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EFFECT OF OXYGEN FREE RADICALS ON CORNEAL COLLAGEN

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Corneal collagen was labeled *in vivo* by injection of ^{14}C-proline into the anterior chamber of rabbit eyes. The isolated corneal collagen was incubated in iron-free phosphate buffered saline (pH 7.4) containing 1 mM ascorbate and 0.1 mM $CuSO_4$ for either 1 hour or 3 hours at 37°. Addition of 2 volumes of 8 M urea-1 mM dithiothreitol and heating for 1 min at 100° solubilized virtually all of the collagen in the control incubations but left a significant amount of insoluble collagen in specimens exposed to the hydroxyl radical generating system. This residue amounted to 19% and 38% of the initial radioactivity in samples incubated for 1 h and 3 h, respectively. The chromatographic profiles (gel filtration on CL-4B) of the soluble fraction showed an increase in both aggregation and degradation products of collagen in the 1 h incubation mixture, whereas after 3 h there was an increase only in degradation products. These observations suggest that additional crosslinking of the soluble collagen aggregates observed at 1 h may be responsible for their subsequent disappearance at 3 h, with concomitant increase of the insoluble fraction. Collagen degradation by $^{\cdot}OH$ may play a role in corneal ulceration, whereas hydroxyl radical-mediated crosslinking is consistent with age-dependent increases in insoluble collagen.

KEY WORDS: Ascorbate, cornea, collagen, eye, copper, hydroxyl radical.

INTRODUCTION

Collagen is a glycoprotein whose basic structural unit is composed of three polypeptide chains associated through most of their length into a triple helix. These structures are linked together to form fibers with high tensile strength. Thin fibers of collagen type I are the main component of the corneal stroma, where they form a scaffold responsible for the high mechanical strength of the anterior portion of the eye.

The cornea is exposed to active oxygen species generated from environmental pollutants, i.e., ozone, nitrogen dioxide, sulfur dioxide, and smoke (including cigarette smoke). Active oxygen species are also generated endogenously in small quantities during electron transport and in larger amounts by activated phagocytic cells. Active oxygen species include hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl radical ($^{\cdot}OH$) and singlet oxygen. $^{\cdot}OH$ is extremely reactive, and it has been implicated in the degradation of corneal glycoconjugates, a process that may lead to corneal ulceration.[1] In addition to degradative reactions by direct scission of covalent bonds, oxidative modification of polypeptide chains renders them susceptible to preferential degradation by intracellular proteases.[2] Therefore, minor oxidative damage, e.g., to a single histidine residue,[3] may have profound effects *in vivo*.

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Recently it has been shown that ascorbate and $CuCl_2$, which produce $^{\cdot}OH$,[4] can cause crosslinking of acid-soluble skin collagen.[5] Decreased solubility of collagen observed during aging and in diabetes is believed to be due to increased crosslinking.[6]

MATERIALS AND METHODS

Collagen, type I, was isolated from rabbit corneas using the method of I.L. Freeman.[7] Briefly, epithelium, endothelium and Descemet's membrane were removed by scraping. The stroma was minced, and washed overnight twice with 1 M NaCl, and twice with 0.4 M acetic acid. The tissue was then digested twice overnight with pepsin, and type I collagen was precipitated with 2.5 M NaCl. The precipitate was dissolved in distilled water, dialyzed and lyophilized. For the preparation of radioactive specimens, collagen was radiolabelled *in vivo* by injection of ^{14}C-proline into the anterior chamber of rabbit eyes.

To determine the effect of copper and ascorbate, 1 mg of collagen (*ca.* 4000 cpm) was incubated at 37° in 1 ml of phosphate buffered saline (pH 7.4) made iron-free by dialysis against conalbumin[8] and containing 0.1% gentamycin and other additions as indicated in Results. Following incubation, the reaction was terminated by addition of 2 volumes of 0.05 M Tris-HCl buffer, pH 7.5, containing 8 M urea and 1 mM dithiothreitol (urea-DTT) and heating for 1 minute at 100°. The samples were then centrifuged at 27,000 × G for 15 minutes and the supernates chromatographed on a CL-4B gel filtration column (1.6 × 100 cm) eluted with urea-DTT. Fractions (3.5 ml) were collected and the elution profiles was monitored by scintillation counting of the fractions. The pellet was dissolved by treatment with 1 M NaOH at 100° for 5 minutes, neutralized with HCl and counted.

