Focal Dermal Hypoplasia (Goltz Syndrome): A Decreased Accumulation of Hyaluronic Acid in Three-dimensional Culture

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We report a 28-year-old female with focal dermal hypoplasia, (Goltz syndrome). We compared the growth kinetics and the production of type I collagen and glycosaminoglycans by fibroblasts from affected and unaffected skin. Fibroblasts were grown in conventional medium supplemented with ascorbic acid 2-phosphate, which makes fibroblasts form a tissue-like structure in vitro. The population doubling time of fibroblasts in affected skin was slightly shorter than that of unaffected skin. There was no difference in the levels of the carboxyterminal propeptide of type I procollagen liberated into the media between affected skin and unaffected skin. However, the cell layer of affected skin fibroblasts revealed a decreased amount of hyaluronic acid-derived disaccharide unit (ΔDi-HA). The abnormal metabolism of glycosaminoglycan in the affected dermis might be involved in the development of skin changes in focal dermal hypoplasia.

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Focal dermal hypoplasia (FDH, Goltz syndrome), is a rare genetic disorder that is often associated with multiple mesenchymal abnormalities (1). Although the pathogenic mechanisms of skin changes in FDH are not well understood, compromised growth potential of dermal fibroblasts (2) and the absence of type IV collagen in the lesion (3) have been reported. In the present report, we describe a case with typical cutaneous manifestations, in which some abnormalities in the glycosaminoglycan metabolism of cultured fibroblasts derived from affected skin are discussed.

CASE REPORT

A 28-year-old female was referred to our department for her skin changes in November, 1993. At birth, disseminated atrophic and hyperpigmented macules with scattered telangiectases were noted. At the age of 16, she was diagnosed as having mixed connective tissue disease and hypothyroidism. Since that time, she had taken oral prednisone,10 mg/day. In 1992, she had an uneventful pregnancy. She was pregnant again in March 1993 and hospitalized for severe thrombocytopenia (4×10^9/l) in our hospital in October.

On physical examination, she had disseminated atrophic and hyperpigmented macules with scattered telangiectases on the neck, trunk and extremities. The skin lesions showed a linear or serpiginous pattern on the extremities, and a cribriform pattern on the mammae. Some round or oval, slightly yellowish erythemas were found on the outer surface of the legs. The patient's nails, scalp, and hair were not affected.

Laboratory tests revealed hemoglobin 10.8 g/dl, white blood cell count 11.8×10^9/l, platelet count 86×10^9/l, total protein 5.6 g/dl, albumin 3.1 g/dl, positive antinuclear antibody titer of 1:640, and positive anti-RNP antibody titer of 1:8. The complement levels (C3 and C4) were normal.

Histological examination of a yellowish erythema on the right lower leg showed normal epidermis. Islands of adipose tissue were scattered within the dermis, which was stained with oil red O. The dermis showed a slight interstitial edema. On alcian blue staining, the affected skin revealed a weaker reaction than the unaffected skin. These clinical and histological findings warranted the diagnosis of FDH.

Fibroblast cultures

Fibroblasts were obtained from the affected and unaffected skin of the patient, and also from a control subject (23-year-old male). Cells were grown in Dulbecco's modified Eagle medium (DMEM; Sanko Junyaku Kogyo,Tokyo, Japan), supplemented with 10% fetal calf serum (FCS, CYTOSYSTEM, NSW) in 5% CO_2 at 37°C. Fibroblasts were passaged serially by trypsinization and used for experiments between the 3rd and 6th passage.

We added L-ascorbic acid 2-phosphate (Asc 2-p, Wako Pure Chemical Industries, Ltd., Osaka, Japan) for the measurement of hydroxyproline and glycosaminoglycans. Asc 2-p can make skin fibroblasts form a three-dimensional structure by stimulating collagen accumulation in vitro (4, 5). We reported that the main disaccharide units produced by normal human dermal fibroblasts in this culture system were similar to those produced by normal human dermis (5).

Growth kinetics

The growth kinetics of fibroblasts was determined by seeding parallel cultures from a single pool at a density of 1×10^4/well in 24-multiwell plates (Costar, Cambridge, USA). Replicate cultures were fed or harvested for cell count twice a week. The mean value was obtained from three separate experiments in triplicate culture.

Assay of the carboxyterminal propeptide of type I procollagen (PICP)

Fibroblasts were grown to confluency in DMEM containing 10% FCS. Media collected 4 days after the last medium change were used for assay. PICP was determined with an enzyme immuno-assay kit (Takara, Tokyo, Japan) according to the manufacturer's protocol (6).

