Oxidative DNA Damage by Vitamin A and Its Derivative via Superoxide Generation*

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Recent intervention studies revealed that β-carotene supplement to smokers resulted in a higher incidence of lung cancer. However, the causal mechanisms remain to be clarified. We reported here that vitamin A (retinol) and its derivative (retinal) caused cellular DNA cleavage detected by pulsed field gel electrophoresis. Retinol and retinal significantly induced 8-oxo-7,8-dihydro-2′-deoxyguanosine formation in HL-60 cells but not in H2O2-resistant HP100 cells, suggesting the involvement of H2O2 in cellular DNA damage. Experiments using 32P-labeled isolated DNA demonstrated that retinol and retinal caused Cu(II)-mediated DNA damage, which was inhibited by catalase. UV-visible spectroscopic and electron spin resonance-trapping studies revealed the generation of superoxide and carbon-centered radicals, although the yield of carbon-centered radicals was not necessarily related to the intensity of DNA damage. These findings suggest that superoxide generated by autoxidation of retinoids was dismutated to H2O2, which was responsible for DNA damage in the presence of endogenous metals. Retinol and retinal have prooxidant abilities, which might lead to carcinogenesis of the supplements of β-carotene.

Many studies have addressed the role of antioxidant vitamins A, C, and E in protection against cancers and cardiovascular diseases (1). It has been suggested that the antioxidant potency of vitamin A and β-carotene may scavenge oxygen radicals and protect against cancer occurrence (1–3). The Alpha-tocopherol, Beta-carotene Cancer Prevention (ATBC) study group (4) and the Beta-carotene and Retinol Efficacy Trial (CARET) (5) supplied study group (4) and the Beta-carotene and Retinol Efficacy Trial (CARET) (5) supplied the chemicals in cigarette smoke and the resultant inflammatory response in the lung (6). The ATBC researchers suspected that β-carotene acts as a promoter of pre-existing latent lung cancers in smokers (7). However, attempts to use retinoids and cartenoids for cancer chemoprevention and therapy are ongoing (8–11). Therefore, the causal mechanisms should be elucidated to establish safe approaches in cancer chemoprevention.

Every antioxidant, including vitamin antioxidants, is in fact a redox (reduction-oxidation) agent, protecting against free radicals in some circumstances and promoting free radical generation in others (12). Several studies have revealed prooxidant effects of vitamin E (13, 14) and vitamin C (15–17) under certain circumstances. Other antioxidants such as N-acetylcysteine (18) and quercetin (19, 20) can cause oxidative damage to cellular and isolated DNA. β-Carotene may also act as both an antioxidant and a prooxidant under various oxygen partial pressures (21–22). Although vitamin A is a good acceptor and donor of electrons in chemical reactions, its properties appear to be very carefully protected by retinol-binding proteins and other endogenous antioxidants in vivo (24). However, pharmacological amounts of the supplements above physiological amounts may perturb key physiological processes.

We report here that low concentrations of vitamin A (retinol) and its derivative (retinal; vitamin A aldehyde) caused cellular DNA cleavage. Furthermore, induction of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) by retinol and retinal was investigated in HL-60 cells and its H2O2-resistant clone HP100 cells. To clarify the mechanism of DNA damage, we performed experiments using 32P-labeled DNA isolated from the human p53 tumor suppressor gene and c-Ha-ras-1 protooncogene. In addition, the time course of 8-oxodG formation was measured by HPLC-EC, and the reactive species was also detected using UV-visible and ESR spectroscopies.

**EXPERIMENTAL PROCEDURES**

Materials—Restriction enzymes (SmaI, EcoRI, Styl, and Apal), calf intestine phosphatase, and proteinase K were purchased from Roche Molecular Biochemicals. Restriction enzymes (HindIII, AvaI, and PstI) and T4 polynucleotide kinase were purchased from New England Biolabs. [γ-32P]ATP (222 TBq/mmol) was from NEN Life Science Products. All-trans retinol (vitamin A) was purchased from Fluka Chemika-Biochemika (Tokyo, Japan). All-trans retinol (vitamin A aldehyde), methionyl, RNase A, bacterial alkaline phosphatase, superoxide dismutase (SOD, 3000 units/mg from bovine erythrocytes), and catalase (45,000 units/mg from bovine liver) were from Sigma. α-(4-Pyridyl-1-oxide)-N-tetra- butyltinonitrile (PBN) was from Aldrich. DNA extractor (lysis buffer model 340A) was from Applied Biosystems. Nuclease P1 was from Yamasa Shoyu Co. (Chiba, Japan). Acrylamide, bisacrylamide, and piperidine were from Wako Chemicals Co. (Osaka, Japan). Diethylenetriamine-N,N,N′,N′,N″,N″-pentaacetic acid (DTPA) and bathocuproinedisulfonic acid were from Dojin Chemicals Co. (Kumamoto, Japan). Ethanol, n-mannitol, and sodium formate were from Nakalai Tesque Inc. (Kyoto, Japan). Ethanol solutions of retinol and retinal were made freshly each time.

