ORIGINAL ARTICLE

Collagen Metabolism in the Fibroblasts Derived from the Skin of a 16-year-old Patient with Marfan Syndrome: A Decreased Rate of Intracellular Degradation of Collagen

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Abstract. The rate of collagen synthesis in the cell layer fraction of the skin fibroblasts derived from a patient with Marfan syndrome was greater than that in the normal fibroblasts. Neither the relative content of type III collagen nor the level of prolyl and lysyl hydroxylations in type I collagen changed. Intracellular degradation rate of collagen in Marfan syndrome fibroblasts was decreased to one fifth of that in the normal fibroblasts. These results suggest that abnormal collagen metabolism may be related to the pathogenesis of clinical manifestations of Marfan syndrome. (Keio J Med 44 (3): 88–92, 1995)

Key words: collagen, collagen degradation, Marfan syndrome

Introduction

Marfan syndrome is a heritable systemic disease which is characterized by arachnodactyly, ectopia lentis, or aortic dissection.¹ Over the years, major classes of connective tissue components including collagen, elastin and proteoglycan have been suggested to be defective in this disease.

From the earlier reports of the increased urinary excretion of hydroxyproline,^{2,3} the increased solubility of dermal collagen^{3,4} and the decreased borohydride-resistant collagen cross-links,⁵ fibrillar collagen has been believed as the cause of Marfan syndrome.

It has been reported that increased synthesis of hyaluronic acid^{6,7} or deficient transcription of decorin, a chondroitin-dermatan sulfate proteoglycan⁸ was found in Marfan syndrome fibroblasts.

Indirect evidence for a defect in elastin in Marfan syndrome came from studies documenting decreased excretion of desmosins, a cross-link structure of insoluble elastin, in the urine of patients⁹ and increased solubility of elastin from the aorta of Marfan patients.¹⁰

Recently it has been demonstrated that Marfan syndrome is caused by mutations in the fibrillin gene, encoding a major protein constituting microfibrillar glycoproteins.^{11–14} However the previous reports de-

scribing the abnormalities in collagen, elastin and proteoglycans in Marfan syndrome suggest that the defects in fibrillin influence the metabolisms of other extracellular matrix components, since fibrillin is thought to interact with other matrix proteins. Marfan syndrome exhibits heterogeneous clinical phenotypes, which can be explained by a defective fibrillin, but its actual pathogenic mechanism remains still speculative.

Intracellular degradation of protein is a fundamental process with important implications for the protein regulation and cell biology. Intracellular degradative rates have been found to correlate with certain physical and chemical properties of the protein, such as charge size proteolytic susceptibility and hydrophobic character.¹⁵

In this study, I found an increased synthesis of collagen in the cell layer of Marfan syndrome fibroblasts which is due to decreased intracellular degradation rate of collagen.

Case Report

A 16-year-old Japanese male referred to dermatology clinic with an extensive linear atrophic skin changes. His height was taller (181 cm) than the mean for his age. Funnel chest (pectus excavatum) was observed but

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vertebral column deformity was not seen. Ophthalmic examination revealed ectopia lentis. Electrocardiogram and echocardiogram showed mitral valve prolapse due to floppy mitral valve. Marked striae distensae on his shoulders, thigh and abdomen were noted. Breast X-p and barium enema were normal. Hypermobility of joints and hyperextensibility of the skin suggesting Ehlers-Danlos syndrome were not observed.

Materials and Methods

Materials

[2,3-³H]Proline (4.0 TBq/mmol), [U-¹⁴C] lysine (12 GBq/mmol) and [³²P]dCPT (110 TBq/mmol) were purchased from Amersham. Multiprime DNA labeling kit was from Amersham, and Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Gibco. Chromatographically purified bacterial collagenase was from Advance Biofactures Company, pepsin was from Cooper Biomedical, and nitrocellulose filters were from Schleicher & Schuell.

Fibroblast culture

Fibroblasts were explanted from skin biopsy samples of the patient and two age-, sex- and site-matched controls. Cells grown in 35-mm Petri dishes in DMEM containing 10% FBS were routinely subcultured every 6 days. Cells in passages 8–10 were used.

Metabolic labeling and collagen synthesis

Confluent cultures were labeled for 24 h with [2,3-³H]proline (20 μ Ci/ml) in the presence of ascorbic acid (50 μ g/ml). The medium was then collected and mixed with a protease inhibitor cocktail to yield 1 mM each of N-ethylmaleimide, EDTA (pH 7.0), and phenylmethylsulfonyl fluoride. The cells were harvested with 0.05% trypsin, and their number was determined with a Coulter counter. After centrifugation, cells were recombined with the medium and stored at -20°C until ready for analysis. The amount of radioactivity incorporated into collagen and total proteins was determined using purified bacterial collagenase¹⁶ as previously described.¹⁷ Syntheses of collagen was expressed as collagenase-sensitive cpm/cell.

