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Review Article

Sequence-Specific DNA Damage by Reactive Oxygen Species: Implications for Carcinogenesis and Aging*

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Abstract

Reactive oxygen species (ROS) generated by environmental chemicals can cause sequence-specific DNA damage, which may lead to carcinogenesis and aging. We investigated the mechanism of DNA damage by environmental chemicals (catechol, propyl gallate and bisphenol-A), homocysteine and UVA radiation using human cultured cell lines and ^{32}P-labeled DNA fragments. Carcinogenic catechol induced piperidine-labile sites frequently at thymine residues in the presence of Cu(II) and NADH. Furthermore, catechol increased the formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), a characteristic oxidative DNA lesion, in human leukemia cell line HL-60, but not in HP100, a hydrogen peroxide (H_2O_2)-resistant cell line derived from HL-60. Thus, it is concluded that oxidative DNA damage through generation of H_2O_2 plays an important role in the carcinogenic process of catechol. In addition, an environmental factor, bisphenol-A, and a dietary factor, propyl gallate, also induced sequence-specific DNA damage via ROS generation.

UVA, as well as UVB, contributes to photoaging. In humans, telomere shortening is believed to be associated with cell senescence. In this study, we investigated the shortening rate of telomeres in human WI-38 fibroblasts exposed to UVA irradiation. The telomere length (as measured by terminal restriction fragment length) in WI-38 fibroblasts irradiated with UVA decreased with increasing the irradiation dose. UVA irradiation with riboflavin caused damage specifically at the GGG sequence in the DNA fragments containing telomere sequence $(TTAGGG)_4$. We concluded that the GGG-specific damage in telomere sequence induced by UVA irradiation participates in the increase of the telomere shortening rate.

In this report, we show our experimental results and discuss the mechanisms of sequence-specific DNA damage in relation to carcinogenesis and aging.

Key words: DNA damage, reactive oxygen species, 8-oxo-7,8-dihydro-2'-deoxyguanosine, carcinogenesis, aging

Introduction

The imposition of oxidative stress by various environmental factors (dietary factors, ultraviolet (UV) radiation and ionizing radiation, etc.) leads to the production of reactive oxygen species (ROS). ROS are involved in a variety of biological phenomena, such as mutation, carcinogenesis and aging (1, 2). In the multistage carcinogenesis model, ROS are associated not only with initiation, but also with promotion and progression. ROS-generating oxidants are known to modulate cell proliferation and apoptosis, and induce synthesis of growth factors that play an important role in tumor growth and invasion. Thus, there is clear evidence that ROS are linked both to carcinogenesis and to tumor behavior.

The oxidative stress hypothesis of aging is currently one of the most popular explanations for the cause of aging. Oxidative stress causes lesions to biomolecules such as DNA, protein and lipids. Recently, it has been reported that oxidative stress increases the amount of telomere length lost per cell population doubling (3–5). Telomeres are the repetitive DNA sequences and telomere shortening has been implicated in cellular

Abbreviations: 8-oxodG, 8-oxo-7,8-dihydro-2'-deoxyguanosine (also known as 8-hydroxy-2'-deoxyguanosine); ROS, reactive oxygen species; O_2^-, superoxide radical anion; •OH, hydroxyl radical; H_2O_2, hydrogen peroxide; $^1O_2$, singlet oxygen; HPLC-ECD; electrochemical detector coupled to high-performance liquid chromatography; PG, propyl gallate; GA, gallic acid; BPA, bisphenol-A; 3-OH-BPA, 3-hydroxybisphenol A.

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senescence. Telomeres are more sensitive to oxidative damage (6), and single-strand breaks in telomeres are less likely to be repaired than elsewhere in the genome.

ROS includes oxygen free radicals, such as superoxide radical anion (O_2^-) and hydroxyl radical (•OH), and non-radical oxidants, such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). These reactive species have different redox potentials, which play important roles in sequence-specific DNA damage. •OH causes DNA damage with no marked site specificity (7, 8). Copper-hydroperoxo complex (Cu(I)OOH) formed by H_2O_2 plus Cu(I) causes DNA damage at thymine residues adjacent to guanine residues, particularly at the 5'-GTC-3' sequence (9). Interestingly, Ohnishi et al. have demonstrated that copper-hydroperoxo complex also caused double base lesions at 5'-TG-3' sequences and the CG of the 5'-ACG-3' sequence complementary to codon 273 of the *p53* gene (10). Furthermore, UVA radiation causes DNA damage at the 5'-G in GG sequence through electron transfer in the presence of certain photosensitizers (9, 11, 12).

