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BIOSYNTHESIS OF COLLAGEN IN PSEUDOXANTHOMA ELASTICUM

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Abstract. Collagen biosynthesis was studied on biopsy specimens from skin lesions of the axillary region and from clinically uninvolved skin of the sacral region of 9 patients with pseudoxanthoma elasticum (PXE), as well as from corresponding areas of 11 control subjects. A significant increase of total ^{14}C(lysine + hydroxylysine) and of (^{14}C)hydroxylysine was observed in collagenase-solubilized peptides of PXE skin lesions when compared with corresponding areas of controls. ^{14}C(proline + hydroxyproline) and (^{14}C)glutamine were unchanged in the PXE lesions. Clinically uninvolved skin of PXE patients showed no abnormalities in the parameters studied. Area differences were observed in the control group with a tendency to higher incorporation of all the amino acids studied in the axillary region than in the sacral. Biosynthesis of an abnormal collagen with an elevated lysine and hydroxylysine content in the PXE lesions seems to take place.

Pseudoxanthoma elasticum (PXE) is a genetically determined connective tissue disease, involving the skin, the eyes and the cardio-vascular system (16). The alterations appear as small yellow papules in the flexural folds of the skin, as angioid streaks in the ocular fundus and as widespread and early arteriosclerosis (4, 10, 16). Calcification in the skin lesions has been demonstrated histochemically (9). The problem as to whether the basic defect in PXE is in elastic or in collagen fibres has been debated over many years (16). Electron-microscopic studies showed changes in true elastic fibres, particularly as deposits of calcium salts inside the fibres (5, 11, 12, 15, 17). The calcification has been reported as the first visible abnormality in PXE (5, 6, 10, 11). In the electron microscope, calcified elastic fibres have been seen to be surrounded by irregularly arranged thick and twisted collagen fibrils and by large areas of a thready material (5).

The morphological finding of abnormal collagen in PXE prompted the biochemical study of this macromolecule. Previous biochemical analyses of collagen in PXE had shown a decrease of insoluble collagen, while the soluble fraction was slightly increased (19), and total skin homogenates had shown a decrease of collagen as measured by the hydroxyproline content (21). The biosynthesis of collagen as measured by the (^{14}C)-hydroxyproline formation in total homogenates had been found unchanged in PXE skin lesions (21). In the present paper, parallel studies of the biosynthesis of the two amino acids characteristic of collagen, i.e. hydroxylysine and hydroxyproline, were carried out on partially purified collagen. In addition, incorporation of (^{14}C)lysine, (^{14}C)proline and (^{14}C)glutamine into collagenase-digested peptides was studied.

Abbreviations

Lys = lysine
Pr = proline
Glut = glutamine
Hylys = hydroxylysine
Hypro = hydroxyproline

MATERIAL AND METHODS

Chemicals

Uniformly labelled (^{14}C)L-lysine 223 μCi/μmole, (^{14}C)-L-proline 180 μCi/μmole and (^{14}C)L-glutamine 150–200 μCi/μmole were purchased from New England Nuclear, Boston.

Papain. E. Merck A.G., Darmstadt.

Collagenase. Worthington Biochemical Corporation (CLSPA).

N-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer. (HEPES) Calbiochem.

Ampicillin. (Pentrexyl®—Lundbeck, Copenhagen, Denmark.)

Skin samples

The tissues studied were age, sex, and area-matched skin biopsies of PXE patients and normal controls. Biopsies

were taken from corresponding sites, i.e. axillary and sacral areas, in patients and normal volunteers.

PXE lesions were present in the axillary, but not in the sacral areas of the patients. One experiment included 5 axillary and 6 sacral biopsies from 6 patients and 6 controls taken on one day, while a second experiment comprised 6 axillary and 8 sacral biopsies from 8 PXE patients and 5 axillary and 6 sacral specimens from 6 controls also taken on one day.

Preparation of samples

In both experiments the tissue specimens were divided into three parts.

Experiment I. Part 1 was used for determination of wet and dry weight. On part 2, studies were made of the uptake of (^{14}C)L-lysine and its hydroxylation, as well as determination of total hydroxyproline in collagen. Part 3 was used for studies of the biosynthesis of (^{14}C)hydroxyproline as well as for determination of the total hydroxyproline content.

