KAWASAKI MED. J. VOL. 13 : NO. 3, 111-116 (1987)

Alterations in Synthesis of Various Connective Tissue Components in Werner's Syndrome Cultured Dermal Fibroblasts

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Accepted for publication on August 11, 1987

ABSTRACT. Werner's syndrome (WS), a premature aging disorder, is known to have scleroderma-like skin changes. We studied both collagen and glycosaminoglycan (GAG) synthesis in dermal fibroblasts derived from two patients with WS. The total synthesized protein was determined by the incorporation of [^{3}H]-proline, and the synthesized collagen was measured as labeled hydroxyproline. The GAG synthesis was measured by the incorporation of [^{3}H]-glucosamine. The synthesis of total and collagenous protein of fibroblasts from WS was increased about threefold respective to controls. The GAG synthesis of fibroblasts from WS patients was also significantly higher than normal controls. These results suggest that the connective tissue synthesis in WS might be common to be that of scleroderma in the active phase.

Key words : Werner's syndrome — collagen synthesis — glycosaminoglycan synthesis — dermal fibroblast — connective tissue

Werner's syndrome (WS), a premature aging disorder, has been considered as a model of normal aging because it has many features in common with normal aging. WS may cause abnormalities of connective tissue metabolism which are seldom seen in normal aging;[1] these changes include scleroderma-like skin, hyaluronic acid in the urine[2,3] and so on. According to these findings, Werner's syndrome is assumed to be a disorder of systemic connective tissue metabolism.[4]

Cultured fibroblasts derived from WS are generally unsuitable for the research in connective tissue metabolism due to their short life span in culture. In this study, we have succeeded in developing fibroblasts which are able to grow in culture. These samples were tested for collagenous protein synthesis and glycosaminoglycan (GAG) synthesis. The levels of synthesis were compared with age, sex matched control samples.

MATERIALS AND METHOD

Cell culture

Fibroblast cultures were established from abdominal skin of two patients with typical Werner's syndrome (35 years old = WF-1, 51 years old = WF-2) and from age, sex matched normal controls (42 years old = NF-1,

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40 years old = NF-2, 53 years old = NF-3). The skin specimens were cut into small pieces, and placed into sterile plastic culture dishes (Falcon). After the tissue attached to the dishes, the culture was initiated with Dulbecco's modified Eagles medium (DMEM, Nissui Seiyaku Co.) containing glutamine, and supplemented with 100 μg/ml kanamycin and 20% fetal bovine serum (FBS, Flow Laboratories). The pH of the medium was controlled by addition bicarbonate. The dishes were placed in a CO_2 incubator (95% air/5% CO_2) and maintained at 37°C. After outgrowth of cells from explant culture, subcultures were established by trypsinization (0.2% trypsin) of primary cultures and placed in 25 cm^2 plastic flasks (Corning). The concentration of FBS in DMEM was then changed to 10%. This medium was used for routine maintenance of the fibroblasts and was changed at intervals of 3 days. Routine subcultivation was performed at a 1:2 or 1:4 split ratio and life span was determined respectively.

In the investigation of collagen and glycosaminoglycan synthesis in fibroblasts from the patients with Werner's syndrome and normal controls, approximately 1×10^5 cells were seeded onto 35×10 mm plastic dish (Falcon) with 2 ml of the medium. The culture was placed in a CO_2 incubator and the medium was changed on day 3, 7, 10 after seeding. On day 10, the cell monolayer had become confluent or preconfluent state and were used for the assay.

Measurement of collagen and total protein synthesis

Four dishes of Werner and control cultures were assayed at confluency. The culture medium was removed and DMEM supplemented with 3.4 μg/ml α-ketoglutarate, and 50 μg/ml ascorbic acid was added for 1 hour. Each culture dish was labeled with 5 μCi/ml L-[2,3-^{3}H] proline (spec. act. 38.5 Ci/mmol, NEN Chemicals) and incubated in a CO_2 incubator for 24 hrs. Parallel cultures were trypsinized and used for determining cell number using a hemocytometer. After labeling, the medium was dialyzed for 48 hrs against distilled water at 4°C to remove free proline. Bovine serum albumin (BSA) was added to a final concentration of 0.02%; Trichloroacetic acid (TCA) was added to a final concentration of 10% and TCA insoluble precipitates were collected by centrifugation at 3000 rpm for 10 min. The cell layer was washed twice with cold phosphate buffer saline (PBS) and sonicated for 1 min on ice. Then BSA was added to a final concentration of 0.02%, and TCA was added to a final concentration of 10%, and the TCA insoluble precipitates were collected (total two times). Each of the precipitates was hydrolyzed, in a sealed Pyrex tube with 2 ml 6 N HCl at 125-135°C for 6 hrs. The hydrolysates were dried in vacuo and dissolved in 4 ml of distilled water. 0.4 ml aliquots were transferred into counting vials, and mixed with 8 ml Aquasol (NEN Chemicals). The radioactivity was considered as incorporation of total [^{3}H]-proline. Labeled hydroxyproline was assayed from the remaining hydrolysate, according to the method of Juva and Prockop,[5] and that was considered as a newly synthesized collagen.