Here we discuss the mechanisms and sequence specificity of DNA damage caused by ROS generated from various environmental factors in relation to carcinogenesis and aging.

The role of reactive oxygen species in carcinogenesis

Site specificity and mechanism of oxidative DNA damage induced by catechol

Catechol is an industrial chemical and is found in certain foods such as onions and coffee. Catechol is also an important constituent of cigarette smoke (13). Furthermore, catechol is a major metabolite of benzene, which is known to cause leukemia in humans and animals (14, 15). Catechol has been shown to be carcinogenic for rodents (16). Recently, the International Agency for Research on Cancer (IARC) has classified catechol as a group 2B carcinogen (17), which is possibly carcinogenic to human. However, the mechanism of DNA damage to elicit carcinogenicity by catechol has not been clarified.

To investigate the ability of catechol to cause oxidative DNA damage, the amount of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), a characteristic oxidative DNA lesion, induced by catechol in a human leukemia cell line, HL-60, and its hydrogen peroxide (H_2O_2)-resistant clone HP100 (18) was measured by using an electrochemical detector coupled to high-performance liquid chromatography (HPLC-ECD). 8-oxodG is known to cause DNA misreplication resulting in mutation or cancer (19, 20). Catechol increased the amount of 8-oxodG in HL-60, whereas the amount of 8-oxodG in HP100 was not increased. Furthermore, to clarify the mechanism of cellular DNA damage, we examined the DNA damage caused by catechol in the presence of metal ions, using ^{32}P-5'-end-labeled DNA fragments obtained from the human *p16* and *p53* tumor suppressor genes and the c-Ha-*ras*-1 protooncogene. Catechol caused damage to DNA fragments in the presence of physiological concentration of Cu(II) (20 μM) (21). When 100 μM NADH was added, the DNA damage was markedly enhanced and clearly observed in relatively low concentrations of catechol (<1 μM). The concentration of NADH in certain tissue was estimated to be as high as 100–200 μM (22). The DNA cleavage was enhanced by piperidine treatment, suggesting that catechol caused not only deoxyribose phosphate backbone breakage but also base modification. Catechol frequently modified thymine residues. Bathocuproine, a specific Cu(I) chelator, and catalase inhibited the DNA damage, indicating the participation of Cu(I) and H_2O_2 in the DNA damage. •OH scavengers did not inhibit catechol plus Cu(II)-induced DNA damage, whereas methional completely inhibited it. These results suggest that ROS derived from the reaction of H_2O_2 with Cu(I) participates in catechol-induced DNA damage. Copper has been found in the nucleus and closely associated with chromosomes and DNA bases to serve physiological functions to maintain DNA structure (23). Copper ions exhibit a very high affinity for DNA, and DNA-bound Cu(II) can undergo Cu(II)/Cu(I) redox cycling in