† Detection of Cellular DNA Damage by Pulsed Field Gel Electrophore-
s—HL-60 cells were grown in RPMI 1640 supplemented with 6% fetal bovine serum at 37 °C under 5% CO2 in a humidified atmosphere. Then the cells were washed, resuspended in 10 ml of RPMI 1640 containing 6% fetal bovine serum (1×106 cells/ml), and incubated with retinoid, which was dissolved in ethanol (final concentration of ethanol, 0.05%) at 37 °C for the indicated duration. Control conditions also contained 0.05% ethanol. After incubation, the medium was removed, and the cells were washed twice with phosphate-buffered saline and resuspended in phosphate-buffered saline. The cell suspension was solidified with agarose, followed by treatment with proteinase K according to the method described previously (25). Electrophoresis was performed in 0.5 × TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA (pH 8.0)) by a CHEF-Mapper pulsed field electrophoresis system (Bio-Rad) at 200 V at 14 °C. Switch time was 60 s for 15 h followed by 90 s switch time for 9 h. The DNA in the gel was visualized using ethidium bromide.

Analysis of 8-oxodG Formation in HL-60 and HP100 Cells Treated with Retinoids—HP100 cells have been derived from HL-60 cells by repeated exposure to H2O2 followed by outgrowth of viable cells and are approximately 340-fold more resistant to H2O2 than HL-60 cells (26, 27). HL-60 and HP100 cells (1×106 cells/ml) were incubated with retinoids in 5 ml of RPMI 1640 supplemented with 6% fetal bovine serum at 37 °C. After the incubation, the medium was removed, and the cells were washed twice with phosphate-buffered saline. The cells were suspended in 0.05 mg/ml RNase A, 0.5 mg/ml proteinase K, and 500 μl of DMSO and digested for 60 min at 60 °C. After ethanol precipitation, DNA was digested to nucleosides with proteinase P2 and bacterial alkaline phosphatase and analyzed by HPLC-ECD, as described previously (28, 29).

Preparation of 32P 5'-end-labeled DNA Fragments—DNA fragments were obtained from the human p53 tumor suppressor gene.2 Fragments from the p53 gene containing exons were prepared as described previously (31). The 5'-end-labeled 470-bp fragment (HindIII* 13038-EcoRI* 13507) was obtained by dephosphorylation with calf intestine phosphatase and rephosphorylation with [γ-32P]ATP and T4 polynucleotide kinase (the asterisk indicates 32P-labeled). The 470-bp fragment was further digested with SstI to obtain the singly labeled 118-bp fragment (HindIII* 13038-Styl 13155), as described previously (31). A DNA fragment was also obtained from the human c-Ha-ras-1 protooncogene (32). A DNA fragment was prepared from plasmid pblCN, which carries a 6.6-kilobase BamHI chromosomal DNA segment containing the c-Ha-ras-1 gene, and the singly labeled 337-bp fragment (Part 2345-Aval* 2681) was obtained according to the method described previously (33). Nucleotide numbering starts with the BamHI site (32).

Detection of Damage to 32P 5'-end-labeled DNA Fragments by Retinoids—The standard reaction mixture (in a microtube, 1.5 ml) contained retinoid, CuCl2 (or FeCl3, FeSO4, MnCl2), 32P 5'-end-labeled DNA fragments and sonicated calf thymus DNA in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. DTPA was added to remove metal ions, which may have been contained in the phosphate buffer. After incubation at 37 °C for the indicated durations, the DNA fragments were heated at 90 °C in 1 X piperidine for 20 min where indicated.

The preferred cleavage sites were determined by direct comparison of the positions of the oligonucleotides with those produced by the chemical reactions of the Maxam-Gilbert procedure (34) using a DNA-sequencing system (LKB 2222 UltraScan XL). The relative amounts of oligonucleotides from the treated DNA fragments were measured with a laser densitometer (LKB 2222 UltraScan XL).

