Free divalent ions of copper (Cu) are capable of generating radical species such as hydroxyl radicals in the presence of hydrogen peroxide or ascorbic acid through Harbord-Weiss-like reactions under physiological conditions. It has been reported that radical-mediated damage to DNA molecules in animal cells leads to programmed cell death. Hence it is important to seek for methods to prevent Cu-mediated DNA damage. In this study we identified on effect of Cu binding of short peptides (chiefly Gly-Gly-His tripeptide) in the prevention of DNA degradation caused by Cu-mediated reactions in the presence of hydrogen peroxide and of ascorbic acid.

Key words: DNA degradation; reactive oxygen species; HO\(^{\cdot}\); copper; peptide

Oxidative damage to DNA is reportedly promoted in the presence of reactive oxygen species (ROS) such as the hydroxyl radical (HO\(^{\cdot}\)), which can be generated thorough Fenton-type or Harbord-Weiss-type reactions in the presence of ions of transition metals (chiefly copper and iron), ascorbic acid (AsA), and/or H\(_2\)O\(_2\).\(^{2,3}\) Among the members of ROS, HO\(^{\cdot}\) is the most highly reactive species. It oxidizes any neighboring molecules. Thus, generation of HO\(^{\cdot}\) in biological systems results in immediate damage to DNA molecules, and the consequent DNA degradation may lead further to apoptotic reaction and carcinogenesis in living cells. Such oxidative stress-mediated DNA fragmentation and chromosomal dysfunction play key roles in mammalian cell death mechanisms.\(^{3,1}\)

According to earlier work,\(^{2,1}\) damage to DNA molecules by HO\(^{\cdot}\) produced through reactions between H\(_2\)O\(_2\) and the Cu(II) ion have reported. It has been proposed that H\(_2\)O\(_2\) reduces Cu(II) to Cu(I), followed by a reaction of Cu(I) with H\(_2\)O\(_2\) and the formation of HO\(^{\cdot}\), as in the following equations:

\[
\begin{align*}
\text{Cu (II)} + \text{H}_2\text{O}_2 & \rightarrow \text{Cu (I)} + \text{HO}_2 + \text{H}^+ \\
\text{Cu (I)} + \text{H}_2\text{O}_2 & \rightarrow \text{Cu (II)} + \text{OH}^- + \text{HO}^{\cdot}
\end{align*}
\]

The generation of HO\(^{\cdot}\) in the reaction cycle of the Cu(II)/AsA system has been identified on the basis of experimental evidence in vitro.\(^2\)

Through DNA-degrading reactions in the Cu(II)/H\(_2\)O\(_2\) and Cu(II)/AsA systems, the production of large amounts of HO\(^{\cdot}\) under physiological pH conditions via the Harbord-Weiss-like reaction has been recorded by monitoring the level of 8-hydroxyguanosine (8-OHG) production, a reliable biomarker for HO\(^{\cdot}\)-dependent oxidative damage to guanosine residues on DNA.\(^{1,4}\)

Due to the hyper-reactivity of HO\(^{\cdot}\) even against water molecules, HO\(^{\cdot}\) hardly migrates even a short distance in aqueous phase, thus the generation of HO\(^{\cdot}\) at the site vicinal to the DNA tends to result in enhanced degradation of or damage to DNA. According to earlier works, Cu\(^{2+}\) binds strongly to the guanosine and cytidine bases at physiological pH, eventually perturbing the A-T base pairs and disrupting the double-helical structure of the DNA.\(^{3,1}\) In addition, Z-DNA structure-like micro-domains, especially at the base guanine, show much higher affinity for the binding of Cu\(^{2+}\).\(^{1}\) Accordingly, a complex between the Cu and the Z-DNA domain readily results in damage to DNA molecules.\(^{5}\)

Taking this together, it is tempting to conclude that the Cu-mediated Fenton-type reaction also occurs on site within the DNA-Cu complex by effectively allowing reactions between HO\(^{\cdot}\) and DNA.

From a gerontological point of view, it is important to seek for methods to prevent Cu-mediated DNA damage. By chelating Fenton catalysts such as the Fe and Cu ions, H\(_2\)O\(_2\)-dependent formation of HO\(^{\cdot}\) can effectively be inhibited. For example, the addition of Cu-chelating agents such as α-phenanthaline or 2-hydroxybenzoic acid to the Cu-catalyzed HO\(^{\cdot}\) generating system in the physiological pH range resulted in complete inhibition of HO\(^{\cdot}\) formation (evaluated by electron spin resonance spectrometry using a spin-trapping agent, 5\(S\)'-dimethyl-1-pyrroline-N-oxide).\(^{7}\) Natural Cu chelating agents are applicable as inhibitors of Cu-dependent DNA cleavage.