Measurement of GAG synthesis

When all cultures had reached confluency or preconfluency, the medium was removed and the cultures were incubated in 2 ml of FBS free DMEM for 1 hr. The cultures were then labeled with D-[1-^{3}H]-glucosamine hydrochloride 5 μCi/ml (spec. act. 10.4 Ci/mmol, CEA) and placed in CO_2 incubator for 24 hrs. After cultivation, the medium and the harvested cells were treated

separately with 0.5 N NaOH at 4°C for 24 hrs. After adjusting the pH to 6–8 by adding 1 N HCl, an equal volume of 0.1 M Tris-HCl buffer pH 7.8 containing 5 mM $CaCl_2$ was added to each sample. The solution was boiled for 30 min and digested by pronase E (Kaken Chemicals) at 50°C for 48 hrs. TCA was added to a final concentration of 10% and the precipitate was removed by centrifugation. The supernatant was dialyzed against distilled water at 4°C for 48 hrs. After dialysis, carrier GAG (hyaluronic acid, chondroitin sulfate A and C, Dermatan Sulfate, Seikagaku Kogyo) was added to each sample followed by cetylpyridinium chloride (CPC, Nakarai Kogyo) to a final concentration of 0.3%. Then the GAG-CPC complex was incubated at 37°C for 1 hr and centrifuged. The pellet was washed twice with 0.04 M NaCl in 0.1% CPC to remove remaining glycopeptide. The GAG-CPC complex was dissolved in 4 M NaCl and the aliquot was taken to measure the radioactivity which was considered as synthesized GAG.

Statistical analysis

All data were statistically analyzed by student's t-test.

RESULTS

The patients' cells showed an increase in overall size, and vacuolation of cytoplasm with respect to fibroblasts from normal controls. The growth of the patients' cells was the same as that of normal cells in early passage. After attaining at 10 population doubling level (PDL), however, they grew more slowly, and they seemed more senescent. The longest life span of patients' cells in this study was 17 (WF-1) and 18 (WF-2) PDL, whereas all normal controls could be maintained for more than 35 PDL.

The collagen synthesis by the patients' cells was studied through four separate experiments. The results are shown in Table 1. The samples derived from patients were compared with age, sex, PDL matched controls. We determined

TABLE 1. Total [^{3}H]-proline incorporation and collagen synthesis by fibroblast derived from patients with Werner's syndrome and normal controls

Series	PDL	Total [^{3}H]-proline incorporation (cell layer, medium) [dpm/10^4 cells]	[^{3}H]-hydroxyproline synthesis (cell layer, medium) [dpm/10^4 cells]
EXP-1			
WF-1	5	35071 ± 1247**** (12431, 22640)	6169 ± 242**** (1272, 4898)
NF-1	5	12900 ± 412 (5003, 7828)	2291 ± 97 (511, 1780)
EXP-2			
WF-1	6	16975 ± 1040**** (7555, 9420)	2756 ± 272**** (585, 1961)
NF-2	6	6535 ± 521 (3260, 3275)	810 ± 45 (282, 514)
EXP-3			
WF-2	9	5843 ± 550*** (1610, 4233)	1158 ± 124*** (181, 977)
NF-2	9	2043 ± 146 (752, 1291)	333 ± 30 (94, 239)
EXP-4			
WF-2	13	6175 ± 334**** (3109, 3065)	810 ± 53**** (407, 402)
NF-3	13	2542 ± 130 (759, 1783)	350 ± 23 (110, 241)

Four dishes were determined. Each value represents mean±SEM.
Difference from control: *** $p<0.005$, ****$p<0.001$

collagen synthesis by adding up values obtained in the cell layer and in the medium per 10^4 cells. In all experiments examined, the total [^{3}H]-proline incorporation were significantly (2.4 to 2.9 times) higher in samples from patients with WS than these in samples from controls. The synthesis of collagen by the WS patients' cells also increased (2.3 to 3.5 times) comparing with that by controls.