Analysis of 8-oxodG Formation in Calf Thymus DNA by Retinoids—Calf thymus DNA fragments were incubated with retinoid in the presence of CuCl2 for the indicated duration at 37 °C. DNA fragments were denatured at 90 °C for 5 min and quickly chilled before incubation, where indicated. After ethanol precipitation, DNA was digested to the nucleosides using nuclease P1 and calf intestine phosphatase and analyzed by HPLC-ECD, as described previously (28).

Detection of O2•− Derived during the Autoxidation of Retinoids—To detect O2•− generation, cytochrome c was added to the reaction mixture, which contained retinoid in 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. A maximum absorption at 550 nm due to ferrocyanochrome c formed by ferrocyanochrome c reduction was measured with a spectrophotometer at an incubation temperature of 37 °C for the indicated duration. A low estimate of O2•− was calculated by subtracting absorbance with SOD from that without SOD at 550 nm (ε = 211×103 M−1 cm−1).

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Fig. 2. Formation of 8-oxodG in cells treated with retinoids. Cells were treated with retinoids as described in the legend to Fig. 1. For detection of 8-oxodG, DNA was extracted and treated as described under “Experimental Procedures.” After ethanol precipitation, DNA was enzymatically digested to the nucleosides and analyzed by HPLC-ECD. ○, retinol; ●, retinal. Means (circles) and S.D. (bars) are the values of 4–6 individual experiments. *, p < 0.05, and **, p < 0.01, indicate significant differences compared with the control group; ##, p < 0.01 indicates a significant difference compared with the same concentration between retinol and retinal using Student’s t test.

Fig. 3. Autoradiogram of 32P-labeled DNA fragments incubated with retinoids in the presence of Cu(II). The reaction mixture contained the 32P-labeled 337-bp DNA fragment, 20 μM per DNA base of sonicated calf thymus DNA, 200 μM retinol or retinal, 20 μM CuCl2 in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. The mixture was incubated at 37 °C for the indicated duration, followed by piperidine treatment. The autoradiogram was obtained by exposing an x-ray film to the gel.

Fig. 4. Effects of scavengers and bathocuproine on DNA damage induced by retinol in the presence of Cu(II). The reaction mixture contained the 32P-labeled 337-bp DNA fragment, 20 μM per DNA base of sonicated calf thymus DNA, 200 μM retinol, 20 μM CuCl2, and scavenger or bathocuproine in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. The mixture was incubated at 37 °C for 3 h, followed by piperidine treatment, and the DNA fragments were analyzed as described in the legend to Fig. 3. The concentration of the scavenger was as follows; 0.1 M mannitol, 0.1 M sodium formate, 0.1 M methional, 5% (v/v) dimethyl sulfoxide (DMSO), 30 units and 60 units of catalase, 30 units of SOD, 50 μM bathocuproine.

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ated DNA damage induced by retinol, suggesting that H2O2 and Cu(I) were required for the DNA damage. The similar effects of scavengers were observed in the case of retinal-induced DNA damage.

Site Specificity of DNA Damage by Retinoids—To examine the DNA damage site, 32P-5-end-labeled DNA fragments treated with retinol plus Cu(II) followed by piperidine treatment were electrophoresed. An autoradiogram was obtained and scanned with a laser densitometer to measure the relative intensity of DNA damage in the human p53 gene and the c-Ha-ras-1 protooncogene as shown in Fig. 5. Retinol and Cu(II) strongly generated piperidine-labile sites at cytosine and thymine residues, especially at the cytosine residues in CC sequences of double-stranded DNA from the p53 tumor suppressor gene (Fig. 5A). Retinol caused Cu(II)-dependent DNA cleavage at thymine residues in fragments from the c-Ha-ras-1 protooncogene (Fig. 5B). Also, the similar patterns were obtained with retinal (data not shown).

Formation of 8-oxodG in Calf Thymus DNA by Retinoids in the Presence of Cu(II)—We measured the time course of 8-oxodG formation in calf thymus DNA incubated with retinoids in the presence of Cu(II) (Fig. 6). Within 2 h, the content of 8-oxodG formation by retinol was higher than that by retinal. No difference between retinol and retinal in 8-oxodG contents was observed at 3 h. After a 6-h incubation, 8-oxodG formation by retinol was higher than that by retinal.