A tripeptide, glycyl-glycyl-histidine (GGH), found in human serum albumin is one of the most active peptides that bind the copper ion.\(^{2,8}\) Hence, we hypothesized that the GGH peptide can be used as a mean of protecting the DNA from Cu-dependent degradation. The nitrogen atoms within the histidine residue located at the C-terminus of the peptide and those on the backbone peptide bonds between amino acids play important roles as anchors for the binding of copper ions and other metals. In general, common copper-binding motifs, known as XXH motifs (where X is any amino acid and H is histidine), contribute to the transportation and

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Note
Prevention of Oxidative DNA Degradation by Copper-Binding Peptides

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homeostasis of copper ions in biological system, thus maintaining free Cu concentrations at lower level to protect living cells and tissues from the toxic impacts of copper ions.8) On the other hand, an earlier study also concluded that the copper-peptide complex (Cu-bound GGH) possibly possesses activity for scission of DNA and protein molecules in the presence of ascorbic acid.9) Since Cu ions alone (in the absence of peptides) show very high DNA-cleaving activity coupled to reducing agents such as ascorbate, the role of the GGH peptide in the promotion of Cu-catalyzed DNA scission is obscure. Most importantly, the study9) gives no experimental data on the requirement of the GGH peptide for copper-mediated reactions. Hence it is worth testing how the GGH peptide behaves during the Cu-catalyzed DNA-scission. The peptide concentration tested was 3 mM. D, Prion-derived copper-binding regions were synthesized and tested. The concentration of each peptide was 3 mM.

Inhibition of DNA degradation by the GGH peptide (glycyl-glycyl-histidine, 3 mM) used as a model His-containing copper-binding peptide was examined (Fig. 2). The peptide concentrations tested were up to 3 mM. As explained above, the GGH sequence is a ubiquitous motif in natural proteins, and the synthetic tripeptide is commonly used as a Cu-binding agent in vitro. The effects of the GGH tripeptide in the DNA/Cu/AsA system (each component, 1 mM; Cu, 1 mM; AsA, 3 mM) were compared to the DNA/Cu/H2O2 system (each component, 1 mM; Cu, 1 mM; H2O2, 1 mM) in which DNA degradation was completely abolished in both systems (Fig. 2A, B). According to an earlier report,8) the dissociation constant for the GGH-Cu complex is 1.18 × 10−16. Hence, one may expect that the amount of free Cu ions in the presence of 1 mM Cu2+ and 1 mM GGH should be at a negligible level not capable of DNA damage, but the molar ratio between Cu2+ and the GGH required for inhibition of DNA damage was 1:3 in the present study. This might reflect competition between DNA and the

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**Fig. 1.** DNA-Breaking Effect of Hydrogen Peroxide and Ascorbic Acid. A. A model 0.5-kDa DNA fragment used to study Cu-mediated DNA degradation. From a commercially available plasmid, pBR322, 501 bp (the region between the 137th and 638th bases) was amplified by PCR, as previously reported. B. 500 bp of the DNA molecule was obtained from the pBR322 plasmid by PCR. CuSO4, H2O2, and AsA (3 mM) were added to the DNA solution (150 μM) as a control experiment. C. Dose-dependency of the Cu/H2O2 system (molar ratio, 1:1) to the 150 μM DNA solution. D. Dose-dependency of the Cu/AsA system (molar ratio, 1:1). At a concentration of 1 mM, the DNA-band disappeared completely, suggesting that the Cu/H2O2 and Cu/AsA systems showed the effects of DNA degradation.

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**Fig. 2.** Action of His-Containing Copper-Binding Peptide against DNA Degradation. A. Effect of Gly-Gly-His tripeptide in the DNA/Cu/H2O2 system. Concentrations were 150 μM, 1 mM, and 1 mM respectively. B. Effect of Gly-Gly-His tripeptide in the DNA/Cu/AsA system. Concentration were 150 μM, 1 mM, and 1 mM respectively. C. A His-containing peptide reported to be metal-binding was examined. The peptide concentration tested was 3 mM. D, Prion-derived copper-binding regions were synthesized and tested. The concentration of each peptide was 3 mM.

EtBr staining. The pBR322 plasmid and Taq polymerase for the PCR reaction were purchased from TakaraBio (Shiga, Japan), and the other chemicals used were from Wako Pure Chemical Industries (Osaka, Japan). Each experiment was repeated at least 3 times.

As Fig. 1B–D shows, Cu-dependent DNA degradation occurring in the presence of H2O2 or AsA can be visualized using the PCR-amplified 500-bp model DNA fragment from pBR322. No DNA degradation was observed when CuSO4, H2O2, or AsA was added alone (at up to 3 mM) to the 150 μM DNA solution (Fig. 1B). In contrast, the DNA bands disappeared completely in the Cu/H2O2 and Cu/AsA systems (each component, 1 mM; Fig. 1C, D).

