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Iron Chelators Modulate the Production of DNA Strand Breaks and 8-hydroxy-2′-deoxyguanosine

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The interaction of chelators and reducing agents is of particular importance in understanding iron-associated pathology since catalytic iron undergoes cyclic reduction and oxidation *in vivo*. Therefore, we treated plasmid DNA with free or chelated Fe(III) in the presence of biological reductants, and simultaneously measured the number of single strand breaks (SSBs) and oxidative base modification (8-hydroxy-2′-deoxyguanosine; 8-OHdG) by quantitative gel electrophoresis and HPLC with electrochemical detection, respectively. Production of SSBs and 8-OHdG was linearly correlated suggesting that these two different lesions share a common chemical mechanism. The levels of both lesions were enhanced when Fe(III) was chelated to citrate or nitrilotriacetic acid. Reducing agents showed different potency in inducing DNA damage catalyzed by chelated iron (L-ascorbate > L-cysteine > H_2O_2). Chelation increased SSB formation by ~8-fold and 8-OHdG production by ~4-fold. The ratio of SSB/8-OHdG catalyzed by chelated iron, which is twice as high as by unchelated iron, indicates that chelation affects iron-catalyzed oxidative DNA damage in a specific way favoring strand breakage over base modification. Since iron is mostly chelated in biological systems, the production of genomic and mitochondrial DNA damage, particularly strand breaks, in diseases involving iron overload is likely to be higher than previously predicted from studies using unchelated iron.

Keywords: Iron, chelate, 8-hydroxy-2′-deoxyguanosine, DNA strand break, reactive oxygen species, carcinogenesis

Abbreviations: DSB, double strand break; Fe-NTA, ferric nitrilotriacetate; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; SSB, single strand break

INTRODUCTION

Iron overload has been associated with carcinogenesis in animal models and also in human diseases such as genetic hemochromatosis and asbestosis.[1–3] The role of iron in carcinogenesis has been associated with oxidative DNA damage including single and double strand breaks (SSBs and DSBs), base modifications and DNA–protein cross-links.[4] Excess iron feeding stimulates

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spontaneous lung tumor promotion in mice concurrently with elevated pulmonary DNA SSBs.[5] Iron also increases the levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a modified DNA base,[6,7] that induces G : C to T : A transversion during DNA replication.[8]

Serum transferrin maintains the levels of catalytic iron to practically nil in the serum of healthy humans.[9] However, saturated transferrin allows excess iron to circulate as low molecular weight chelates.[10] Serum catalytic iron in the severe cases of genetic hemochromatosis exists largely as complexes with citrate.[11] Therefore, it is suspected that ferric citrate-induced oxidative DNA damage plays a central role in the high incidence of primary hepatocellular carcinoma observed in genetic hemochromatosis patients.[1,12] This warranted the inclusion of citrate as a relevant chelator in the present study.

Another biologically relevant iron chelator is nitrilotriacetic acid (NTA) that forms a soluble iron chelate complex (Fe-NTA)[13] with high catalytic activity at neutral pH.[14] Intraperitoneal injection of Fe-NTA to rodents induces acute renal tubular necrosis and apoptosis revealing oxidative damage that eventually leads to a high incidence of renal cell carcinoma.[15–17] Single administration of Fe-NTA, but not Fe(III) alone, to rats increased 8-OHdG levels in the DNA of kidney.[18] The finding confirmed the participation of NTA in iron-mediated oxidative DNA damage. Further, we recently found that one of the critical genetic changes in Fe-NTA-induced renal carcinogenesis is hemizygous or homozygous deletion of $p15^{INK4B}/p16^{INK4A}$ tumor suppressor genes that code cyclin-dependent kinase inhibitors.[19] Genetic alteration of this kind might be induced by iron-mediated DNA strand breaks in the carcinogenic process. Therefore, NTA was also included in this study.

We have previously shown that iron chelates, including Fe-NTA and Fe-citrate can induce SSBs and DSBs in plasmid DNA *in vitro*.[20,21] However, due to the fragility of genomic DNA, DNA strand breakage resulting specifically from iron-mediated damage is difficult to measure. We previously proposed to estimate DNA strand breakage *in vivo* by the measurement of 8-OHdG.[22] Namely, we have shown that free Fe(III) in the presence of reductants produces DNA SSBs and 8-OHdG via common chemical pathways. In the present study we evaluated whether the association between SSBs and 8-OHdG would be maintained after iron chelation. Furthermore, we studied whether chelation could alter the ratio of strand breakage over base modification mediated by iron and hence the underlying mechanism of oxidative DNA damage.