Generation of O2•− Derived from the Autoxidation of Retinoids—Using the cytochrome c reduction method, O2•− generated during the autoxidation of retinoids was detected. Within a 2-h incubation, the O2•− yield by retinol was higher than that by retinal. The yield of O2•− was very similar between retinol and retinal at 3 h. After a 6-h incubation, the O2•− yield by retinol was higher than that by retinol (Fig. 7).

Generation of Radicals Derived from Retinoids—ESR spectroscopic measurement with a trapping agent POBN detected retinoid-derived radicals with six-line signals (Fig. 8). The hyperfine splitting constants of the radical derived were aN = 1.56 mT, aH = 0.27 mT for retinol, and aN = 1.56 mT, aH = 0.25 mT for retinal. These radicals were both assigned as carbon-centered radicals by reference to the reported hyperfine splitting constants (36). Retinol-derived radicals could be detected immediately after mixture, whereas retinal-derived radicals were detected only after the longer incubation. After a 6-h incubation, the radical signals from retinol were much larger than those from retinal. Under anaerobic conditions, the POBN-trapping radicals were much less accumulated (data not shown).
DISCUSSION

We report here that both retinol and retinal caused oxidative damage to cellular and isolated DNA. Retinoids significantly induced 8-oxodG formation in HL-60 cells but did not significantly increase 8-oxodG in H2O2-resistant HP100 cells. It is suggested that the generation of H2O2 plays a critical role in oxidative DNA damage by retinol and retinal. When the isolated DNA was used, the existence of Cu(II) was required for the DNA damage. Copper exists in the nucleus and is closely associated with chromosomes and DNA bases in vivo (37). In cell-free systems, catalase and bathocuproine inhibited Cu(II)-mediated DNA damage, suggesting that H2O2 reacts with Cu(I) to produce active species causing DNA damage. DNA-bound Cu(II) can undergo Cu(II)/Cu(I) redox cycling; also, O2 is reduced to O2−, which is dismutated to H2O2 resulting in the DNA-Cu(I)-H2O2 complex (33). Typical free -OH scavengers showed little or no inhibitory effect on the DNA damage. Therefore, it is considered that the DNA damage is caused by a reactive oxygen species such as Cu(II)OOH rather than free -OH. Site-specific DNA cleavage supports this hypothesis, because it is known that free -OH causes DNA damage without site specificity (38). Thus, it is speculated that H2O2 reacts with endogenous metals to form a metal-oxygen complex causing oxidative DNA damage in vivo.

ESR spectroscopic studies using a trapping agent POBN have demonstrated retinol- and retinal-derived radicals with six-line signals, assigned as carbon-centered radicals. Retinoic acid was shown to be oxidized to 5,6-epoxyretinoic acid via formation of carbon-centered radicals (aH = 0.27 mT, aN = 1.55 mT) trapped by POBN (39). In addition, Tesoriere et al. (40) suggest that retinol reacts with radical addition to the cyclohexenyl ring and was further oxidized to 5,6-epoxyretinol. These carbon-centered radicals from retinol and retinal do not appear to be the main species to react with DNA, because neither retinol nor retinal caused DNA damage even though it was detected using the ESR spin-trapping method when Cu(II) was omitted. Using the cytochrome c reduction method, generation of O2− derived from the autoxidation of retinoids was detected. Within a 2-h incubation, higher generation of O2− from retinol was observed than that from retinal, and the yield became very similar at 3-h. After a 6-h incubation, O2− from retinal was higher than that from retinol. The generation of

![Fig. 5](image-url)

**Fig. 5.** Site specificity of DNA cleavage induced by retinol in the presence of Cu(II). The reaction mixture contained the 32P-labeled 118-bp DNA fragment (HindIII* 13038-Sty1 13155) obtained from the p53 tumor suppressor gene (A) or 337-bp fragments from the c-Ha-ras-1 protooncogene (B). 20 µM per DNA base of sonicated calf thymus DNA, 100 µM retinol, 20 µM CuCl2 in 200 µl of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 µM DTPA. The mixture was incubated at 37 °C for 18 h, followed by piperidine treatment. The horizontal axes show the nucleotide number of the human p53 tumor suppressor gene or the c-Ha-ras-1 protooncogene starting with the BamHI site (32).

![Fig. 6](image-url)

**Fig. 6.** Cu(II)-mediated formation of 8-oxodG in calf thymus DNA by retinoids. Calf thymus DNA fragments (20 µM per DNA base) were incubated with 200 µM retinoid and 20 µM CuCl2 in 400 µl of 4 mM sodium phosphate buffer (pH 7.8) containing 1 µM DTPA for the indicated durations at 37 °C. After ethanol precipitation, DNA was enzymatically digested to nucleosides and analyzed by HPLC-ECD. The symbols indicate the following: open, retinol; closed, retinal; circles, native DNA; squares, denatured DNA.

