HYDROXYLYSINE GLYCOSIDES IN THE COLLAGEN OF NORMAL AND SCARRED RABBIT CORNEAS

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Summary: Rabbit corneal scar collagen contains a lower content of glucosylgalactosylhydroxylysine than normal corneal collagen. Although the ratio of lysine to total hydroxylysine in normal and scar collagen is approximately the same, the relative proportion of glycosylated hydroxylysine to non-glycosylated hydroxylysine is different. These observations relate to the disposition of glycoside residues in scar and normal collagen and to the ultrastructural characteristics of the collagen fibrils.

INTRODUCTION

Recent studies on collagen have demonstrated heterogeneities in the primary and secondary structure of this protein in the same tissue as well as in different tissues (1-3). In particular, differences have been noted in the degree of hydroxylation of lysine residues and the quantity of carbohydrates in the form of monosaccharides and disaccharides linked to hydroxylysine of the peptide chain (1,4-6). Furthermore, differences in the carbohydrate content of collagen have been, in a broad manner, correlated with fibril diameter suggesting that hydroxylysine-glycosides may play a role in regulating fibrillogenesis (6).

Given that the problem of opacity in corneal scar resides in the presence of abnormal collagen fibril size (7), it becomes important to understand the regulation of collagen fibrillogenesis in normal cornea and its apparent defects during scar formation.

The present investigation examines the degree of hydroxylation of lysine and glycosylation of hydroxylysine in collagen from normal rabbit corneal stroma and corneal scar tissue.

MATERIALS AND METHODS

Collagen extraction and analysis

Albino rabbits, weighing 2-3 kg., were anesthetized and central corneal 2.0 mm full thickness excision wounds were made as described by Cintron, et al. (8) and allowed to heal without further treatment. Fresh tissue from 2 or 16 week old corneal scars or normal corneal stroma was frozen in liquid nitrogen and fragmented with a frozen tissue pulverizer (Thermovac Industries, Capiague, N.Y.). The tissue was then transferred to a 4.0 ml polypropylene test tube with 1.0 ml of glass distilled H_2O, and placed in a boiling water bath for 10 minutes to denature endogenous proteolytic enzymes. The mixture was dialyzed against 10 volumes of 0.01 ml M Tris HCl, pH 7.6 containing 0.01 M $CaCl_2$ and 5.0 mM N-ethylmaleimide (NEM) using an Amicon diafiltration system with a PM-30 membrane filter (Amicon Corp., Lexington, MA). The retentate was centrifuged at 20,000 x g for 10 minutes, and the pellet incubated with 0.2 ml (500 units) of chromatographically purified bacterial collagenase-Form III (Advance Biofactures Corp., Lynbrook, N.Y.) in 0.01 M Tris HCl pH 7.6 containing 0.01 M $CaCl_2$ and 2.5 mM NEM at 37°C for 72 hours under an atmosphere of chloroform. Estimated enzyme to substrate ratio was approximately 400 units/mg collagen. Enzymatically hydrolyzed tissue was dialyzed against 10 volumes of glass distilled H_2O using the Amicon system described above.

The analytical method used to determine lysine, hydroxylysine and its glycosides in the dialyzable fraction of enzymatically-hydrolyzed tissue was similar to that developed by Moczar and Moczar (9). The dialysate, containing collagen peptides, was hydrolyzed in a polypropylene tube with 0.5 ml of 2 M NaOH at 105°C for 24 hours. The alkaline hydrolysate was neutralized with 2.2

Abbreviations: glucosylgalactosylhydroxylysine, Hyl(GlcGal); galactosylhydroxylysine, Hyl(Gal); N-ethylmaleimide, NEM.

volumes of 1N acetic acid and desalted in a Dowex 50W-8X 200-400 mesh column (0.5x8.0 cm) using 0.1 N HCl. Amino acids were eluted from the column with 1.5 N NH_4OH and the sample dried down in a 40°C sandbath under a flow of air. The residue was then dissolved in a small volume of water and aliquots used to spot Whatman 3 MM Chromatography paper for high voltage electrophoresis. Electrophoresis was run with a pyridine-acetate-water buffer (1:10:89), pH 3.8 at 4000 V for 1.5 hours. Amino acids were stained with ninhydrin-cadmium reagent as described by Blackburn (10) and the location of each amino acid was based on its relative mobility to lysine. Spots were eluted with absolute methanol and optical densities read at 505 mμ. As a standard, known quantities of lysine were spotted on paper and processed similarly to unknown samples. The data were expressed as the number of residues/1000 amino acid residues assuming that the quantity of lysine plus its derivatives from alkaline hydrolyzed corneal collagen is 3.7% of the total number of amino acids (11).

