Cross-Linking of Collagen by Singlet Oxygen Generated with UV-A

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Singlet oxygen ($^1O_2$), a highly reactive and toxic intermediate, may play a role in photo-induced aging. We examined singlet oxygen generation from hematoporphyrin (HP) with UV-A, by monitoring the emission at 1268 nm corresponding to $^1O_2 \rightarrow {}^3O_2$. Singlet oxygen was formed HP-dose-dependently in this system. We then investigated the reaction of singlet oxygen generated by UV-A irradiation with collagen, which is related to skin elasticity and softness. Collagen from skin was rapidly and dose-dependently cross-linked by singlet oxygen. The reaction was inhibited by NaN_3, a selective quencher of singlet oxygen. In contrast, SOD (superoxide dismutase) and mannitol had no effect. These results suggested that cross-linking of collagen was caused by UV-A-generated singlet oxygen, not by any other reactive oxygen species. Compared with another multisubunit protein, alcohol dehydrogenase, collagen was cross-linked much more efficiently. Further, the finding that semicarbazide inhibited cross-linking of collagen showed that cross-links were formed between photooxidized histidyl residues and amino groups. Singlet oxygen generated by UV-A irradiation may contribute to cross-linking of collagen in the process of skin photoaging.

Key words singlet oxygen; UV-A; photoaging; collagen; cross-link

Reactive oxygen species (singlet oxygen ($^1O_2$), superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\cdot OH$)) are known to cause a number of oxidative injuries such as inflammation, cancer, ischemia, *etc.*[1,2] It has also been proposed that reactive oxygen species generated by UV irradiation may play an important role in photo-induced skin damage, such as sunburn reaction, phototoxicity and photoallergy. In addition to UV-B, UV-A is considered to be important, especially in relation to sensitization reactions.[3–5] However, the mechanism leading to the skin damage remains to be elucidated.

Singlet oxygen has usually been detected by monitoring weak visible chemiluminescence, chemical reaction products, and deuterium kinetic effects, as well as by chemical scavenger techniques. Compared with these nonspecific detection methods, direct observation of singlet oxygen emission at 1268 nm is most reliable.[6,7] We have constructed a near-infrared emission spectrometer with an ultrasensitive germanium (Ge) detector.[8] In this study, we used this apparatus to examine the reactivity with collagen of singlet oxygen generated from UV-A-irradiated hematoporphyrin (HP), and we showed that collagen from skin was rapidly cross-linked by singlet oxygen thus generated.

Experimental

Materials Acid-soluble collagen extracted from calf skin was purchased from Elastin Product Co. Inc., U.S.A. Concentrations of collagen were determined according to the method of Lowry *et al.*, using bovine serum albumin (BSA) as a standard.[9] HP and alcohol dehydrogenase from baker's yeast were purchased from Sigma Chemical Co., U.S.A. The other chemicals used were commercial products of reagent grade.

Singlet Oxygen Emission Measurements Our experimental setup consisted of an argon (Ar) laser (Innova 70-4; Coherent Inc., U.S.A.) and a near-infrared Ge detector (model 403HS; Applied Detector Co., U.S.A.) cooled by liquid nitrogen and connected to the exit slit of a monochromator (model CT10; JASCO, Japan) with a blaze wavelength at 1250 nm to minimize grating loss. An IR-80 cutoff filter with 0% transmittance below 750 nm and 35% transmittance at 800 nm was placed at the entrance slit of the monochromator. A collecting lens focused the monochromator output onto the detector crystal. The Ar laser output in the UV-A region (351.1, 351.4, 363.8 nm) was chopped at 800 Hz by an acousto-optic modulator (model ASM-702-8; Intra Action Corp., U.S.A.) driven by a driver (model ME-70JT; Intra Action Corp., U.S.A.). The signal output from the Ge detector was fed to a model 124A lock-in amplifier *via* a model 116 preamplifier (both from E.G. & G. Princeton Applied Research, U.S.A.) and synchronized with the internal reference signal of the lock-in amplifier. The signal output from the lock-in amplifier was fed to an XY recorder, and the emission spectrum corresponding to the $^1O_2 \rightarrow {}^3O_2$ transition was recorded by scanning the grating with a motor.[8]

Irradiation Procedure UV irradiation in this study was performed with a solar simulator (XB-25T1W1, WACOM R & D Co., Tokyo, Japan) in the UV-A region light (320—400 nm). UV-A output power, measured by an Eppley thermopile (The Eppley Lab., Inc., U.S.A.), was 2 mW/cm^2. Collagen solutions in 50 mM Tris–HCl buffer (pH 7.5), with or without HP, various quenchers for reactive oxygen species (100 mM NaN_3, 10 mg/ml SOD (superoxide dismutase) and 10 mg/ml mannitol) and 50 mM semicarbazide were irradiated with UV-A. Anaerobic conditions were set up using an Anaero Pack® (Mitsubishi Gas Chemical Co., Inc., Japan).