![Fig. 7](image-url)

**Fig. 7.** Generation of O2− derived from the autoxidation of retinoids. To detect O2− generation, 100 µM cytochrome c was added to the reaction mixture with 200 µM retinoid in 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 µM DTPA. The reaction mixture was incubated at 37 °C for the indicated duration. The content of O2− at a low estimate was calculated by subtracting absorbance with SOD from that without SOD at 550 nm (ε = 21.1 × 10^3 M^−1 cm^−1) measured at 37 °C using a UV-visible spectrophotometer. Open circle, retinol; closed circle, retinal.
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was significantly correlated with 8-oxodG formation. In other circumstances, it is suggested that antioxidants in some circumstances but also act as prooxidants. Palozza et al. (21) report that retinol and retinal (B) trapped using 150 mM POBN in 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA was detected by ESR spectroscopy (JEOl model JES-TE100) with 100-kHz field modulation. Reaction mixtures were incubated for the indicated durations at 37 °C and then taken up in a flat cell, and the spectra were measured at room temperature. Spectra were recorded with a microwave power of 8 mW, a modulation amplitude of 0.1 mT, and a receiver gain of 200.

\[ \text{O}_2^- \text{ was significantly correlated with 8-oxodG formation (} r = 0.9824, p < 0.0001). \text{ Therefore, it is concluded that the DNA damage observed here occurs through the generation of O}_2^- \text{ rather than carbon-centered radicals, although the radicals may participate in the generation of O}_2^- \].

Halliwell (41) indicated that so-called “antioxidants” act as antioxidants in some circumstances but also act as prooxidants in other circumstances. Palozza et al. (21) report that β-carotene at high oxygen partial pressure lost its antioxidant activity in normal cells and exhibited a prooxidant effect in tumor cells. Caffeic acid, which has antioxidant properties and carcinogenicity, inhibited oxidative DNA damage, whereas caffeic acid by itself caused oxidative DNA damage at certain concentrations (42). Such antioxidant compounds like caffeic acid may have the dual function of carcinogenic and anticarcinogenic potentials. If antioxidants have a prooxidant property, they may have carcinogenic effects. Similarly, it was reported, using a simplified in vitro model, that an antioxidant vitamin E (α-tocopherol) can act as a potent oxidative DNA-damaging agent in the presence of Cu(II) (14). Virtually all putative chemopreventive antioxidants may have potential carcinogenicity (18, 20).

It is noteworthy to find that the addition of such low doses of retinoids can induce cellular DNA damage. β-Carotene is converted to two molecules of retinal principally by central cleavage. Retinal is further oxidized to retinoic acid or reduced to retinol. We confirmed using isolated DNA that other derivatives of vitamin A such as retinoic acid and β-ionone and β-carotene itself induced slight DNA damage, including 8-oxodG in the presence of Cu(II), but not efficiently in comparison with retinol and retinal (data not shown). If excessive intake or supplements of vitamin A and β-carotene saturate binding protein, free compounds may have cytotoxicity. This has been suggested by several studies (43-44). Excess amounts of vitamin A increased chromosomal aberration in a lymphocyte culture system (43). Rats treated with retinol had increased incidence of pheochromocytomas in a dose-related manner (44). The supplements of β-carotene and retinol resulted in higher incidences of lung cancer in the intervention groups (4, 5).

It is known that reactive oxygen species are related to tumor-promoting potencies (45, 46). Reid and Loeb (30) indicated that oxidative stress caused tandem double CC → TT mutations. It is very interesting that retinoids strongly generated piperidine-sites at the CC sequences in the p53 tumor suppressor gene observed in our study with 32P-labeled DNA fragment. On the basis of the finding that excessive retinol and retinal induced oxidative DNA damage via O2− generation, it is suggested that the oxidative DNA damage may be responsible for initiation and/or tumor promotion/progression in multistage carcinogenesis. Retinol and retinal, β-carotene metabolites, have a stronger ability to induce DNA damage and to generate O2− than β-carotene itself. Therefore, it is suggested that both retinol and retinal play important roles in carcinogenesis in the intervention studies using excess amounts of β-carotene. Further studies will be required for evaluation of safety and efficacy before recommending use of retinoid supplements for cancer chemoprevention.

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