Experiment II. Part 1 was used for determination of wet and dry weight as well as for total hydroxyproline and total hydroxylysine assays. On part 2 the uptake of (^{14}C)-L-glutamine by collagen was studied. Part 3 was used for determination of the incorporation of (^{14}C)L-proline into collagen.

Dry weight

To determine dry weight, both parts 1 were weighed on an ultramicro Mettler balance immediately after excision. The samples were then defatted with acetone, acetone–ether (1 : 1) and ether, and desiccated to constant weight by storing for 3 days in a stainless-steel vacuum desiccator (Nikortanks, Cat. No. 800). The dry samples were weighed, and the contents of water and lipid were calculated as percentage of the wet weight of the skin.

Biosynthesis of (^{14}C)hydroxylysine and (^{14}C)hydroxyproline and total incorporation of (^{14}C)labelled amino acids

To study the biosynthesis of the two amino acids characteristic of collagen and related collagen-like glycoproteins, incubations with the precursors (^{14}C)L-lysine and (^{14}C)L-proline were performed under parallel and similar conditions, as described for proline by Uitto (20). To determine the incorporation of (^{14}C)L-proline and (^{14}C)L-glutamine, incubations were carried out under similar and comparable conditions. Minced samples were prepared by scissors-cutting to obtain fragments of 2–3 mm in diameter. Samples were preincubated at 37°C for 1 hour. Five μCi of each labelled amino acid was added to each sample. Incubations were carried out for 10 hours at 37°C for parts 2 and 3 of Experiment I, and for 24 hours for the corresponding parts of Experiment II. Preincubations and incubations were performed under continuous shaking. To end the incubations, the samples were rapidly cooled and frozen. After thawing, the samples were digested with papain.

In the first experiment, after the enzymatic digestion, the samples were dialysed against running tap water for 24 hours at room temperature, then against distilled water, and finally against 10^{-3} M $CaCl_2$. The latter two steps were performed at 4°C (1). The samples were then submitted to collagenase digestion.

In the second experiment, the samples were lyophilized after dialysis against distilled water, then suspended in 10^{-3} M $CaCl_2$ and submitted to collagenase digestion.

Before digestion the undialysed material from Experiment I and the suspended material from Experiment II were adjusted to pH 7.2 with 1 M Tris buffer (pH 7.2) and then 200 μg collagenase in 10^{-3} M $CaCl_2$ was added to each tube. Enzymatic digestion was performed overnight at 37°C under continuous shaking. The samples were then heated at 56–60°C for 30 min to precipitate insoluble proteins. After cooling, the samples were centrifuged for 20 min in a Servall centrifuge at 13 000 g at 4°C. The supernates containing "collagenase-solubilized peptides" were separated from the undigested pellets, and the (^{14}C) total uptake (dpm) determined in the former. Hydrolysis of the "collagenase-solubilized peptides" was carried out in 6 N HCl at 110°C through 16 hours. After hydrolysis, HCl was evaporated at 65°C under vacuum. The evaporated samples were dissolved in distilled water. Aliquots of the hydrolysed and evaporated samples of Experiment I were used for determination of (^{14}C)hydroxylysine and (^{14}C)hydroxyproline and assayed according to Blumenkrantz & Prockop (1) and Juva & Prockop (13), respectively.

Other aliquots were used for the determination of total hydroxyproline according to Kivirikko et al. (14), and total hydroxylysine according to a modification of the method elaborated by Blumenkrantz & Prockop (2). Aliquots of the hydrolysed collagenase-solubilized peptides, were counted in a liquid scintillation counter after dissolution in distilled water, and the value of total (^{14}C) incorporation established.

Statistical analysis

Results are expressed as means ± S.E. Student's *t*-test was used to determine the significance of the differences observed.

RESULTS

Total skin

Weight of dry and defatted skin. Comparison of dry weight as percentage of wet weight of skin biopsies removed from PXE patients and normal controls revealed no significant differences between the groups (Table I).

