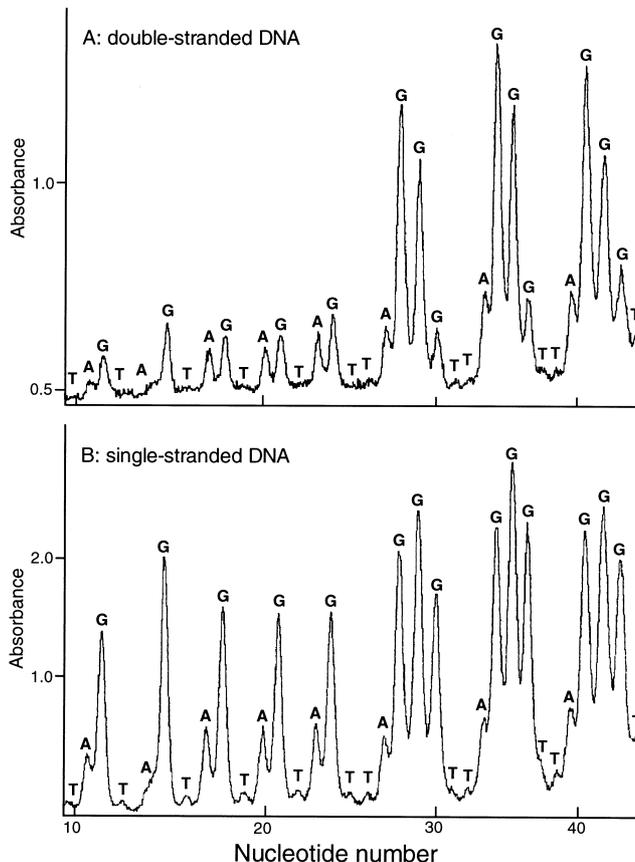


Fig. 2. Site specificity of DNA cleavage induced by SIN-1. The <sup>32</sup>P 5' end-labeled 48-bp fragment (5'-(TAGTAG)<sub>4</sub>-(TTAGGG)<sub>4</sub>-3') in 200  $\mu$ l of 10 mM sodium bicarbonate buffer at pH 7 containing 5  $\mu$ M DTPA and 1  $\mu$ M per base of sonicated calf thymus DNA was incubated with 1 mM SIN-1 at 37°C for 60 min. After piperidine treatment, DNA fragments were analyzed as described in the legend to Fig. 1. The horizontal axis shows the nucleotide number.

5' site of 5'-GGG-3' in the telomere sequence (Fig. 1A). Even without piperidine treatment, oligonucleotides were formed, suggesting the breakage of the deoxyribose phosphate backbone. The increased amount of oligonucleotides detected following piperidine treatment (data not shown) suggests that base alteration was induced by H<sub>2</sub>O<sub>2</sub> in the presence of Cu(II). The extent of DNA damage in the telomere sequence region (5'-TTAGGG-3')<sub>4</sub> was approximately six times stronger than that in the non-telomere sequence region (5'-TAGTAG-3')<sub>4</sub>. When single-stranded DNA was used, the site specificity of DNA cleavage was changed. Cleavage at every guanine was increased (Fig. 1C).

SIN-1 was used as a model for the continuous release of NO and O<sub>2</sub><sup>-</sup> [36]. SIN-1 efficiently caused DNA cleavage at the 5' site of the 5'-GGG-3' sequence in the telomere sequence (Fig. 2A). Although SIN-1 caused no DNA cleavage without piperidine treatment, piperidine treatment led to chain cleavage (data not shown). This result indicated that SIN-1 caused base alteration without breakage of the deoxyribose phosphate backbone. The extent of DNA damage in the telomere sequence region was approximately four times stronger than that in the non-telomere sequence region. When single-stranded DNA was used, DNA damage occurred mostly at every guanine residue (Fig. 2B). Although cleavage at adenine occurred slightly, no or little cleavage was observed

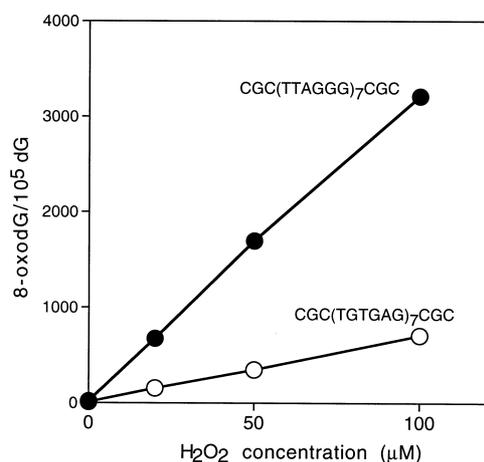


Fig. 3. Formation of 8-oxodG in DNA fragments induced by oxidative stress. DNA fragments (10  $\mu$ M per base) containing the telomere sequence (5'-CGC(TTAGGG)<sub>7</sub>CGC-3') (●) or the non-telomere sequence (5'-CGC(TGTGAG)<sub>7</sub>CGC-3') (○) were incubated with H<sub>2</sub>O<sub>2</sub> in the presence of 25  $\mu$ M Cu(II) in 50  $\mu$ l of 10 mM sodium bicarbonate buffer (pH 7) containing 5  $\mu$ M DTPA at 37°C for 30 min. After ethanol precipitation, the DNA was subjected to enzyme digestion and analyzed by an HPLC-ECD as described in Section 2. These results are obtained from two independent experiments.

at thymine (Fig. 2). A similar DNA cleavage pattern was observed with a combination of an NO-generating agent (NOC-7) and an O<sub>2</sub><sup>-</sup>-generating system (BQ+NADH) (data not shown). It has been reported that O<sub>2</sub><sup>-</sup> is continuously generated through NADH-mediated BQ reduction [37].