We have shown that oxidative DNA damage mediated by iron or iron chelates requires the participation of reducing agents.[14] Several reductants have been associated with iron-mediated oxidative DNA damage *in vivo*. Hydrogen peroxide is believed to play a role in the production of oxidative DNA damage *in vivo* due to its cellular production[23,24] and its diffusion through membranes.[24] L-ascorbic acid (vitamin C) is a strong reductant and antioxidant which might nonetheless cause serious side effects when catalytic iron such as Fe(III)-citrate is present in the serum of genetic hemochromatosis patients.[25] L-cysteine has been a candidate reductant in the renal proximal tubular injury induced by Fe-NTA.[26] Therefore, we studied oxidative DNA damage catalyzed by iron chelates in the presence of H_2O_2, L-ascorbate or L-cysteine.

The interaction of chelators and reducing agents of biological relevance is of particular importance in understanding iron-associated pathology. The results of this study show that iron chelation enhance oxidative DNA damage and change the ratios of strand breaks to base modification.

MATERIALS AND METHODS

DNA and Chemicals

Double-stranded supercoiled plasmid pZ189 (5504 bp) DNA was prepared and purified as

previously described.[27] Ferric chloride and 30% (w/v) H_2O_2 were obtained from Mallinkrodt (Paris, KN). Nitrilotriacetic acid disodium salt, citric acid trisodium salt, L-ascorbic acid, L-cysteine, deferoxamine mesylate, 2′-deoxyguanosine and chelating resin, sodium form (50–100 mesh) were purchased from Sigma Chemical Co. (St. Louis, MO). 8-Hydroxy-2′-deoxyguanosine was a kind gift from Dr. Miral Dizdaroglu (NIST, Gaithersburg, MD). All the chemicals used were of analytical grade, and doubly distilled water was used throughout. To minimize metal contamination, plasmid DNA, phosphate buffer and NaCl solutions were pretreated with one-fifth volume of chelating resin and the pH was readjusted to 7.4. Solutions of Fe(III) chelates and reductants were prepared immediately before use.

DNA Treatment

DNA (10 μg) in 10 mM phosphate-buffered 137 mM NaCl, pH 7.4 was incubated at 37°C with 1–100 μM Fe(III) chelate (Fe-NTA or Fe-citrate) plus different concentrations (20 μM–10 mM) of either H_2O_2, L-ascorbate or L-cysteine in a total volume of 80 μl under ambient oxygen pressure. The reactions were stopped by the addition of 2 μl of 100 mM deferoxamine mesylate. Thereafter, the DNA solution was divided into two aliquots.

SSB Analysis

One-tenth of the DNA (1 μg) was analyzed by 0.8% agarose gel electrophoresis and densitometry as previously described.[20,21] Detection sensitivity in agarose gels stained with ethidium bromide was 1 ng of DNA. Intact plasmid DNA (supercoiled form, form I), DNA with one or more SSBs (relaxed form, form II) and DNA with DSBs (linear form, form III) migrated as previously reported,[28] with a characteristic pattern shown in the inset of Figure 1. A total number (N) of SSBs produced by each treatment was calculated using the equation $N = s\,(\ln 10)\,[2\text{-}\log(y)]$ where (y) is the