Hydroxyproline and hydroxylysine. No significant differences in hydroxyproline and hydroxylysine content, expressed as μg/mg dry defatted skin, were found between the PXE patients and controls. However, rather low values of hydroxyproline were found in both axillary and sacral regions of the PXE patients. As a consequence, when μg Hylys/μg Hypro were considered, a slight relative increase of the former amino acid of the lesions was seen (Table II).

Table I. *Dry and defatted tissue as percentage of wet weight*

Diagnosis	No.	Skin region	DDS[a] %
Exp. 1			
PXE	5	Axillary	26.42 ± 0.9
	6	Sacral	29.96 ± 0.9
Controls	5	Axillary	28.56 ± 1.2
	6	Sacral	28.3 ± 1.0
Exp. 2			
PXE	6	Axillary	36.4 ± 5
	8	Sacral	26.1 ± 2
Controls	5	Axillary	27.6 ± 3
	6	Sacral	25.5 ± 2

[a] DDS = Dry defatted skin.

Collagenase-solubilized peptides

Total ^{14}C(lysine + hydroxylysine) per μg hydroxyproline. Total ^{14}C(Lys + Hylys) dpm per μg Hypro in the axillary samples of the PXE patients were significantly higher than in the corresponding areas of controls (Table III). There was no difference in total ^{14}C(Lys + Hylys) dpm per μg Hypro between the sacral areas of the groups. Significant area differences were found in the control group as well as in the PXE patients ($P < 0.001$), the axillary areas showing the highest values (Table III).

Total ^{14}C(proline + hydroxyproline) per μg hydroxyproline. When ^{14}C(Pr + Hypro) was related to μg Hypro content (as determined by J. Uitto, M.D.), no difference between PXE patients and control groups was observed. The axillary areas showed a higher uptake than the sacral areas in both groups (not shown).

Total ^{14}C(proline + hydroxyproline) and (^{14}C)-glutamine per mg wet weight of skin. No significant differences between corresponding areas of patients and controls were found, either for (^{14}C)-Glut or for total ^{14}C(Pr + Hypro) (Table IV). Comparison of ^{14}C(Pr + Hypro) values for axillary and sacral areas of controls revealed no statistically significant differences. The (^{14}C)Glut content in collagenase-digested peptides from axillae of controls was significantly greater than that of the sacral area ($P < 0.01$).

Table II. *Hydroxylysine and hydroxyproline in skin biopsies of PXE patients and normal controls*

Skin region	No.	Hylys (μg/mg DDS[a])	Hypro (μg/mg DDS[a])	μg Hylys / μg Hypro
PXE				
Axillary	6	6.92 ± 0.38	75.12 ± 5.0	0.092
Sacral	8	6.23 ± 0.39	73.96 ± 5.0	0.084
Control				
Axillary	5	6.82 ± 0.6	84.03 ± 4.0	0.081
Sacral	5	7.62 ± 0.4	85.18 ± 4.0	0.089

[a] DDS: Dry defatted skin.

Table III. *Total (^{14}C)lysine + (^{14}C)hydroxylysine per μg hydroxyproline in "collagenase-solubilized peptides" from skin of PXE patients and normal controls*

Diagnosis	No.	Skin region	(^{14}C)Lys + (^{14}C)Hylys (dpm per μg Hypro)
PXE	5	Axillary	12.121 ± 958
	6	Sacral	1.882 ± 405
Controls	5	Axillary	3.851 ± 306
	6	Sacral	1.912 ± 191

Synthesis of (^{14}C)hydroxylysine and (^{14}C)-hydroxyproline by human skin. If hydroxylation of (^{14}C)Lys and (^{14}C)Pr was expressed as dpm (^{14}C)-Hylys or (^{14}C)Hypro per 50 000 dpm total ^{14}C, no deviations in the former were noticed, while a slight increase in the (^{14}C)Hypro formation was found in the lesions. If, however, the synthesis of (^{14}C)Hylys and (^{14}C)Hypro was related to μg Hypro content in the correspondent aliquots, a significant increase of (^{14}C)Hylys was found, while a slight but non-significant increase of (^{14}C)Hypro was noticed in the lesions (Table V). No significant differences in the biosynthesis of (^{14}C)Hypro and (^{14}C)Hylys in relation to the two parameters considered were observed between the sacral areas of both groups.

