Radiation-induced Double-strand Modification in Calf Thymus DNA in the Presence of 1,2-Dihydroxy-9,10-anthraquinone and Its Cu(II) Complex

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When cells or DNA is exposed to ionizing radiation, the radicals produced in the irradiated sample will modify the base-pair region of the double strands. Effects of 1,2-dihydroxy-9,10-anthraquinone (DHA) and its Cu(II) complex on the radiation-induced modification of double-strands in calf thymus DNA were studied using ethidium bromide as a fluorescent probe. Our results show that the Cu(II)-DHA complex is more efficient in modifying the base-pair region in double-stranded DNA compared to free DHA. — Environ Health Perspect 105(Suppl 6):1459-1462 (1997)

Key words: DNA double-strand modification, calf-thymus DNA, ethidium bromide, anthra-cyclines, 1,2-dihydroxy-9,10-anthraquinone, Cu(II)-DHA (copper complex of 1,2-dihydroxy-9,10-anthraquinone)

Introduction

Anthracine antibiotic drugs (1-3) show cardiotoxicity and cytotoxicity that may be reduced by complexation with suitable metals (4-6). To understand the mechanism of cytotoxicity we undertook studies using 1,2-dihydroxy-9,10-anthraquinone (DHA) as the model compound. The compound catalyzes the formation of superoxide radical anion through an enzymatic reduction pathway. However, its formation through the same reductive pathway is reduced when the compound is complexed with metal ions such as Cu(II), Fe(III), and Ni(II) (7,8). Because of the presence of the quinone moiety, the molecules could be used as suitable radiosensitzers (9) in radiotherapy.

A literature survey shows little evidence that anthraclines are effective radiosensitzing agents during radiotherapy. It is well established (10,11) that the response of living cells to ionizing radiation involves various types of DNA damage in aerated and deaerated conditions (12). Of the DNA constituents, pyrimidine and purine bases are more easily attacked than the aliphatic deoxyribose backbone. Radicals (13-16) localized on bases can interact with ribose units to produce ribose radicals, which then leads to strand break (17). The actual isolation and identification of bases damaged in the DNA chain in irradiated solutions light in advanced understanding of various forms of radiation-induced DNA damage. It should be noted that the radiation chemistry of free bases may be quite different from that of bases covalently linked to the phosphate-sugar backbone of the DNA chain (18). However, prior knowledge of the interaction of free bases with radiation is certainly an advantage. The radiation chemical reactions of nucleic acid bases, e.g., uracil and thymine, have been studied in detail (19-21). Also, we have studied the effects of DHA and its metal complexes on the radiosensitivity of uracil and thymine and have shown that DHA and metal complexes are good radiosensitizing agents (7,22). Though the results obtained in isolated base molecules could be extrapolated to nuclear DNA with some limitations, it is more relevant to study macromolecular DNA.

Ethidium bromide (ETB) forms a highly fluorescent complex with native DNA by intercalation between base pairs (23). As stated earlier (13-17), radicals produced in irradiated solution react at the base site of the DNA and destroy the hydrogen bonds, which leads to the breakage of the strands of the DNA. As a result, the fluorescent intensity of the ETB-DNA complex would decrease, which would give a measure of the change in the base pair region due to irradiation. In this investigation we studied the sensitizing effect of DHA and its Cu(II)-complex on radiation-induced strand scission and modification of the base pair site of double-stranded calf thymus DNA in dilute aqueous solution. In an earlier investigation (24) high doses were used to study the effects of radiation on DNA. However, for more radiobiological relevance, we used low doses to study such effects.

This study shows that a quinone DHA and its Cu(II) complex are able to enhance the radiation-induced double-strand modification in calf thymus.