Electrophoresis Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed under denaturing conditions with 2-mercaptoethanol, adopting the method proposed by Laemmli.[10] The UV-irradiated or unirradiated samples were subjected to SDS-PAGE using 4% polyacrylamide gels. The gels were stained with 0.25% Coomassie Brilliant Blue.

Results

Detection of Singlet Oxygen Generated by UV-A Irradiation Figure 1a shows a singlet oxygen emission spectrum in the near-infrared region, obtained with 20 μM HP in methanol exposed to Ar laser light. The spectrum clearly showed a peak at 1268 nm corresponding to the $^1O_2 \rightarrow {}^3O_2$ transition. The emission intensity at 1268 nm was dependent on the concentration of HP (Fig. 1b), suggesting that UV-A-irradiated HP dose-dependently generates singlet oxygen.

Cross-Linking of Collagen by UV-A Irradiation Collagen is separated into monomer (α(α1, α2)), dimer (β) and trimer (γ) species by SDS-PAGE under denaturing conditions. When irradiation was carried out at pH 7.5 with a 2 mW/cm^2 output of UV-A, gel electrophoresis of the

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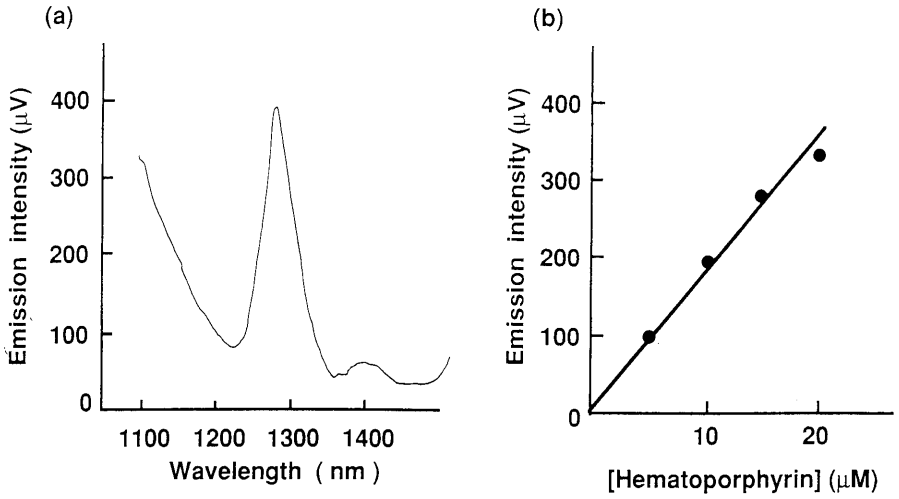

Fig. 1. Singlet Oxygen Emission Spectrum in 20 μM HP Solution Excited with Ar Laser Light in the UV-A Region at 100 mW Output Power (a), and the Effect of HP Concentration on the Emission Intensity at 1268 nm (b)