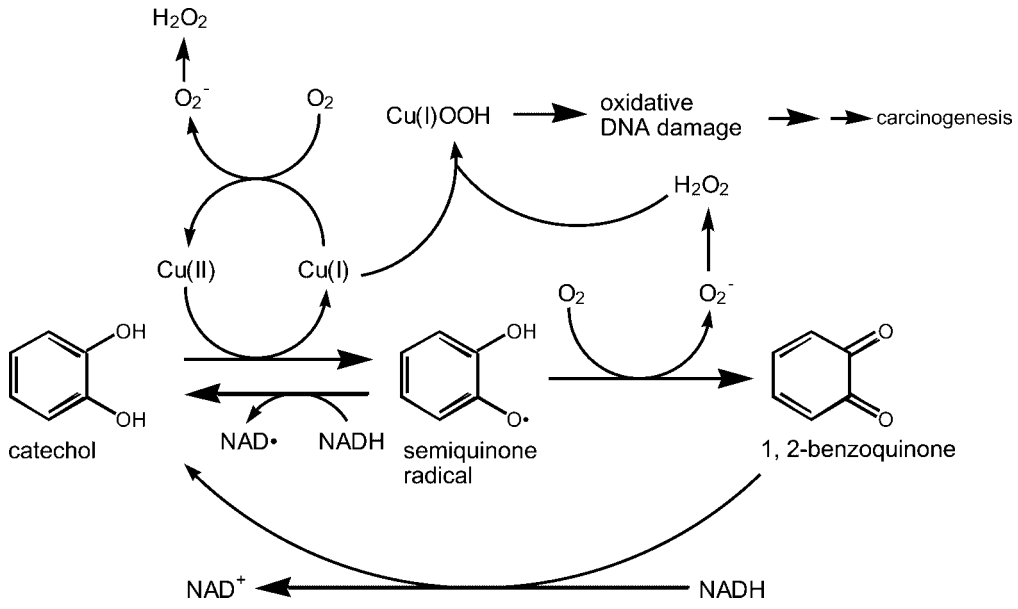

Fig. 1. Possible mechanism of oxidative DNA damage induced by catechol and NADH plus Cu(II).

reducing environment, and also reduce O_2 to O_2^-, generating H_2O_2. On the basis of these results, a possible mechanism of oxidative DNA damage by catechol and NADH in the presence of Cu(II) has been proposed in Fig. 1. Interestingly, the ROS can be produced abundantly through the reduction of the oxidized form, such as semiquinone radical or 1,2-benzoquinone, by NADH non-enzymatically. Collectively, it is concluded that the oxidative DNA damage by catechol through generation of ROS plays an important role in the carcinogenic process of catechol and benzene (24, 25).

Metal-mediated oxidative damage to cellular and isolated DNA by gallic acid, a metabolite of antioxidant propyl gallate

Propyl gallate (PG) is widely used as an antioxidant in the food industry. PG has been investigated as a potential chemopreventive agent in several animal experiments (26). In contrast, National Toxicology Program (NTP) has reported that PG (27, 28) is carcinogenic to mice and rats. However, the mechanism leading to carcinogenesis has not yet been clarified. To clarify the carcinogenic mechanism of PG, we investigated DNA damage using human cultured cell lines.

PG increased the amount of 8-oxodG, a characteristic oxidative DNA lesion, in HL-60, but not in HP100 (Fig. 2). Although PG induced no or little damage to ^{32}P-5'-end-labeled DNA, DNA damage was observed with esterase treated PG. HPLC analysis of the products generated from PG incubated with esterase revealed that PG converted into gallic acid (GA). Relevantly, it has been reported that PG in the hepatocyte suspensions is converted to GA (29). GA induced DNA damage in a dose-dependent manner in the presence of Fe(III)EDTA or Cu(II). In the presence of Fe(III) complex such as Fe(III)EDTA or Fe(III)ADP, GA caused DNA damage at every nucleotide. Fe(III) complex-mediated DNA damage by GA was inhibited by •OH scavengers, catalase and an iron chelating agent. These results suggest that the Fe(III) complex-mediated DNA damage caused by GA is mainly due to •OH generated via the Fenton reaction. In the presence of Cu(II), DNA damage induced by GA occurred at thymine and cytosine. Although •OH scavengers did not prevent the DNA damage, methional inhibited the DNA damage. Cu(II)-mediated DNA damage was inhibited by catalase and a Cu(I) chelator. These results indicated that copper-hydroperoxo complex formed by the interaction of Cu(I) and H_2O_2 participates in the DNA damage. This study suggested that site-specific DNA damage caused by GA plays an important role in the carcinogenesis by PG (30).