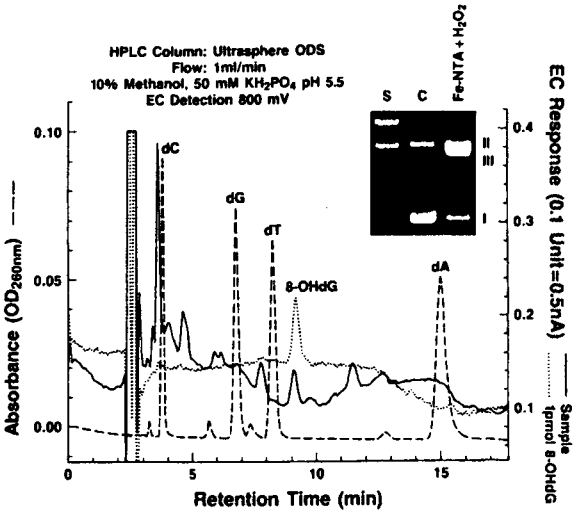

FIGURE 1 Production of 8-OHdG and SSBs after exposure of plasmid DNA to Fe(III)-NTA and H_2O_2. The figure shows a representative result of DNA treatment for 20 min at 37°C with a combination of reagents: 0.1 mM Fe(III), 0.4 mM NTA and 0.1 mM H_2O_2 (ratio = 1 : 4 : 1). HPLC profiles were obtained by simultaneous measurement of absorbance at 260 nm (dashed line) and electrochemical detection (solid line) of plasmid DNA enzymatically hydrolyzed as described in the text. The dotted line is the response obtained with 1 pmol of standard 8-OHdG. The inset shows strand break analysis of the same specimen by electrophoresis in 0.8% agarose gel after staining with ethidium bromide as described in the text. Lane S shows the migration of an HindIII digest of lambda phage DNA (from top, 9416, 6557 and 4361 bp, respectively) included as a size marker. Lane C corresponds to the migration of untreated control DNA, and the lane labeled as Fe-NTA + H_2O_2 shows the migration of DNA treated with these reagents at the concentration indicated above. The migration of DNA fragments corresponds to forms I, supercoiled; II, relaxed; and III, linear form plasmid DNA; respectively.

percentage of remaining supercoiled DNA and "s" is the number of plasmid molecules in the system.[21]

Measurement of 8-OHdG

The remaining reaction mixture (9 μg DNA) was extensively dialyzed against water, precipitated with ethanol and lyophilized. DNA samples were hydrolyzed to deoxyribonucleosides with a mixture of DNase I (200 mU/μg DNA), snake venom exonuclease (0.5 mU/μg DNA) and alkaline phosphatase (10 mU/μg DNA) (Boehringer Mannheim) and analyzed by HPLC with

simultaneous electrochemical and spectrophotometric detection as previously described.[22] The amount of 8-OHdG was obtained by comparing the electrochemical response of a sample with 1 pmol of standard 8-OHdG.

RESULTS AND DISCUSSION

Treatment of DNA as described above with 0.1 mM Fe-NTA and 0.1 mM H_2O_2 produced 0.8×10^{12} DNA SSBs (Figure 1, inset) as shown by a decrease in the supercoiled form (form I) and a concomitant increase in the bands corresponding to the nicked circular form (form II) and linear form (form III) of plasmid DNA. The same treatment produced 0.45×10^{12} 8-OHdG as indicated by area integration of the corresponding peak shown in Figure 1.

Fe(III) alone, or chelated in the absence of a reducing agents did not produce SSB nor increased the level of 8-OHdG (data not shown). When SSB production was plotted against the number of 8-OHdG produced by each treatment, a linear correlation was obtained for Fe-NTA or Fe-citrate in the presence of any of the reducing agent employed (Figure 2). The relationship between SSB and 8-OHdG production fitted regression lines: $Y = a + bX$ with correlation coefficients r (indicated in Table I) where X is the number of 8-OHdG and Y is the number of SSBs. The regression parameters "b" that correspond to the ratio SSB/8-OHdG are summarized in Table I. Simultaneous measurement in our plasmid DNA system demonstrated that DNA SSBs catalyzed by chelated Fe(III) are linearly associated with the generation of 8-OHdG.

The regression parameters "a" (y-intercept) were relatively small, ranging from −0.6 (Fe-NTA + L-ascorbate) to 0.24 (Fe + L-ascorbate) and are shown in Table I. The regression lines between SSBs and 8-OHdG which intercept each axis near the origin rule out a "threshold effect" in the production of either lesion. The strong correlation ($0.88 < n < 0.99$, see Table I) between SSBs