Activity of contaminating proteolytic enzymes

Eight one-week old corneal wounds were exposed to DL-tryptophan (side chain-3-^{14}C), 48.2 mCi/mM (New England Nuclear, Boston MA.) by injecting a 0.2 ml saline solution containing 10 μc of label into the anterior chamber of anesthetized rabbits immediately after removal of an equal volume of aqueous humor. Rabbits were sacrificed 24 hours later and wound healing tissue removed for analysis. Non-radioactive "carrier tissue" from one-week old corneal wounds were added to the sample. Tissues were then processed as described above. The dialysate and retentate obtained after treatment with collagenase were hydrolyzed with 2 N NaOH at 105°C in sealed test tubes for 24 hours. Hydrolysates were diluted and neutralized with 1.0 ml of 1 N HCl prior to liquid scintillation counting. Aliquots

from each sample were added to 10.0 ml of Toluene-liquiflor solution and counted in a Beckman LS-230.

RESULTS

Activity of contaminating enzymes

Comparison of the dialyzable versus non-dialyzable radioactivity following collagenase-Form III digestion of (^{14}C)-tryptophan labeled scar tissue for 72 hours, revealed that approximately 4% is liberated into the dialyzable fraction. Since collagen from various tissues do not contain tryptophan (12), the result suggests that the activity of proteolytic enzymes on non-collagenous protein in corneal scar tissue is negligible.

Quantification of lysine and its derivatives

The number of lysine residues in collagen from normal corneal stroma in comparison to that in 2 week old scar tissue is approximately the same (Table 1). Furthermore, no difference was noted in

TABLE 1: Quantity of lysine and some of its derivatives in collagen from normal rabbit corneal stroma and corneal scar tissue.

Residue	Stroma	2 week Scar	16 week Scar
Lys	24.8	24.5	22.8
Hyl	5.5	8.5	8.4
Hyl(Gal)+	1.8	1.5	2.5
Hyl(GlcGal)+	4.9	2.7	3.6

+Deviation in the number of glycoside residues from triplicate determinations was no more than 0.4 residues/1000 residues.

the number of galactosylhydroxylysine (HylGal) residues between normal and scar tissue. In contrast to these, 2 week old scar tissue collagen contains 3.0 more residues of unsubstituted hydroxylysine per 1000 amino acid residues and 2.3 less glucosylgalactosylhydroxylysine [Hyl(GlcGal)] residues than that in normal corneal collagen. The degree of hydroxylation of collagen lysine residues

TABLE 2: The relative quantities of lysine, hydroxylysine and hydroxylysine-glycosides from rabbit corneal collagen and scar tissue collagen.

Collagen Source	Lys: Total Hyl+	Hyl(Gal)+Hyl(GlcGal): Hyl	Fibril‡ Diam. Å
Normal cornea	2.2	1.21	~ 300
2 week corneal scar	1.9	0.50	100-500
16 week corneal scar	1.6	0.73	

+Total Hyl = Hyl(Gal) + Hyl(GlcGal) + Hyl

‡(See Ref. 13)

in normal and two week scar is about the same (Table 2). The ratio of carbohydrate-substituted hydroxylysine to unsubstituted hydroxylysine in normal stromal collagen in almost two and one half times greater than that in scar tissue collagen (Table 2).

The number of lysine residues in 16 week old scar collagen is slightly less than that found in 2 week old wounds (Table 1). Although no change in unsubstituted hydroxylysine content was noted, each glycoside derivative of hydroxylysine was increased by one residue relative to that in the 2 week old scar (Table 1).

DISCUSSION

The ratio of hydroxylysine glycosides to unsubstituted hydroxylysine as determined by Spiro (4) from gelatinized rabbit corneal collagen is 1.33. The present data, using collagenase extracted collagen peptides are in agreement with these findings. This strongly suggests that all of the collagen peptides containing substituted and unsubstituted hydroxylysine residues were released from the tissue during enzymatic digestion.

The present observations suggest that the scar collagen contains a deficiency of Hyl(GlcGal) in comparison to normal stromal

collagen. This deficiency is disaccharide residues is reflected in the increased level of non-glycosylated hydroxylysine in scar collagen.

It has been suggested that there may be some relationship between collagen fibril diameter and carbohydrate content of collagen (6). Collagen with low carbohydrate content have a large mean cross sectional diameter and a distribution of fibril size highly variable whereas collagen with a high carbohydrate content, have a small and relatively constant fibril size. Corneal scar tissue contains collagen fibrils having large and variable cross sectional diameters (13). The data presented in this paper is consistent with the above correlation (Table 1, Table 2). Although a quantitative and dimensional correlation between two events do not necessarily constitute a causal relationship, the present observations are consistent with hydroxylysine-glycosides playing a role in regulating fibrillogenesis during corneal scar formation.

The present data suggest that there are changes in scar collagen between the second and sixteenth week of healing with respect to hydroxylysine glycoside content.

Scar tissue in skin has been shown to be metabolically active in the synthesis and breakdown of its collagenous components (14). Ross and Benditt (15) described two populations of collagen fibrils in guinea pig scar tissue differing in the size of their fibrils. Furthermore, these two populations of fibrils change with time resulting in two new populations with larger cross sectional diameters. Recently, Forrest, et al. (16) also noted changes in cross sectional diameters of scar collagen from skin. These observations have suggested a remodelling of the scar tissue. If corneal scar tissue undergoes remodelling during the period studied, it may well be expressed in the quantity of sugar residues attached to the new collagen molecules.

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