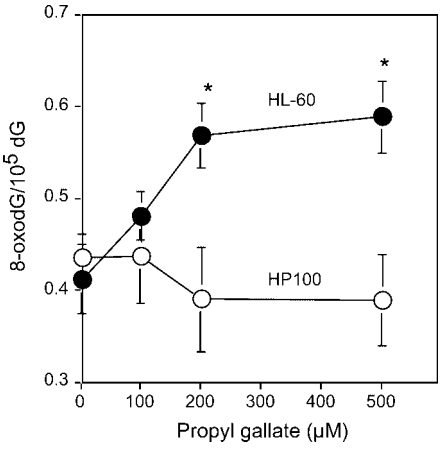

Fig. 2 Comparison of 8-oxodG formation in HL-60 and HP100 cells treated with PG. HL-60 (closed circle) and HP100 (open circle) cells (10^6 cells/ml) were incubated with PG for 2 h at 37°C and the DNA was extracted immediately. DNA was digested to nucleosides enzymatically and 8-oxodG content was analyzed by HPLC-ECD. Results are expressed as mean±SE of values obtained from 6 independent experiments. Symbols indicate significant differences compared with control (*; $p<0.05$) by *t*-test.

Oxidative DNA damage induced by bisphenol-A

Bisphenol-A (BPA; 4,4'-isopropylidenediphenol) is a monomer of polycarbonate plastics and a constituent of epoxy and polystyrene resins that are widely used in consumer products, including composite dental sealants and the inner coating of food cans. BPA is a well-known endocrine-disrupting chemical found in the environment. In BPA bioassay report, the National Toxicology Program (NTP) showed a marginally significant increase in leukemias in male rats (31). Huff (32) reported that BPA increased in the combined incidence of lymphomas and leukemias in male mice. These reports suggest that BPA may be associated with increased cancers of the hemopoietic system.

To clarify whether BPA has potential carcinogenicity, we investigated DNA damage induced by 3-hydroxybisphenol A (3-OH-BPA), a metabolite of BPA, using ^{32}P-labeled DNA fragments obtained from genes that are relevant to human cancer. 3-OH-BPA caused damage to ^{32}P-labeled DNA fragments in the presence of Cu(II). When NADH was added, the DNA damage was markedly enhanced and clearly observed of low concentrations of 3-OH-BPA (<0.1 μM). 3-OH-BPA induced DNA damage frequently at thymine, cytosine and guanine residues in the presence of Cu(II) and NADH. Interestingly, 3-OH-BPA formed piperidine-labile lesions at cytosine residue of the 5'-ACG-3' sequence, complementary to codon 273, a well-known hotspot of the *p53* gene (Fig. 3). The guanine residue of this sequence was significantly cleaved with Fpg treatment. 3-OH-BPA also caused double base lesions at 5'-TG-3' sequences (data not shown). Taken together, 3-OH-BPA caused double base lesions at 5'-ACG-3' and 5'-TG-3' sequences. Furthermore, bathocuproine, a Cu(I) chelator, and catalase inhibited the DNA damage, indicating the participation of Cu(I) and H_2O_2 in the DNA damage. These results suggest that copper-hydroperoxo complex derived from the reaction of H_2O_2 with Cu(I) participates in 3-OH-BPA-induced DNA damage.

The role of reactive oxygen species in aging

Mechanism of telomere shortening by oxidative stress

Eukaryotic telomeres have important roles in cellular processes including chromatin organization and control of cell proliferation. Telomere shortening has been implicated in cellular aging (33). A study on aging in animals has revealed that telomeres shorten in the rat kidney, liver, pancreas and the lung in an age-dependent manner. In human beings, telomere shortening contributes to mortality in many age-related diseases (34). Notably, it is reported that some alleles show almost