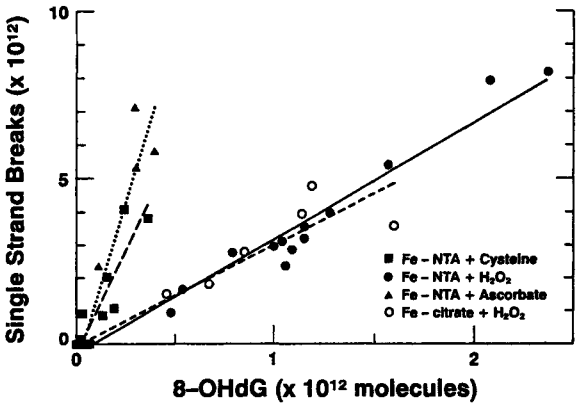

FIGURE 2 Linear regression analyses of SSB versus 8-OHdG produced after treatment of DNA with Fe(III)-NTA in the presence of H_2O_2, L-cysteine or L-ascorbate, and Fe(III)-citrate + H_2O_2. DNA was treated for a variable incubation time (1–60 min) at 37°C with 0.1 mM Fe(III) chelated to either NTA (0.4 mM, full symbols) or to citrate (0.4 mM, open circles) and 0.1 mM of L-ascorbate, L-cysteine or H_2O_2. The number of SSBs was calculated using the equation described in the text and the number of 8-OHdG was obtained in comparison to the electrochemical signal generated by an 8-OHdG standard.

and 8-OHdG produced by chelated iron agrees well with the correlation coefficients previously determined for free unchelated iron.[21] These findings confirm that SSBs and 8-OHdG share a common generation mechanism that is independent of the chelation state of iron.

The strong correlation between stand breakage and 8-OHdG formation mediated by iron in the presence of biologically relevant chelators and reducing agents suggest that SSBs *in vivo* could be estimated by the measurement of 8-OHdG. This could be useful in pathologic conditions where oxidative DNA damage mediated by iron has been either suspected or confirmed, such as in genetic hemochromatosis and asbestosis[29] since precise quantitative evaluation of SSBs in genomic DNA could be clinically important.

Oxidative DNA damage, either SSBs or 8-OHdG, was highly enhanced when Fe(III) was chelated (Table I). Furthermore, the data in Table I shows that chelation results in twice as much production of SSBs as that of 8-OHdG. Chelation could enhance the catalytic activity of iron by increasing the solubility of Fe(III) at neutral

TABLE I DNA damage by different iron chelate systems

Reductant	Ratio (SSB/8-OHdG)[a]			8-OHdG formation[b]			SSB formation[c]		
	Fe-NTA	Fe-citrate	Fe alone	Fe-NTA	Fe-citrate	Fe alone	Fe-NTA	Fe-citrate	Fe alone
H_2O_2	3.5 (−0.31) [22, 0.99]	3.1 (−0.07) [15, 0.96]	1.7 (0.03) [16, 0.98]	4.0	4.0	1.0[b]	14.1	12.4	1.7
L-cysteine	12.5 (−0.32) [17, 0.88]			4.1	—[d]	—	51	—	—
L-ascorbate	19.5 (−0.59) [14, 0.95]		9.4 (0.24) [17, 0.92]	3.9	—	1.0	77	—	9.2

[a]Ratio of SSB/8-OHdG corresponds to the slope of regression lines shown in Figure 2. Y-intercept in each regression line is shown in parenthesis. Number of data points and correlation coefficient are shown in brackets.
[b]The number of 8-OHdG was determined under the same experimental condition for each combination as described in materials and methods, then the relative number was calculated using the number for Fe alone and H_2O_2 as a standard of 1.0.
[c]The relative number of SSBs was calculated from each regression line and the corresponding number of 8-OHdG.
[d]Not done.

pH.[13] In any case, the enhancement appears to be independent of the chelator since two different chelating agents (NTA or citrate) produced similar increase in strand breakage (8.3- and 7.3-fold over free iron, respectively) and identical augmentation in base modification (4.0-fold) when DNA damage was produced in the presence of H_2O_2 (Table I). In contrast, the reducing agent present in the system affected the relative intensity of the two DNA lesions. The order of enhancing effects for SSBs formation by reducing agents was L-ascorbate > L-cysteine > H_2O_2. This order is similar to the ranking previously reported for Cu(II)-catalyzed DNA damage.[22]

Iron is mostly chelated in biological systems. We observed that chelation increases the production of oxidative DNA damage mediated by iron. The contribution of iron on the production of genomic and mitochondrial DNA damage, especially strand breaks, in diseases involving iron overload is likely to be higher than that previously predicted from the studies using unchelated iron.

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