Synthesis type I and III collagens

Confluent cultures were labeled as described above. The culture medium was collected and the cell layer was scraped and homogenized with 0.5 M acetic acid, and the homogenate was centrifuged at $13000 \times g$ for 30 min at 4°C . The resulting supernatant was combined with the

culture medium, dialyzed against 0.1% acetic acid at 4°C , and lyophilized. The material was digested with pepsin (50 μ g/ml) for 18 h at 4°C . After precipitation with ammonium sulfate (176 mg/ml), the pepsin resistant proteins were resolved on 4–15% SDS-PAGE without reduction and subjected to autoradiography. The intensities of the bands corresponding to $\alpha 1(\text{III})$, $\alpha 1(\text{I})$ and $\alpha 2(\text{I})$ were quantitated with a scanning densitometer (Cliniscan Helena Laboratories).

Collagen prolyl and lysyl hydroxylation levels

Confluent cultures were labeled for 24 h with [³H] proline (25 μ Ci/ml) and [¹⁴C]lysine (1.25 μ Ci/ml) in lysine-free DMEM supplemented with dialyzed FBS (0.5%) and ascorbic acid (50 μ g/ml). The culture medium was precipitated with ammonium sulfate (176 mg/ml). The cell layer was homogenized with 0.5 M acetic acid and centrifuged. The resulting supernatant was dialyzed against 0.1% acetic acid and lyophilized. The proteins in the culture medium and cell layer were combined and pepsinized as described above, then resolved on 4% SDS-PAGE under reducing condition. The bands corresponding to $\alpha 1(\text{I})$, $\alpha 2(\text{I})$ and $\alpha 1(\text{III})$ were cut out and hydrolyzed with 6 M HCl for 18 h at 110°C . The radioactive hydroxyproline, proline, hydroxylysine, and lysine were separated by HPLC (AA-pak Na⁺ type, JASCO) and quantitated with a Beckman LS9800 liquid scintillation spectrometer.¹⁸

Northern blot analysis

Total RNA was isolated from cells.¹⁹ RNA samples were denatured in deionized 1 M glyoxal/10 mM phosphate buffer, pH 6.5 at 50°C for 1 h, then resolved on 1% agarose gel electrophoresis (10 μ g/lane) and blotted to nitrocellulose filters. The membranes were baked at 80°C for 2 h and hybridized to ³²P-labeled probes in 50% formamide, $5 \times \text{SCC}$, $5 \times \text{Denhardt's}$ solution, 0.1% SDS, 250 μ g/ml sonicated salmon sperm DNA at 42°C for 18 h. The following cDNA probes radioactively labeled by random priming were used; pro $\alpha 1(\text{I})$ collagen cDNA (Hf677),²⁰ pro $\alpha 1(\text{III})$ (Hf934)²¹ and β actin cDNA (Oncor Inc) (specificities $\sim 10^8$ cpm/ μ g DNA). The filters were washed at a stringency of $0.1 \times \text{SSC}$ at 42°C for 1 h and exposed to X-ray film at -80°C with an intensifying screen. The autoradiograms were scanned with a densitometer.

Determination of intracellular degradation of collagen

The proportion of collagen synthesized by the fibroblasts that was degraded within the cells prior to secretion was determined as previously described.^{22,23} Briefly, cultures were shortly-pulsed with [³H]proline in the

presence of ascorbic acid (50 μ M) for 30 min during the period the labeled collagen was still within the cells. The medium was aspirated and washed with PBS five times, then the cells were chased for 1 h in the medium containing unlabeled proline (1 mM). The cell layers were scraped off from the plate using 10 ml 0.5 M acetic acid, then sonicated and heated at 100°C for 5 min to inactivate proteases. An aliquot was taken for quantitation of [3 H]hydroxyproline using acid hydrolysis (6 M HCl, 110°C, 18 h).¹⁸ Another aliquot was dialyzed against 1% acetic acid containing protease inhibitor cocktail at 4°C for 48 h, then the retentate was hydrolyzed for quantitation of [3 H]hydroxyproline as described above. Percent dialysable 3 H-hydroxyproline was calculated from the formula: % dialysable 3 H-hydroxyproline = (c.p.m. of total 3 H-hydroxyproline in the sample) – (c.p.m. of 3 H-hydroxyproline in the sample after dialysis) $\times$ 100 / (c.p.m. of total 3 H-hydroxyproline in the sample).

Results

The rate of collagen synthesis in the cell layer of the patient fibroblasts was greater than that in the control fibroblasts, whereas that in the medium counterpart was essentially the same (or unchanged) between the patient and control fibroblasts (Table 1).

There were no significant changes in the ratio of type III collagen to type I collagen, suggesting that syntheses of both type I and III collagens which were major collagens synthesized by skin fibroblasts were equally elevated (Table 2).