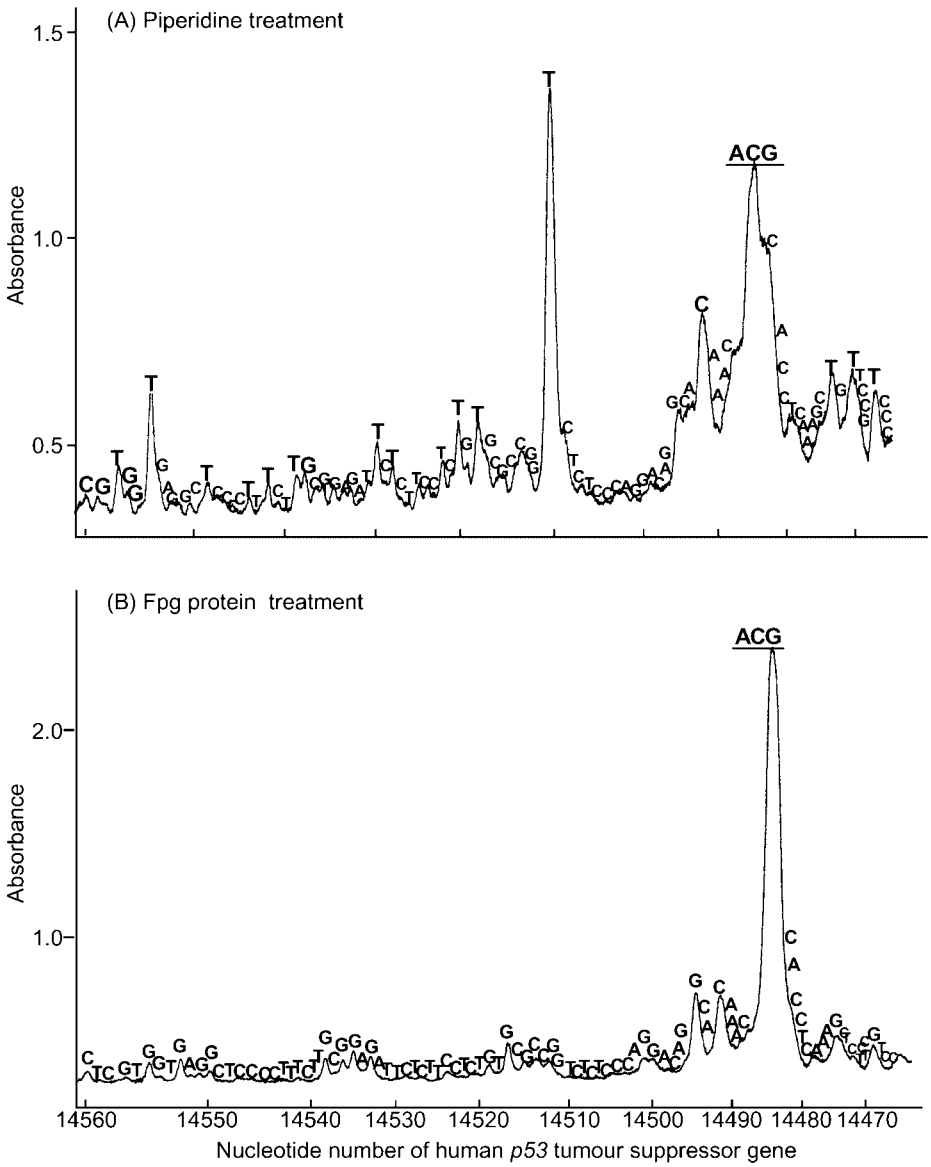

Fig. 3 Site specificity of DNA cleavage induced by 3-OH-BPA and NADH in the presence of Cu(II). Reaction mixtures containing the ^{32}P-5'-end labeled 443-base pair DNA fragment, 20 μM calf thymus DNA, 20 μM $CuCl_2$ and 1 μM 3-OH-BPA and 100 μM NADH in 10 mM phosphate buffer (pH 7.8) containing 5 μM DTPA were incubated at 37°C for 60 min. After piperidine treatment (A) or Fpg protein treatment (B), the DNA fragment was electrophoresed on an 8% polyacrylamide/8 M urea gel and the autoradiogram was obtained by exposing x-ray film to the gel.

complete loss of TTAGGG repeats in senescence (35). Thus, telomere shortening has been suggested to be a 'molecular clock' of the aging process. Recently, Zglinicki et al. reported an increase of the rate of telomere shortening by oxidative stress in human fibroblasts (3, 4). However, the mechanism for the increase in telomere shortening rate by oxidative stress remains to be clarified.

Repeated exposure of human skin to solar UV irradiation leads to skin carcinogenesis and photoaging, which involves cell senescence. Increasing evidence demonstrates that UVA, as well as UVB, contributes to photoaging (36). The molecular mechanisms of photodamage by UVA, the sunlight's major ultraviolet constituent, are poorly understood. Kawanishi et al. have demonstrated that UVA irradiation induced oxidative DNA damage in the presence of endogenous photosensitizers (9, 11, 12, 37–39). In this study, we investigated shortening rate of telomeres in human WI-38 fibroblasts exposed to UVA irradiation. We also examined the formation of 8-oxodG in human cultured cells by using HPLC-ECD. Furthermore, we investigated the mechanism for increase of telomere shortening induced by UVA irradiation using ^{32}P-5'-end labeled DNA fragment including the telomeric sequence.