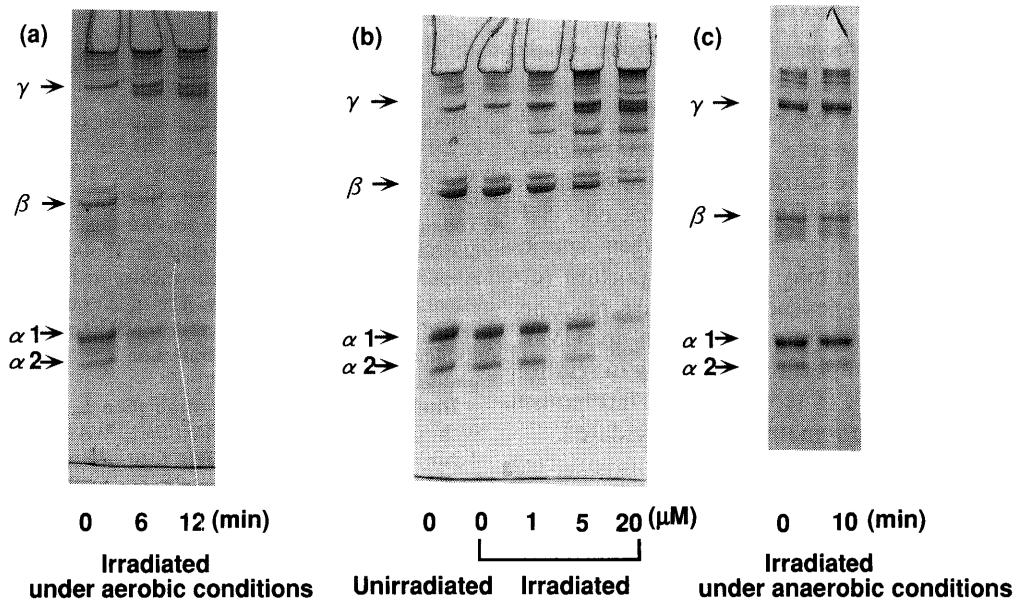

Fig. 2. Cross-Linking of Collagen by Singlet Oxygen Generated with UV-A

(a) A collagen solution was irradiated for the indicated times with 20 μM HP. (b) Collagen solutions were irradiated for 10 min with the indicated concentrations of HP. (c) A collagen solution was irradiated for 10 min with 20 μM HP under anaerobic conditions.

irradiated collagen showed a decrease of α and β, while γ and other cross-linked polymers increased (Fig. 2a).

This result indicated that α and β were converted into γ and other cross-linked polymers. UV-A-induced cross-linking of collagen was observed only in the presence of HP, and the amount of γ and other cross-linked polymers was dependent on the concentration of HP (Fig. 2b). Under anaerobic conditions, collagen was not cross-linked, which suggested that cross-link formation was dependent on oxygen (Fig. 2c). These results indicate that singlet oxygen generated by UV-A irradiation induced increasing amounts of cross-links in collagen.

UV-A-induced cross-linking was significantly inhibited by NaN_3, a selective quencher of singlet oxygen. However, SOD and mannitol, a hydroxyl radical scavenger, had no effect on the cross-linking of collagen (Fig. 3). These results suggest that cross-linking of collagen is caused by singlet oxygen generated by UV-A irradiation, not by other reactive oxygen species.

Further, we compared the photodynamic cross-linking of collagen with that of a multisubunit protein, alcohol dehydrogenase. Under similar conditions of irradiation, it was found that cross-linking occurred much more efficiently on collagen than on alcohol dehydrogenase (Fig. 4a). When collagen was denatured at 60 °C before UV-A irradiation with HP, collagen was not cross-linked, suggesting that the highly efficient cross-linking of collagen is a consequence of its particular conformation (Fig. 4b).

Inhibition of Cross-Linking of Collagen by Semicarbazide during UV-A Irradiation We next examined the mechanism of cross-link formation of collagen. In the presence of semicarbazide, cross-linking of collagen by UV-A irradiation was inhibited (Fig. 5). Semicarbazide is proposed to be bound to an oxidative intermediate of histidine and to protect against nucleophilic addition to another amino acid (Fig. 6).[11,12] This result suggests that cross-linking

of collagen by singlet oxygen may be dependent on photooxidized histidyl residues.

Discussion

It is widely assumed that solar radiation directly damages epidermal cells (keratinocytes, Langerhans cells, and melanocytes) and dermal cells (fibroblasts, endothelial cells, mast cells, *etc.*).[13-15] Recently there has been great concern about the potential long-term effects associated with repeated exposure to sunlight, especially UV components. It is well known that UV-B causes direct cleavage of DNA, delayed sunburn and tanning. UV-A accounts for the greatest proportion of UV solar light and penetrates deeply into the dermis. Exposure to UV-A may induce degenerative changes in the dermal connective tissue macromolecules, especially collagen, which is related to skin elasticity and softness, and accumulation of cross-linked collagen is considered to lead to various signs of skin aging, such as wrinkles and loss of elasticity.[16-19] Photoaging may involve not only the direct action of UV light on DNA, but also reactive oxygen species, especially singlet oxygen, which is highly reactive and toxic. Many attempts have been made to demonstrate the involvement of singlet oxygen in photo-induced cross-linking reactions in proteins, but nonspecific methods, such as chemical scavenger or deuterium kinetic techniques have failed to clarify the situation. Singlet oxygen can be detected most reliably by monitoring the emission at 1268 nm corresponding to the $^1O_2 \rightarrow {}^3O_2$ transition.[8]