Table 1 Collagen Synthesis in the Medium and Cell Layer Fractions of Cultured Fibroblasts from Marfan Syndrome and Control Subjects

Cell Strain	Relative Collagen Synthesis collagen cpm total protein cpm	
	medium	cell layer
Patient	0.40	0.07
	0.37	0.08
Control 1	0.38	0.03
	0.41	0.03
Control 2	0.44	0.02
	0.40	0.03

Cells were labeled with [3 H]proline for 18 h in the presence of ascorbic acid (50 μ M). Collagen synthesis was determined with purified bacterial collagenase digestion. Relative collagen synthesis was calculated by collagenase-sensitive counts/total protein counts. Values indicate data obtained from duplicate experiments.

The levels of type I and III collagen mRNA were unaltered between the patient and controls (Fig 1), indicating that enhanced collagen synthesis is due to post translational events including prolyl and lysyl hydroxylation of collagen and intra- or extracellular degradation of collagen.

Prolyl and lysyl hydroxylation levels of type I and III collagens produced by patient fibroblasts were essentially

Table 2 Relative Synthesis of Type III Collagen in Marfan Syndrome Fibroblasts

Cell Strain	Type III $\times$ 100/type I + type III
Patient	12.8
	14.1
Control 1	15.1
	13.3
Control 2	15.0
	13.6

Cells were labeled with [3 H]proline for 18 h in the presence of ascorbic acid (50 μ M). The proteins from medium and cell layer were combined and pepsinized for 18 h at 4°C (50 μ M). Pepsin resistant proteins were resolved on SDS-PAGE without reduction followed by autoradiography. Relative synthesis of type III collagen was expressed by type III collagen $\times$ 100/type I collagen + type III collagen from the autoradiograms. The values are from duplicate experiments.

Fig 1 Levels of type I and III collagen mRNAs in the patient fibroblasts. RNA was extracted from patient (lane 1) and controls (lanes 2 and 3) fibroblasts, resolved by electrophoresis on 1% agarose gel and blotted to membranes. The filters were hybridized with α 1(I) or α 1(III) cDNA probes.

Table 3 Prolyl and Lysyl Hydroxylation Levels of Collagens in Patient and Control Fibroblasts

Cell Strain	$\alpha 1(I)$		$\alpha 2(I)$		$\alpha 1(III)$	
	Pro/Hyp	Hyl/Lys	Pro/Hyp	Hyl/Lys	pro/Hyp	Hyl/Lys
Patient	0.90	0.41	0.85	0.87	1.16	0.45
Control 1	0.97	0.42	0.81	0.88	1.16	0.48
Control 2	0.93	0.48	0.80	0.90	1.20	0.51

Cells were dual-labeled with [^3H]proline and [^{14}C]lysine in lysine-free DNEM for 18 h. The proteins in the medium and cell layer were combined and pepsinized at 4°C for 18 h. Pepsin resistant proteins were resolved on SDS-PAGE. The bands corresponding to $\alpha 1(I)$, $\alpha 2(I)$ and $\alpha 1(III)$ were cut out and hydrolyzed with 6 M HCl for 18 h at 110°C. Labeled hydroxyproline, proline, hydroxylysine and lysine were separated on HPLC and counted with liquid scintillation counter.

Table 4 Intracellular Degradation of Collagen in Patient and Control Fibroblasts

Cell Strain	% Degradation
Patient	5.4 1.5
Control 1	23 27
Control 2	37 29

Cells were labeled with [^3H]proline for 30 min and chased for 1 h. The cell layer was disrupted with 0.5 M acetic acid and dialyzed exhaustively against 1% acetic acid. The samples prior or after dialysis were hydrolyzed in 6 M HCl for 20 h at 110°C. ^3H -Hydroxyproline was determined by HPLC column. Percent degradation was calculated as described in Materials and Methods. Values are from duplicate experiments.

unchanged compared to those in control fibroblasts (Table 3).

Intracellular degradation rate of collagen in the fibroblasts from Marfan syndrome was significantly lower than that in control cells (Table 4), indicating that the enhanced collagen synthesis may be caused by the reduced intracellular degradation rate of collagen.

Discussion

Intracellular degradation of collagen in control fibroblasts was ~20–30%, the values were in good agreement with the previously reported data.^{22,23} Intracellular protein degradation is believed to be controlled by the conformation of the proteins, but it is unclear what

structural features in fact determine protein half-lives.¹⁵

Several abnormalities in collagen metabolism in Marfan syndrome have been previously reported. These include increased urinary excretion of hydroxyproline^{2,3} increased solubility of dermal collagen^{3,4} and decreased cross-links.⁵ In this study, reduced intracellular degradation of collagen was found in our case. These abnormal metabolism of collagen may be a secondary defect rather than a primary defect. Although the significance of the reduced intracellular degradation of collagen in Marfan syndrome is unclear at present time, this abnormality will result in the abnormal structural architecture of connective tissue because fine balance between synthesis and degradation is required for the physiological function of connective tissue.

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