The terminal restriction fragment (TRF) length, as telomere length, from WI-38 fibroblasts irradiated with UVA (365-nm light) decreased with increasing irradiation doses (Table 1). Furthermore, UVA irradiation dose-dependently increased the formation of 8-oxodG in WI-38 fibroblasts. In order to clarify the mechanism of the acceleration of telomere shortening, we investigated site-specific DNA damage induced by UVA irradiation in the presence of endogenous photosensitizers using ^{32}P-5'-end labeled DNA fragments containing telomeric oligonucleotide $(TTAGGG)_4$. UVA irradiation with riboflavin induced

Table 1 TRF length and 8-oxodG formation in WI-38 fibroblasts irradiated with UVA

UVA (J/cm^2)	TRF[a)] (kb)	8-oxodG/10^5 dG[b)]±S.E.
0	9.48	0.44±0.07
2	8.53	0.72±0.03
5	7.98	0.98±0.16

[a)] WI-38 fibroblasts (1.0×10^6 cells) were irradiated with indicated dose of UVA light (365 nm). After the irradiation, the cells were lysed, and DNA was extracted. Genomic DNA was digested with *Hinf* I and *Rsa* I, separated by electrophoresis on a 0.8% agarose gel and hybridized to telomeric probe using a Telo TAGGG telomere length assay kit (Roche).

[b)] WI-38 fibroblasts (2.0×10^6 cells) were irradiated with indicated dose of UVA light (365 nm). After the irradiation, the cells were lysed, and DNA was extracted and subjected to enzyme digestion and analyzed by an HPLC-ECD.

8-oxodG formation in the DNA fragments containing telomeric sequence, and Fpg protein treatment led to chain cleavages at the central guanine of 5'-GGG-3' in telomere sequence. Fpg protein catalyzes the excision of piperidine-resistant 8-oxodG (40). The amount of 8-oxodG formation in DNA fragment containing telomere sequence (5'-CGC(TTAGGG)$_7$CGC-3') was approximately 5 times more than that in DNA fragment containing non-telomere sequence (5'-CGC(TGTGAG)$_7$CGC-3'). Catalase did not inhibit these oxidative DNA damage, indicating little or no participation of H_2O_2 in the DNA damage. These results indicate that the photoexcited endogenous photosensitizer specifically oxidizes the central guanine of 5'-GGG-3' in telomere sequence to produce 8-oxodG probably through an electron transfer reaction. It is concluded that the site-specific damage in telomere sequence induced by UVA irradiation participates in the increase of the telomere shortening rate (41, 42).

Oxidative damage to cellular and isolated DNA by homocysteine

Homocysteine, generated by the catabolism of the essential amino acid methionine, is a naturally occurring thiol amino acid. Increased plasma total homocysteine, a risk factor for cardiovascular disease, is related to genetic, environmental, and nutritional factors (43). Evidence is accumulating that elevated levels of homocysteine are correlated with higher risk for cancer (44–47). Furthermore, a recent report has suggested that by keeping homocysteine levels low, folic acid can protect cerebral vessels and prevent the accumulation of DNA damage in neurons caused by oxidative stress and facilitated by homocysteine (48). In addition, homocysteine increases the amount of telomere length lost per population doubling (5). Thus, elevated homocysteine levels may also render the brain vulnerable to age-related neurodegenerative disorders.

In this study, we investigated site-specific DNA damage induced by homocysteine using ^{32}P-labeled DNA fragments. There were two mechanisms by which homocysteine caused DNA damage in the presence of Cu(II) (49). A low concentration of homocysteine (20 μM) frequently induced piperidine-labile sites at thymine residues, whereas a high concentration of homocysteine (100 μM) resulted in damage principally to guanine residues. Catalase inhibited DNA damage by 20 μM homocysteine, indicating the participation of H_2O_2, but was ineffective in preventing DNA damage by 100 μM homocysteine. Experiments using a singlet oxygen probe showed that 100 μM homocysteine enhanced chemiluminescence intensity in deuterium oxide (D_2O) more efficiently than that in H_2O. These results suggest the involvement of $^1O_2$, but not H_2O_2, in Cu(II) mediated-DNA damage at higher concentrations of homocysteine. Furthermore, homocysteine increased the amount of 8-oxodG in HL-60, whereas the amount of 8-oxodG in HP100 was not increased. This study demonstrated that homocysteine caused sequence-specific DNA damage via ROS generation in the presence of Cu(II). Therefore, it is concluded that elevated plasma homocysteine contributes to aging and carcinogenesis through metal-mediated oxidative DNA damage.