Materials and Methods

1,2-Dihydroxy-9,10-anthraquinone (DHA) (British Drug House, U.K.) was purified by recrystallization from an alcohol-water mixture (8). The concentration of solutions was 10^{-6} M. The compound DHA is sensitive to both light and air. Hence solutions were prepared just before use and were stored in the dark until needed. The Cu(II) complex of DHA was prepared by the method described earlier (8). Phosphate buffer, pH 7.0, was used during the experiments. Calf thymus DNA was purchased from Sigma Chemical Company (St. Louis, MO). It was dissolved in phosphate buffer which was stirred and stored overnight. To calculate the DNA concentration, an absorption coefficient of 13,200 M^{-1}cm^{-1} was used at 260 nm. ETB was purchased from Sisco Research Laboratories (Bombay, India).

Calf thymus DNA was mixed with sensitizer molecules DHA and Cu(II)-DHA, and subjected to γ-irradiation with 60Co source. Hypoxic conditions were achieved by passing pure nitrous oxide gas through the solutions before performing the radioysis experiments. The dose rate (4.28 Gy/min) was monitored using a Fricke dosimeter.
Absorption spectra were recorded on a Shimadzu UV-2101 spectrophotometer (Shimadzu, Japan). Fluorescence was measured on a Hitachi 4010 fluorescence spectrophotometer (Hitachi, Japan). In this experiment change in double-stranded DNA and modification of the base-pair region due to γ-irradiation was monitored using ETB as a fluorescent probe that binds strongly at the base-pair site of the double-stranded DNA forming a highly fluorescent complex.

It was reported earlier (24) that the fluorescent intensity of the ETB–DNA complex is dependent on the concentration of ETB taken for binding. To get maximum binding we have used an ETB concentration of 87 M that is approximately 22-fold higher than the DNA concentration so that it saturates the binding with DNA.

**Results**

A neutral solution of ETB shows a strong absorption band with \( \gamma_{\text{max}} \) at 470 nm; but when complexed with DNA, its absorption maxima shifts to 510 nm. When excited at 510 nm, the dye shows a small fluorescence emission at its emission \( \gamma_{\text{max}} \) of approximately 590 nm. However, when it is complexed with double-stranded DNA, the emission intensity is greatly increased. Again, when DNA solution irradiated with γ-rays is allowed to bind with ETB, its fluorescence emission intensity decreases when compared to that of the unirradiated control.

The fluorescent intensity of the ETB–DNA complex formed with nonirradiated and irradiated DNA measured at 590 nm was corrected by subtracting the fluorescent intensity of free ETB at the same wavelength.

Heat-denatured DNA also gives appreciable fluorescence with ETB (24). But if the intramolecular hydrogen bonding in single-stranded DNA destabilizes, there will be negligible increase in fluorescence due to ETB binding. In fact, Morgan and Pulleyblank (23) reported that at pH 12 where intramolecular hydrogen bonding in single-stranded DNA destabilizes, double-stranded DNA in the presence of single-stranded DNA could be estimated by measuring the fluorescence of the ETB–DNA complex. During radiation the OH· also destabilizes intramolecular hydrogen bonding in single-stranded DNA, with base release and base modification. As a result, under our experimental conditions the fluorescence observed with ETB in the irradiated DNA sample might be due to binding of ETB with double-stranded DNA at the base-pair region only. To verify this, we measured the fluorescence of the ETB complex with irradiated DNA at pH 12 and noted that fluorescence due to the complex formed between ETB and irradiated DNA is identical with that observed in our method. As a result, we can assume that the fluorescence observed with ETB in irradiated DNA is a measure of double-stranded DNA that is not modified at the base-pair site. For convenience we have expressed the results in terms of percentage of double-stranded DNA with absorbed dose, where fluorescence increase in unirradiated control DNA is taken as 100% of double-stranded DNA.