Area differences in the control group between axillary and sacral areas were observed for both (^{14}C)Hylys and (^{14}C)Hypro when expressed as dpm/μg Hypro.

DISCUSSION

The presence of abnormal collagen in PXE is suggested by the increased total ^{14}C(Lys + Hylys) with a parallel increase in (^{14}C)Hylys, when both were related to Hypro content, while total ^{14}C-(Pr + Hypro) and (^{14}C)Hypro showed normal values in relation to the same parameter.

The differences noted between the highly sig-

Table IV. *Changes in (^{14}C)glutamine and ^{14}C(proline+hydroxyproline) content in collagenase-digested peptides from skin of PXE patients and normal controls*

Diagnosis	No.	Skin region	(^{14}C) Glut dpm per 100 mg wet weight of skin	^{14}C (Pr+Hypro) dpm per 100 mg wet weight of skin	(^{14}C) Glut / ^{14}C (Pr+Hypro)
PXE	6	Axillary	13 491±3 524	90 483±18 451	0.15
	8	Sacral	6 700± 892	44 101± 8 191	0.15
Control	5	Axillary	13 660±1 541	81 378±16 953	0.16
	6	Sacral	6 606±1 247	51 718±10 191	0.13

nificant increase of (^{14}C)Hylys and the slight increase in the Hylys content as related to Hypro in PXE skin lesions might be due to the fact that the biosynthesis was studied on partially purified collagen, while Hylys was determined on total dry defatted skin. In dry defatted skin the parameter of reference, Hypro, represents collagen plus elastin, while that of the collagenase-solubilized peptides is only indicative of collagen. The above-mentioned differences may also be explained by suggesting an increased degradation of the newly synthesized collagen and/or a defect in collagen maturation. This hypothesis would be in agreement with the finding of Smith et al. (19), who reported a decrease in total collagen due to a decrease in the insoluble fraction, with a slight increase of the soluble collagen in PXE lesions. It is well known that Lys and Hylys participate in the normal process of collagen cross-linkage (18). A decrease in cross-linkage is found in correlation with increased soluble collagen (8, 18). The lack of Lys and Hylys cross-linkage formation in PXE may be related to the increased (^{14}C)Hylys found. Recent electronmicroscopic studies suggest that the large areas of thready material in PXE may represent malformed collagen (7). This finding, together with the observation of twisted collagen fibrils in PXE (5), would tally with the presence of an abnormal collagen, with a high content of Lys and Hylys per μg Hypro, suggesting an incompletely cross-linked collagen being synthesized at the lesions.

In contrast to the finding in elastin (3), collagen showed normal (^{14}C)Glut incorporation. The unchanged total ^{14}C(Pr+Hypro) and (^{14}C)Glut in PXE lesions shows normal contents of these amino acids in the newly synthesized collagen. The presence of Ca^{++} in the lesions may influence our results and conceal an increased synthesis, as the wet as well as the dry weights of the connective tissue of the lesions may actually be lower than those of the controls, if calcium is subtracted. The question as to whether the tendency to an incorporation of all the labelled amino acids studied that is higher in the axillary regions of the controls than in their sacral areas may be related to the fact that PXE lesions often develop in the axillary and never in the sacral region, remains unanswered. The area difference demonstrated in the controls also stresses the im-

Table V. *Synthesis of (^{14}C)hydroxylysine and (^{14}C)hydroxyproline by human skin*

Skin region	No.	Total (^{14}C) Hylys dpm per 50 000 dpm total (^{14}C)	(^{14}C) Hypro dpm per 50 000 dpm total (^{14}C)	Total (^{14}C) Hylys dpm per μg Hypro	(^{14}C) Hypro[a] dpm per μg Hypro
PXE					
Axillary	5	599±68	6 140±895	145±8	243±52
Sacral	6	458±33	6 525±915	25±7	103±26
Control					
Axillary	5	649±108	4 606±795	50±9	198±55
Sacral	6	568± 53	6 593±648	26±8	106±43

[a] These determinations were carried out by Jouni Uitto, M.D. (Home address: University of Helsinki).

portance of considering corresponding areas in connective tissue studies. Of utmost importance is the parallel determination of Hylys and Hypro, whenever collagen is studied.

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