In order to examine synthesis of another connective tissue component, we assayed further the GAG synthesis under the same culture conditions used in determination of the collagen synthesis. Newly synthesized, total GAG in both cell layer and medium by patients' cells were examined in three separate experiments. The data is shown in Table 2. In all experiments examined, fibroblasts derived from WS were more active (about 2–4 times) than normal controls with respect to total GAG synthesis in the cell layer and the medium but no statistically significant difference was noted in EXP1 (medium) and in EXP2 (cell layer). In regard to cell layer/medium ratio, no obvious difference was shown beween patients' cells and normal controls.

TABLE 2. Glycosaminoglycan synthesis by fibroblasts derived from patients with Werner's syndrome and normal controls

Series	PDL	Cell layer [dpm/10^4 cells]	Medium [dpm/10^4 cells]	Ratio of cell layer to medium
EXP-1				
WF-1	4	89 ± 4**	180 ± 23	0.49
NF-1	4	50 ± 1	145 ± 15	0.34
EXP-2				
WF-1	5	75 ± 9	316 ± 45*	0.24
NF-2	5	47 ± 19	194 ± 22	0.24
EXP-3				
WF-2	15	38 ± 3**	145 ± 12**	0.26
NF-3	15	10 ± 1	35 ± 3	0.29

Four dishes were determined. Each value represents mean±SEM.
Difference from control: *$p<0.05$, **$p<0.01$

DISCUSSION

Although it has been recognized that Werner's syndrome may cause abnormalities of connective tissue metabolism, the research of the cell culture *in vitro* has not been done sufficiently. This is due to the difficulty of culturing fibroblasts derived from WS because of their slow growth and short life span. Hence such this disease must be investigated, case by case. We obtained fibroblasts derived from two WS patients and succeeded in culturing them. These samples were tested for synthesis of both collagen and GAG, the main components of connective tissue matrix, comparing with age, sex matched control samples.

In this study, the synthesis of total protein, collagenous protein, and GAG by fibroblasts from our patients were obviously higher than normal controls. In regard to collagen synthesis, Tajima and his colleagues[7] had reported the increase of collagen synthesis in fibroblasts derived from the thigh accompanying

skin changes like scleroderma. Gawkrodger and his colleagues[8] had also reported an increase of collagen synthesis in fibroblasts derived from the forearm of a 29-year-old male WS patient. These results are almost consistent with ours. This increase of collagen synthetic activity is assumed to be parallel with the increase of total protein synthesis.

On the other hand, as regard to synthesis of GAG by fibroblast from WS Tajima *et al.*[9] reported an increased total GAG synthesis and accumulation on the cell surface in two patients of WS's fibroblasts. In contrast, Gawkrodger *et al.*[8] noted low production of GAG in fibroblasts from a male patient and Maekawa *et al.*[10] reported that the synthesis of GAG per cell per hour by WS fibroblasts was quantitatively the same as the healthy control's. Like the above, there have been various reports that revealed different results. However, both of our two cases, appeared an increased GAG synthesis in cell layer and medium, and that is in accord with Tajima *et al.*'s report.[9] It is a well-known fact that normal human diploid fibroblasts show a reduced synthesis of collagenous protein[11,12] and GAG[13] after continued *in vitro* aging. These facts would indicate that the connective tissue metabolism in fibroblasts from WS is different from that of cultured aging fibroblasts.

The other main component in extracellular connective tissue matrix, besides collagen and GAG, which is synthesized by fibroblasts is fibronectin (FN), which is a high molecular weight glycoprotein. We have determined that the amount of FN released into the medium by WF-1 was fivefold as much as that by NF-2 using enzyme linked immunosorbent assay (Data not shown). Such a finding does not prove increased synthesis of FN directly, but it suggests an enhancement of FN producing ability in patients' cells.

To summarize the findings obtained in this study, it is shown that the synthetic activity in connective tissue of WS patients' fibroblasts is enhanced. That is similar to the features of fibroblasts derived from scleroderma patients, namely increased production of collagen and other components of connective tissue, such as GAG and FN, which is reported by LeRoy.[14] However, the possibility still remains that such features of WS fibroblasts are the secondary effects from a primary disorder, as Tajima *et al.* had reported. Further study of protein degradation in WS fibroblasts should be done to clarify the connective tissue metabolism of WS. Measurement of collagenase activity will play an important role in the study.

Acknowledgment

The authors wish to thank Yoko Jinno for technical assistance and Paul Galumbek for critical reading of the manuscript. This work was supported by a grant from the Japanese Ministry of Public Welfare for Scleroderma in 1985.

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