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**Notes:**

8) K. Y. OKAWA, H. ETU, and S. TADA. (1998). The dissociation constant for the GGH-Cu complex is 1.18 × 10−16. Hence, one may expect that the amount of free Cu ions in the presence of 1 mM Cu2+ and 1 mM GGH should be at a negligible level not capable of DNA damage, but the molar ratio between Cu2+ and the GGH required for inhibition of DNA damage was 1:3 in the present study. This might reflect competition between DNA and the...
peptide for binding to Cu$^{2+}$ since it has been reported that DNA possesses a number of Cu-binding sites, especially at the guanosine and cytidine bases.\(^5\,\)\(^6\)

To determine the role of the Cu-anchoring His residue in the GGH peptide, we examined the effect of the His-lacking Gly-Gly-Gly (GGG) tripeptide in both the DNA/Cu/H$_2$O$_2$ and the DNA/Cu/AsA systems. The GGG tripeptide showed no catalytic or DNA-protecting activity (data not shown).\(^1\)

In addition to GGH tripeptides, the effects of other His-containing tripeptides reportedly active as metal-binding peptide sequences, KGH, RGH,\(^10\) and QPH (a putative copper-binding motif found in the prion octarepeat region), were examined under conditions comparable to those shown in Fig. 2A and B (3 mM GGH; Fig. 2C). As expected, almost identical results were obtained with these Cu-binding tripeptides, confirming that tripeptides containing the His residue at the C-terminal are generally active in the removal of Cu. As a consequence they show high performance in protecting DNA from Cu-mediated degradation. Among the tripeptides examined, the behavior of the QPH tripeptide was slightly different, possibly due to its "imino acid" residue, which is different from the other model peptides used.

It has been found that human prion protein is rich in Cu-binding sequences and the peptide sequences isolated from or mimicking the prion-derived Cu-binding sequence possess various redox activities upon binding to Cu ions.\(^11\)\(^-\)\(^13\) Thus, prion-derived sequences might form a pool of natural putative DNA-protecting peptides to be tested. In order to assess the effect of the prion-derived copper-binding peptide sequence, three peptides (GGGTH, KTNMKHMA, and VNITKQHTVTTTT, 3 mM each) were synthesized as previously reported\(^14\) and used in the test in the same manner as for XHX peptides.

The KTNMKHMA and VNITKQHTVTTTT sequences but not the GGGTH sequence showed a DNA-protecting effect, similarly to the GGH tripeptide (Fig. 2D). These results suggest that biochemical aspects may differ among the XXH Cu-binding motif-containing peptides, possibly depending on peptide length or the combination of amino acids.

As Fig. 3A suggests, the involvement of HO$^*$ in damaging the DNA molecule by the addition of dimethylthiourea (DMTU; 1 mM or 10 mM), an HO$^*$-specific scavenger, to the systems (DNA/Cu/H$_2$O$_2$ or DNA/Cu/AsA, each consisting of 150 μM 500-bp DNA and 1 mM cofactors). After incubation for 1 h, Cu-dependent DNA degradation in the presence of H$_2$O$_2$ or AsA was inhibited by 10 mM DMTU.

Finally, in Fig. 3B we illustrate one of the likely structures for the Cu-GGH complex (1:1 peptide-Cu ratio) and propose a model mechanism for the DNA-protecting action of the peptide, suggesting that the Cu-GGH complex is not active in H$_2$O$_2$-dependent and/or AsA-dependent enhancement in HO$^*$ formation and thus protects DNA from oxidative degradation. In addition to the proposed structure of the Cu-peptide complex shown in Fig. 3B, additional structures involving multiple peptides per Cu ion should be considered, since the ratio of peptides over Cu ions successfully preventing the degradation of DNA was 3:1 (Fig. 2A, B).

In this study, we demonstrated that the addition of Cu-binding peptides with Cu-chelating activity resulted in inhibition of Cu-mediated damage to DNA, which is known to involve the generation of ROS in the presence of electron donors or acceptors. We observed that Cu-dependent DNA degradation in the presence of H$_2$O$_2$ or AsA was effectively prevented by both the peptides (which contained Cu-binding XXH motifs) and the HO$^*$ scavenger tested. Hence we conclude that multiple members of the Cu-binding peptides sharing a common structure (XXH motifs) show Cu-chelating activity in a form not active in the generation of HO$^*$, which attacks DNA. We expect that a wide variety of natural and synthetic proteins and peptides exposing the copper-binding motifs in the hydrophilic micro-environments can be used for DNA protection from damaging copper toxicity under physiological conditions.

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