Determination of hydroxyproline

Fibroblasts were grown in 100-mm diameter plastic dishes and fed with DMEM supplemented with 10% FCS and 1.0 mM Asc 2-p twice a week. After 3 weeks' culture, the cell layer was washed with cold PBS three times and scraped with a rubber policeman, and further washed with PBS three times. The sample suspended in PBS was sonicated for 5 min at 20 mW (Model 450 Sonifier, Branson, Danbury, USA) and centrifuged at $10,000 \times g$ for 20 min. The pellet containing neutral insoluble collagens was lyophilized. The content of hydroxyproline was measured according to the method described by Woessner (7). A hydroxyproline standard solution at a concentration of 7.63–38.13 μmol/ml was run in parallel. The hydroxyproline values were determined directly from the standard curve.

Preparation of glycosaminoglycans (GAGs)

The cell layer was prepared as in the hydroxyproline determination. After 3 weeks' incubation, the cell layer was washed with PBS three times and scraped. Crude GAGs were isolated using a modification of the previously described method (8). Briefly, a sample treated with 2% NaOH overnight at 4°C was neutralized with HCl. Then, the sample was digested with pronase, followed by deproteinization with 10% trichloroacetic acid and centrifugation. The supernatant was dialyzed against running water for 2 days and GAGs were precipitated with 0.1% cetylpyridinium chloride in the presence of 0.012 N sodium sulfate. After centrifugation, the precipitate was washed with 95% ethanol saturated with NaCl twice and then with pure ethanol, and dried. The crude GAGs were dissolved in water at the concentration of 1 mg/100 μl (w/v) and used for further disaccharide analysis.

Table I. *Growth kinetics of focal dermal hypoplasia fibroblasts*

Culture	Population doubling time* (hr)
Patient, affected skin	64.8 ± 20.2**
Patient, unaffected skin	84.1 ± 21.2

* Values expressed as mean ± SD. Values represent the results of 3 experiments.
** Not significant as compared with unaffected skin.

Disaccharide analysis

Fifty μl of the sample solution was evaporated and digested with chondroitinase-ABC or with chondroitinase-AC II (Seikagaku Kogyo, Tokyo, Japan), as described elsewhere (9). The precolumn labeling with 1-phenyl-3-methyl-5-pyrazolone (PMP) was carried out according to the modified method described by Honda et al. (10). The samples or commercially available chondroitin sulfate (CS)-, dermatan sulfate (DS)- and hyaluronic acid (HA)-derived disaccharides (Seikagaku Kogyo, Tokyo, Japan) were dissolved in 20 μl of 0.3 M NaOH. An equal volume of 0.5 M PMP in methanol was added and the mixture was kept at 70°C for 30 min. Xylose was added as an internal standard. After PMP labeling, 60 μl of 0.1 M HCl was added to neutralize, and the mixture was extracted with 50 μl of chloroform twice. The aqueous layer was evaporated to dryness, then the residue was dissolved in 100 μl of water, and an aliquot was applied to high performance liquid chromatography (Model L-6200, Hitachi, Japan) equipped with a CHEMCO 3C18 column (6 × 100 mm). Elution was performed with a linear-gradient from 5 to 30% of acetonitrile/water (3 : 1, v/v) in 20 mM phosphate buffer, pH 7.5 containing 5% acetonitrile at a flow rate of 1 ml/min at 50°C. Peaks were detected at 245 nm. ΔDi-6S (CS), ΔDi-4S(CS), the main disaccharide unit of chondroitin sulfate A, and ΔDi-HA were determined by unsaturated disaccharides liberated with chondroitinase AC digestion. ΔDi-4S (DS), dermatan sulfate-derived disaccharide, was calculated by deducting the above ΔDi-4S (CS) from total ΔDi-4S liberated with chondroitinase ABC digestion.

Statistical analysis was performed by Student's t-test or unpaired t-test.

RESULTS

Fibroblasts from affected skin revealed a slightly shorter population doubling time than those from unaffected skin (Table I).

The PICP level and the hydroxyproline content were slightly decreased in the fibroblasts from affected skin, as compared with those from unaffected skin or normal control. However, the difference was not statistically significant (Tables II, III).

The amount of main disaccharide units in the cell layer is shown in Table IV. The total amount was remarkably decreased in the cell layer from affected skin, as compared with that from unaffected skin or control skin. This decrease was mainly due to the decrease in ΔDi-HA derived from

Table II. *Carboxyterminal propeptide of type I procollagen (PICP) levels in the culture media*

Culture	PICP* (×10⁻² ng/ml)
Patient, affected skin	17.5 ± 2.0**
Patient, unaffected skin	21.1 ± 1.7
Normal skin (23y, male)	22.2 ± 1.9

* Values expressed as mean ± SD. Values represent the results of 3 experiments.
** Not significant as compared with unaffected skin or normal control.