DNA solution (4 μM in base pair) in phosphate buffer was irradiated with \(^{60}\)Co \( \gamma \)-rays at different absorbed doses in the presence and in the absence of 1-μM sensitizer molecules in \( \text{N}_2\text{O} \)-saturated conditions. It was observed in \( \text{N}_2\text{O} \)-saturated medium that radiation-induced modifications in calf thymus DNA are increased in the presence of the sensitizer molecules DNA and Cu(II)–DHA. Figure 1 shows the plot for the amount of double-stranded DNA remaining unchanged against doses both in the absence and in the presence of the sensitizer molecules. The dose–effect curve for modification of double-stranded DNA is exponential with dose; \( D_{37} \) is calculated from the initial slope of the curves. The percentage loss of double-stranded DNA due to radiation was calculated using \( [\text{DNA}]_0 / D_{37} \), where \( [\text{DNA}]_0 \) is the initial concentration of DNA solution that is irradiated. The yield value \( [\text{DNA}]_0 / D_{37} \) is independent of DNA concentration in the range of 2 to 20 μM in base pair in this investigation.

The ability of the sensitizer molecules to enhance radiation-induced modification of double-stranded DNA is expressed in terms of enhancement ratio—the ratio of the slope of the dose–effect curve obtained in the presence of sensitizer to that in the absence of sensitizer. Results are shown in Table 1. The enhancement ratio was higher in the Cu(II)–DHA complex compared to free DHA.

**Discussion**

It is well established that cell death by radiation is the result of physicochemical alterations directly or indirectly produced by radiation on cellular DNA. However, it is difficult to reconcile that observation with the finding that the inactivation of DNA irradiated extracellularly is not comparable to that observed when DNA is irradiated intracellularly. A series of studies on the molecular mechanisms of radiation-induced DNA strand breakage offers a clear description of the radiation chemistry of a macromolecular structure. In these studies the main event leading to strand breakage in DNA irradiated in dilute solution is the formation of a C-4' radical in deoxyribose (25). The C-4' radical is produced through hydrogen abstraction by OH· in the absence of O₂, this leads to a heterolytic cleavage of one of the sugar–phosphate bonds at C-5' or C-3' leading to DNA chain break.

Studies on *Escherichia coli* DNA with \(^{14}\)CH₃ labeled on the thymine moiety indicates that irradiation with \(^{60}\)Co rays produced free thymine and its radiolyzed products (18) in addition to the formation of a modified DNA chain. It was suggested that the release of the thymine fragment (26) involves an OH· attack at C-4' and C-1'. Hydrolysis (18) of the modified DNA chain yielded \(^{14}\)CH₃-labeled thymine, 5,6-dihydroxythymine, cis- and trans-5,6-dihydroxy-5,6-dihydrothymine, etc., indicating that radiation damages the constituent bases exposed to it. Interaction

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**Table 1.** Radiation-induced modification of double-stranded DNA in the presence of DNA and its Cu(II) complex under \( \text{N}_2\text{O} \)-saturated conditions.

| Sensitizer | % Double-strand modification, Gy⁻¹ | Enhancement ratio |
|------------|-----------------------------------|------------------|
| —          | 2.00                              | —                |
| DHA        | 3.01                              | 1.51             |
| Cu(DHA)    | 5.28                              | 2.64             |

Maximum error, 10%; DNA, 4; sensitizer, 1 μM.
of radiation with DNA can occur in essentially two major ways, leading to either breakdown of the sugar-phosphate backbone or modification of the individual DNA bases. Both can result in breakdown of strands in DNA and lead to modification of the base pair region that holds the strands together.

In the presence of molecules that sensitize radiation-induced DNA damage, there occurs either a direct or an indirect mechanism that enhances such damage. Reports are available on the kinetics and reaction mechanisms of some cupric complexes of 1,10-phenanthroline (OP) 5-NO₂-1,10-phenanthroline (5-NO₂-OP), or 2,2-bipyridine (bpy) with O₂⁻, H₂O₂, and O₂ in the presence of calf thymus DNA. They indicate that these complexes are effective cleaving DNA (27). Results indicate that the cuprous complex of these ligands formed during the course of reaction binds to DNA and that subsequent oxidation by H₂O₂ causes site-specific damage due to formation of the OH· at the binding site (27–29).