Conclusion

We investigated the mechanisms of DNA damage induced by environmental chemicals, homocysteine and UVA radiation. We found that ROS mediate DNA damage in sequence-specific manners. The DNA damage causes mutations, which can lead to activation of protooncogenes and inactivation of tumor suppressor genes, resulting in carcinogenesis. Particularly, 8-oxodG is an important oxidative product of guanine, and 8-oxodG

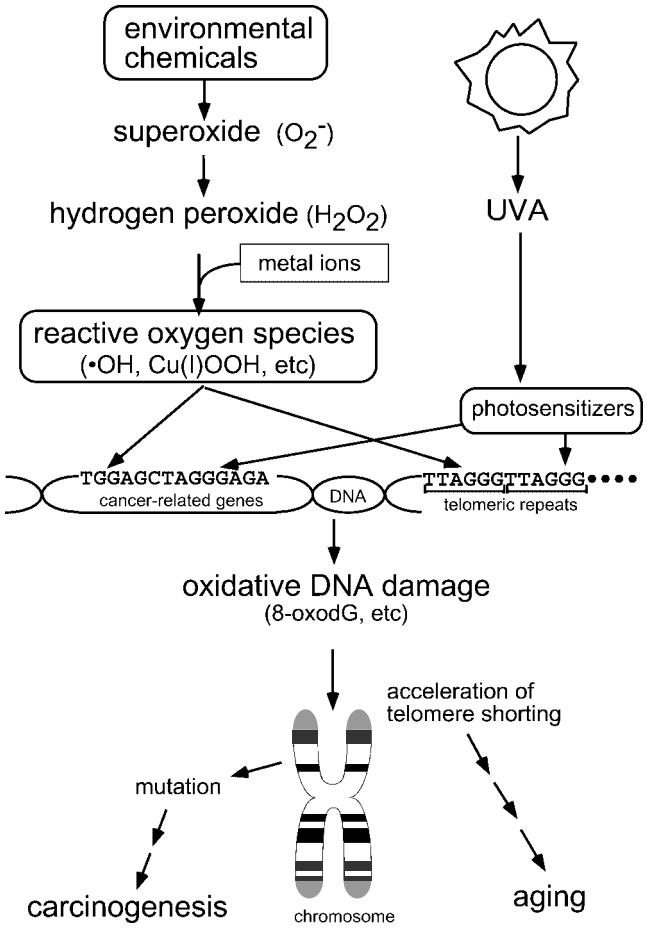

Fig. 4 Mechanism of DNA damage caused by ROS generated from environmental factors and its role in carcinogenesis and aging.

formation can cause DNA misreplication that may lead to mutation such as G•C→T•A transversion, and carcinogenesis (36, 50).

Oxidative stress may function as a common trigger for activation of the senescence program. The GGG-specific DNA damage by oxidative stress in telomere sequence may play an important role in increasing of the rate of telomere shortening (42, 51). UVA radiation plus riboflavin induced 8-oxodG formation specifically at the GGG sequence in telomere through electron transfer. Human 8-oxodG-DNA glycosylase introduces a chain break in a double-stranded oligonucleotide specifically at an 8-oxodG residue (52). Therefore, the formation of 8-oxodG at the GGG triplet in telemore sequence induced by oxidative stress could participate in acceleration of telomere shortening. Telomeres shortening might impact the regenerative capacity of human tissues during aging and chronic diseases (53). These results require further study to clarify whether telomere dysfunction contributes to aging and chronic diseases. Finally, it is concluded that sequence-specific oxidative damage to DNA plays important roles in not only carcinogenesis but also aging (Fig. 4).

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