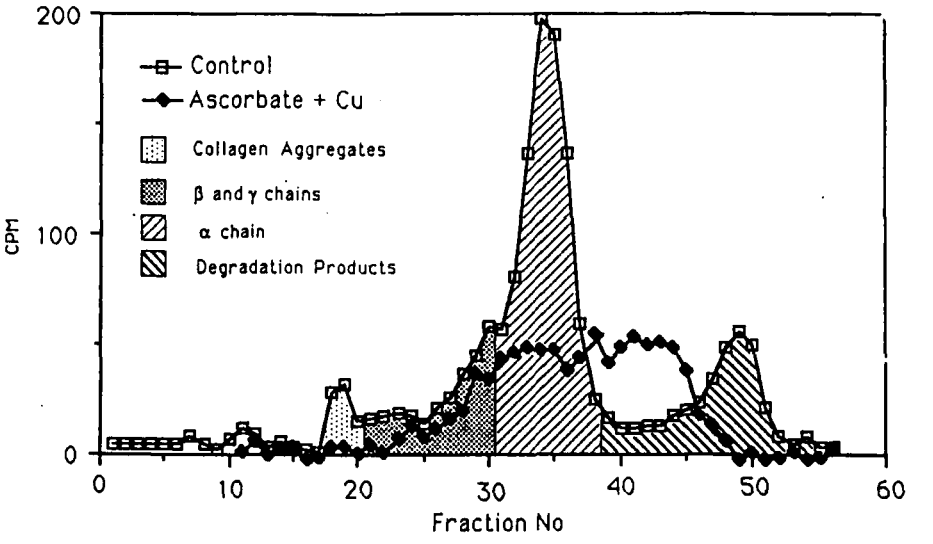

FIGURE 1. Elution profiles of the urea-DTT-soluble fractions from control and $^{\cdot}OH$-treated-, ^{14}C-labelled-corneal collagen. Following a 3 h incubation at 37° the specimens were chromatographed on a column of CL-4B as described under Methods. Polyacrylamide gel electrophoresis was utilized to establish the position of α, β, and γ chains.

RESULTS AND DISCUSSION

Incubation of ^{14}C-labelled type I corneal collagen in 1 mM ascorbate and 100 μM $CuSO_4$ for 3 hours resulted in formation of a gel which was only partially soluble in urea-DTT. Chromatography of the supernate indicated that much of the collagen in this fraction had been degraded (Figures 1 and 2). When the collagen was incubated in either ascorbate alone or copper alone, no changes were seen (Figure 2). Addition of 0.1 M mannitol to the complete incubation system inhibited the increase of collagen degradation products, but not of insoluble aggregates. Since mannitol is a quencher of $^{\cdot}OH$, these results are consistent with degradation of collagen by $^{\cdot}OH$ formed in the reaction medium, whereas $^{\cdot}OH$ responsible for the crosslinks may be formed on collagen-bound copper, and therefore be inaccessible to mannitol. When the collagen was incubated with ascorbate and copper for 1 hour, the relative amount of soluble aggregates of collagen was larger than in controls, while the insoluble pellet was smaller than that seen in the 3 hour incubation (Figure 2). Therefore, soluble collagen aggregates are likely to be a precursor of the insoluble collagen aggregate.

The dual effect of $^{\cdot}OH$ on corneal collagen *in vitro*, i.e., degradation and aggregation, is relevant to collagen changes observed *in vivo*. Collagen degradation is consistent with the acute pathological changes of collagen observed during corneal ulcera-

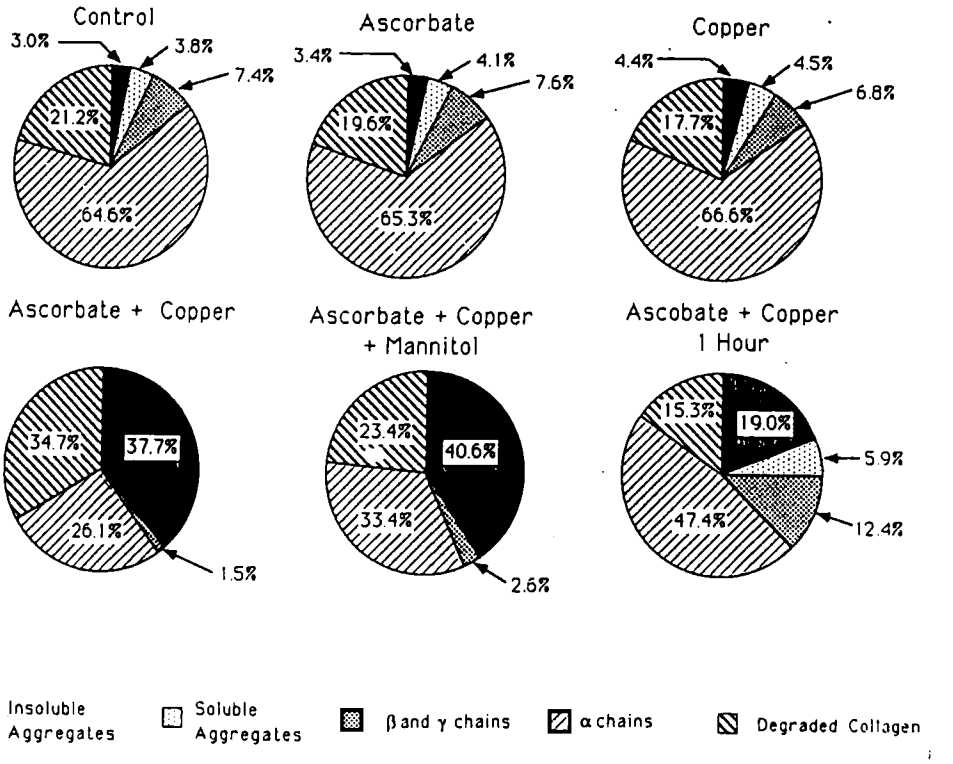

FIGURE 2. Relative distribution of the urea-DTT-insoluble fraction and of the subfractions obtained by column chromatography (see Figure 1) of the urea-DTT-soluble fraction. ^{14}C-labelled corneal collagen was incubated for 3 h (except where indicated) in iron-free phosphate buffered saline containing, where indicated, one or more of the following: 1 mM ascorbate, 100 μM $CuSO_4$, 100 mM mannitol.

tion,[1] whereas the formation of collagen aggregates is relevant to the chronic age-related crosslinking of collagen.[6]

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