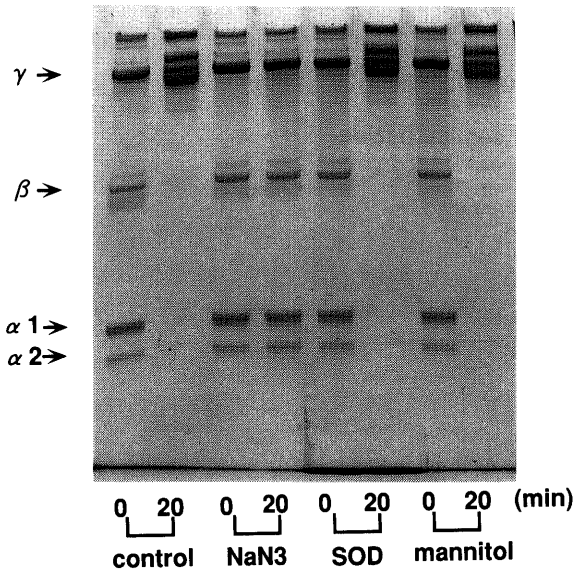

Fig. 3. Effect of Various Quenchers on Cross-Linking by Reactive Oxygen Species

Collagen solutions with various quenchers of reactive oxygen species (100 mM NaN_3, 10 mg/ml SOD or 10 mg/ml mannitol) were irradiated for 10 min with 20 μM HP.

In this study, we firstly confirmed the generation of singlet oxygen from HP exposed to UV-A by monitoring the 1268 nm emission of singlet oxygen. Then we examined in detail the effect of singlet oxygen on cross-linking of collagen. Only in the presence of HP was collagen cross-linked by UV-A irradiation, and the reaction was not inhibited by SOD or mannitol. The reaction did not occur under anaerobic conditions. The results suggest that the cross-link formation is mediated by singlet oxygen, not by other reactive oxygen species, which have been reported to degrade collagen as well as many other proteins (Figs. 2, 3).[20,21]

Protein oxidation by singlet oxygen frequently occurs at one or more of only five amino acid residues: cysteinyl, histidyl, methionyl, tryptophanyl, and tyrosyl.[22] Collagen does not contain tryptophan or cysteine, and oxidation of tyrosine occurs only in the phenolic anionic form at alkaline pH. These facts, together with the result that semicarbazide protected against the cross-linking (Fig. 5), suggest that cross-linking by singlet oxygen occurs between photooxidized histidyl residues and other amino groups.

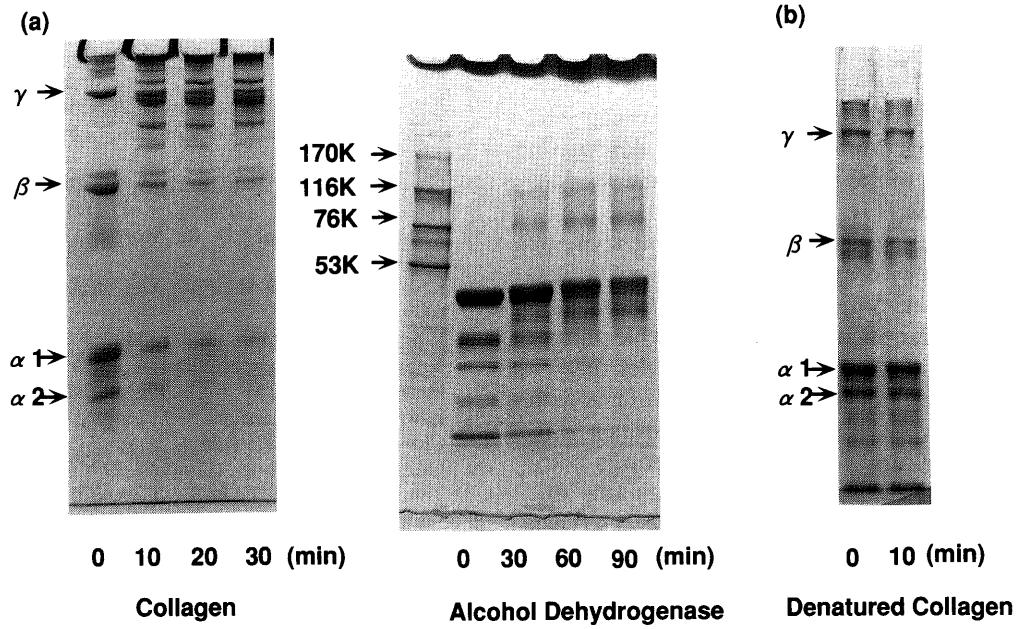

Fig. 4. Cross-Linking of Multisubunit Protein by Singlet Oxygen

(a) Collagen and alcohol dehydrogenase solutions were irradiated for the indicated times in the presence of 20 μM HP. (b) A solution of collagen denatured by heating at 60 °C for 10 min was irradiated for 10 min in the presence of 20 μM HP.