We found in the present investigation that radiation-induced modification of the base-pair region in calf thymus DNA increases in the presence of DHA and its Cu(II) complex Na[Cu(DHA)₃]²⁺. It is well known that radiolysis of water leads to the formation of the following primary species in solution:

\[ H₂O → H⁺, OH⁻, e⁻_{aq}, H₂O₂, H₂, H₂O²⁻. \]

In the presence of N₂O the e⁻_{aq} formed are completely converted to OH⁻. As our experiments were carried out in a nitrous oxide-saturated solution, the damage inflicted on DNA was primarily due to OH⁻ attack. Studies with model pyrimidine nucleic acid bases indicate that in the case of uracil, thymine, and cytosine, the 5,6 positions of the ring are most susceptible to attack by OH⁻ (30,31). This results in the generation of a radical center either at C-5 or at C-6. If this radical center transfers an electron to the sensitizer molecule, there is every possibility that a carbocation will be generated either at C-5 or C-6. Once that happens the nucleic acid base becomes susceptible to attack by water molecules, leading to the formation of glycol or ring-cleaved products. However, the transfer of the electron from the radical center at C-5 or C-6 certainly depends on the redox couple of the quinone and its corresponding semiquinone.

\[ B + OH⁻ → BOH⁺ \]  \[ 1 \]

\[ BOH⁺ + Q → BOH⁺Q⁻ \]  \[ 2 \]

\[ BOH⁺ + H₂O → B(OH)₂ + H⁺. \]  \[ 3 \]

The presence of an electron acceptor quinone molecule (Q) such as DHA would for obvious reasons increase the possibility of modification of the nucleic acid bases without giving them much time to recover from the attack by OH⁻ which could have happened in their absence. This would therefore result in more deformation of the nucleic acid bases, leading to modification in the strands as more interactions are destroyed because of modification of the base pair. In the presence of the Cu(II)–DHA complex, it is expected that the possibility of electron transfer from the C-5 or C-6 radical center to the complex would be higher because of the presence of the metal in the complex. Electron transfer may occur either at the free quinone site or directly to the metal center. The electron accepted by the complex from the radical center can be more effectively delocalized in the complex because of the presence of the metal ion and three quinone molecules, thereby increasing the possibility that redox potential would increase in the case of the complex. So base damage in the presence of the complex is likely to increase.

\[ BOH⁺ + Cu⁴Q₃ → BOH⁺ + Cu⁴Q₅. \]  \[ 4 \]

Moreover, because of electron acceptance, the Cu(II)–DHA complex will eventually be converted to a Cu(I)-DHA complex that will react with H₂O₂ present in the system to regenerate Cu(II)–DHA, forming more OH⁻ at the same time (27–29).

\[ Cu⁴Q₃ + H₂O₂ → Cu⁴Q₃ + OH⁻ + OH⁻. \]  \[ 5 \]

Metal complexes of Cu(II) and Cu(I) show a tendency to bind to DNA (27) so in this case, if the complexes are DNA bound then the OH⁻ formed in Equation [5] will be present in the vicinity of the binding site thereby inflicting more site-specific base damage. Although Goldstein and Czapski (27) and Prutz (32) have suggested the formation of a discrete OH⁻ the vicinity of the reaction site, that formation is questioned by Johnson and Nazhat (33). They suggest that instead of an OH⁻, a species closely approximating OH co-ordinated with Cu⁰, and/or a Cu³⁺ species co-ordinated with OH⁻ is formed that react in a way similar to that of the OH⁻. However, it can be argued that whether the discrete OH⁻ or a Cu(II)-bound OH⁻ as proposed by Johnson and Nazhat is formed, it will react further with the base molecule in the DNA at the site of their OH⁻ formation. As a consequence it is likely that modification of DNA would increase in the presence of the metal complex and that this would be higher than might be expected with quinone.

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