### 3.2. Hydroxylation of deoxyguanosine in the telomeric DNA fragment by oxidative stress

To confirm the oxidative DNA damage, we measured the content of 8-oxodG in the 48-bp DNA fragment containing the telomere sequence (5'-CGC(TTAGGG)<sub>7</sub>CGC-3') or the non-telomere sequence (5'-CGC(TGTGAG)<sub>7</sub>CGC-3') induced by oxidative stress. H<sub>2</sub>O<sub>2</sub> plus Cu(II) increased the 8-oxodG content in the DNA fragment (Fig. 3). The formation of 8-oxodG in the DNA fragment containing the telomere sequence efficiently increased with increasing H<sub>2</sub>O<sub>2</sub> concentration. The amount of 8-oxodG produced in the DNA fragment containing the telomere sequence was approximately 4.5 times more than that produced in the DNA fragment containing the non-telomere sequence. The yields of 8-oxodG were approximately 70% of the total guanine alteration in the DNA fragment containing the telomere sequence under the conditions employed.

## 4. Discussion

This study demonstrated for the first time that oxidative stress efficiently induced DNA damage at the 5' site of 5'-GGG-3' in the telomere sequence. H<sub>2</sub>O<sub>2</sub> plus Cu(II) caused predominant DNA cleavage at the 5' site of 5'-GGG-3' in the telomere sequence region in the DNA fragment (5'-(TAGTAG)<sub>4</sub>(TTAGGG)<sub>4</sub>-3'). SIN-1, which leads to simultaneous generation of both NO and O<sub>2</sub><sup>-</sup>, and an NO-generating agent plus an O<sub>2</sub><sup>-</sup>-generating system efficiently caused base alteration at the 5' site of the 5'-GGG-3' sequence in the

telomere sequence. In contrast, when single-stranded DNA was used, the damage induced by oxidative stress occurred at every guanine. The difference of site specificity of DNA damage between double-stranded DNA and single-stranded DNA could be explained in terms of the lower ionization potentials of stacked guanine base pairs in double-stranded DNA on the basis of theoretical calculations [38]. Relevantly, Henle et al. have reported that when Fe(II) and a high concentration of H<sub>2</sub>O<sub>2</sub> were used, preferential cleavages occurred at the nucleotide located 5' to the sequence 5'-RGGG-3' [39]. In addition, it has been demonstrated that oxidative damage to DNA by H<sub>2</sub>O<sub>2</sub> is sufficient to promote instability of repetitive sequences [40].

Fraga et al. reported that the age-dependent accumulation of 8-oxodG was observed in DNA from rat organs [41]. 8-oxodG is a biomarker for oxidative stress on DNA, a common lesion in mammalian cells. The present study demonstrated that H<sub>2</sub>O<sub>2</sub> plus Cu(II) efficiently increased 8-oxodG content in the DNA fragment containing the telomere sequence. Since the yields of 8-oxodG were approximately 70% of the total guanine alterations in the DNA fragment containing the telomere sequence, it can be estimated that H<sub>2</sub>O<sub>2</sub> plus Cu(II) induces 8-oxodG formation at the 5' site of 5'-GGG-3' sequence-specifically. Recently, it has been reported that human 8-oxodG-DNA glycosylase introduces a chain break in a double-stranded oligonucleotide specifically at an 8-oxodG paired with cytosine [42]. Therefore, it is considered that formation of 8-oxodG induced by H<sub>2</sub>O<sub>2</sub> may participate in the increase of the telomere shortening rate in vivo. Aging is associated with increased rates of mitochondrial free radical production [17–19]. Our finding is noteworthy in relation to the report that the rate of mitochondrial oxygen free radical production increases with aging [17–19]. In this study, SIN-1 did not induce 8-oxodG formation in the DNA fragment containing the telomere sequence in bicarbonate buffer. This result is similar to those reported by previous studies [43,44]. On the other hand, several reports have demonstrated that ONOO<sup>-</sup> and SIN-1 induced 8-nitroguanine formation in calf thymus DNA [45,46]. Therefore, it is considered that reactive nitrogen species may produce piperidine-labile 8-nitroguanine in GGG sequences.

Franceschi and Ottaviani have predicted that inflammatory response might increase with aging [47]. Activated inflammatory cells such as phagocytes produce O<sub>2</sub><sup>-</sup> and NO through the induction of nitric oxide synthase (NOS). In the cerebral cortex of the aged senescence-accelerated mouse (SAM), of which the SAMP8 sub-strain is inferior in acquisition of learning, NOS activity was increased compared with that of young SAMP8 [48]. The present results show that NO plus O<sub>2</sub><sup>-</sup> efficiently cause base alteration at the 5' site of 5'-GGG-3' in the telomere sequence in the DNA fragment (5'-(TAGTAG)<sub>4</sub>(TTAGGG)<sub>4</sub>-3'). In summary, oxidative stress can cause cleavage specifically at polyguanosine sequences in the telomere sequence. It is concluded that the site-specific DNA damage in the telomere sequence may play an important role in increasing the rate of telomere shortening with aging.

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