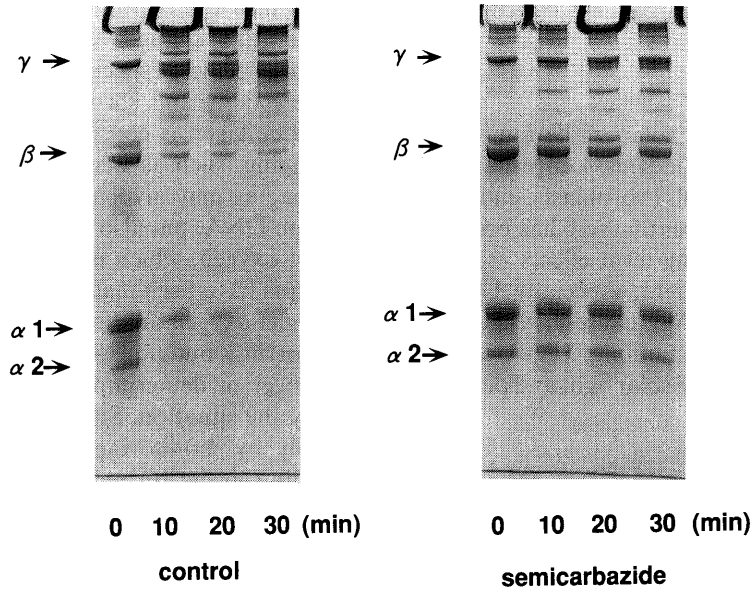

Fig. 5. Inhibition of Cross-Linking of Collagen by Semicarbazide

Collagen solutions with or without semicarbazide were irradiated for the indicated times with 20 μM HP.

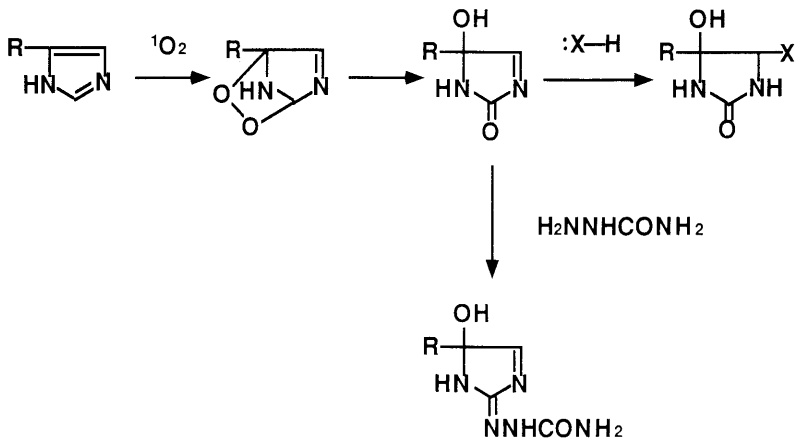

Fig. 6. Inhibition of Nucleophilic Addition of Oxidized Histidyl Residues to Other Amino Acids by Semicarbazide

Collagen was cross-linked by singlet oxygen more efficiently than alcohol dehydrogenase. However, denatured collagen was not cross-linked by singlet oxygen. The high efficiency of the reaction on collagen may depend on a specific conformation of the reactive amino acid residues in the collagen molecule (Fig. 4).

In long-lived proteins such as collagen, nonenzymatic polymerization occurs with aging. Cross-links are transformed into more stable forms during maturation, and pentosidine has been identified as one of the stable mature cross-links.[23,24] Cross-links generated by singlet oxygen were different from these mature cross-links. Patients with psoriasis or vitiligo who receive PUVA (psoralen plus UV-A) photochemotherapy for prolonged periods manifest sings of photo-induced skin aging, presumably due to the production of singlet oxygen.[2] Thus, singlet oxygen generated by UV-A irradiation may cause great damage to matrix components, accelerating skin photoaging.

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