Table III. *Hydroxyproline contents in the three-dimensional fibroblast culture*

Culture	Hydroxyproline (nmol/mg dry weight)
Patient, affected skin	144.5 ± 0.076*†
Patient, unaffected skin	121.0**
Normal skin (23y, male)	115.1 ± 0.033*

* Values expressed as mean ± SD. Values represent the results of 3 experiments.
** Values expressed as mean of two experiments.
† Not significant as compared with unaffected skin or normal control.

hyaluronic acid. The unaffected skin revealed an intermediate value between the affected and normal skin.

DISCUSSION

FDH is a rare genodermatosis affecting tissues of ectodermal and mesodermal origin (1). Presumed to be transmitted in an X-linked dominant fashion with lethality in males, approximately 95% of reported cases are sporadic (11). Skin involvement is essential, including hypoplastic and atrophic skin changes, linear and reticular areas of hypo- and hyperpigmentation, lipomatous lesions, periorificial and mucous membrane papillomas, and telangiectases. The clinical spectrum of the disease is wide, from multisystem FDH to FDH limited to the skin (12). We diagnosed our patient as limited type without extracutaneous abnormalities.

The pathogenic mechanisms of FDH are not fully understood. On the basis of the histological findings characterized by hypoplasia of connective tissue replaced by adipose tissue, some investigators postulated that the cutaneous defects of FDH might be due to ectodermal dysplasia (13). Tsuji, however, suggested an active adipose tissue proliferation rather than connective tissue involution alone based on the microscopical presence of young adipocytes (14). Uitto et al. (2) indicated an abnormality in the growth kinetics of fibroblasts and normal synthesis of collagen. In the present study, we found no significant difference between PICP and hydroxyproline levels in fibroblasts from affected skin and those from unaffected skin or normal skin. We did not, however, observe any abnormal growth kinetics in fibroblasts from affected skin.

In the present case, we found a decreased accumulation of ΔDi-HA, one of the main disaccharide units, in the cell layer of fibroblasts from affected skin, while it was regrettable that we could not directly analyze the disaccharide units in the patient's skin. The altered synthesis of HA, an essential constituent in the skin, has been presumed to be critical in the regulation of cell proliferation and the metabolism of other extracellular matrices (15–17). Higuchi et al. (18) reported a decreased amount of ΔDi-HA in the atrophic skin of patients with Werner's syndrome. They speculated that the decrease in ΔDi-HA might be responsible for the atrophic skin change through HA-associated dehydration and shrinkage of the connective tissue.

In conclusion, it is possible that the decreased accumulation of HA may be partly responsible for the development of atrophic skin lesions in FDH, though it is uncertain whether abnormal HA metabolism is primary or secondary event in FDH.

Table IV. *The content of main disaccharide units in the cell layer of the three-dimensional culture*

Culture	ΔDi-6S	ΔDi-4S(CS)	ΔDi-4S(DS)	ΔDi-0S	ΔDi-HA	Total
Patient affected skin	2.19 ± 0.18#	2.93 ± 0.30**	14.84 ± 3.51†	0.89 ± 0.07*†	25.20 ± 4.03***††	46.06 ± 7.95***††
Patient unaffected skin	1.93 ± 0.62	3.84 ± 0.69*	7.03 ± 3.37	0.49 ± 0.46	89.27 ± 10.26**	102.56 ± 6.07**
Normal skin (23y, male)	2.99 ± 0.47	8.91 ± 1.94	10.51 ± 1.89	1.38 ± 0.27	131.53 ± 11.68	155.31 ± 12.28

\# Values expressed as mean ± SD. Values represent the results of 3 experiments.
* $p < 0.05$ vs normal skin. ** $p < 0.01$ vs normal control. *** $p < 0.001$ vs normal skin.
† $p < 0.05$ vs unaffected skin. †† $p < 0.01$ vs unaffected skin.
ΔDi-6S; main disaccharide unit of chondroitin sulfate C, ΔDi-4S(CS); main disaccharide unit of chondroitin sulfate A, ΔDi-4S(DS); main disaccharide unit of dermatan sulfate, ΔDi-0S; main disaccharide unit of chondroitin, ΔDi-HA; main disaccharide unit of hyaluronic acid.

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