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Werner's Syndrome: No Difference in *In Vitro* Life Span of Dermal Fibroblasts from Proximal and Distal Parts of the Body

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Recently it has been reported that fibroblasts from distal parts of the body of a patient with Werner's syndrome grew poorly in vitro as compared with those from the proximal part of the same patient. To confirm this observation, cultures of fibroblasts from different parts of the body were set up in 2 cases of Werner's syndrome, but no significant difference in life span was observed. Fetal calf serum (FCS) and fibroblast growth factor (FGF) stimulated growth of fibroblasts from different body parts equally well. These data indicate that there is no difference in growth activity of fibroblasts from proximal and distal body parts in patients with Werner's syndrome. Moreover, the growth rate of epidermal outgrowths did not differ significantly between proximal and distal parts of these patients. ***Key words: Ageing of skin; Fibroblast culture; Comparison of life span; Scleroderma-like skin.***

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Werner's syndrome shares several features with normal ageing, but the primary retardation of growth and multiple phenotypic effects which occur imply that it is a partial copy rather than a full model of accelerated ageing. A characteristic feature of Werner's syndrome is the presence of a stocky trunk and slender extremities, with scleroderma-like alterations in acral areas. Culture growth in vitro of skin fibroblasts from patients with Werner's syndrome is said to be more difficult than that of normal skin fibroblasts (1). Furthermore, in Werner's syndrome, fibroblasts often have a reduced in vitro life span and lengthened mean population doubling time (2). Recently, it was reported that fibroblasts obtained from acral areas of a patient grew poorly and could not be subcultured, whereas those obtained from skin on the trunk could be subcultured (3).

We have investigated the in vitro life span of derrmal fibroblasts obtained from different body areas of patients with Werner's syndrome, and suggest that there is no significant difference between fibroblasts from proximal vis-à-vis acral areas of the body.

MATERIAL AND METHODS

Patients

Case 1 (Fig. 1*a–c*). A 30-year-old female Japanese patient visited our clinic in January 1978 because of pain in the soles of her feet which she had been suffering from for 10 years. Examination revealed that she had a short stature (145 cm, 33 kg), poliosis, baldness, and thick keratoses and painful ulcers on her soles. She had a beak-shaped nose, a stocky trunk and slender extremities. Her voice was high-pitched and raspy. The skin of her extremities, especially of the hands and feet, had a sclerodermoid and poikilodermatous appearance. The patient had been suffering from cataracts, but did not have diabetes mellitus.

Case 2 (Fig. 1*d–f*). A 54-year-old male Japanese patient visited our clinic in December 1987, because of concern about his short stature (154 cm), hoarseness, poliosis, baldness and clavus. Examination revealed that he had had poliosis since the age of 12. He had a beak-shaped nose, stocky trunk and slender extemities. His voice was high-pitched and raspy. His extremities, especially the hands and feet, had a sclerodermoid and poikilodermatous appearance. He had been suffering from cataracts and diabetes mellitus. Thick keratoses had developed on his soles.

Family history

In Case 1, there was no intermarriage. The patient had 4 brothers and 3 sisters. Propositus was the 6th child. Her youngest male sibling, who had died in a traffic accident, seemed to have had characteristics similar to those of our patient.

In Case 2, there was also no history of intermarriage. The patient had 2 brothers and 2 sisters. He was the 2nd child. The elder of his 2 sisters (decreased) had suffered from cataracts and diabetes mellitus and had had a similar characteristic appearance.

Materials

Dulbecco's modified minimum essential medium (DMEM), and fetal calf serum (FCS) were purchased from Osaka Dainihon Pharmaceutical Co. (Osaka, Japan). Fibroblast growth factor basic form (FGF) was from R & D Systems Inc. Disposable tissue culture dishes (35 mm) and 24-well multiplates were from Corning®.

Culture

Punched-out skin specimens (1½ mm) were obtained from different parts of the patients' bodies. Each piece was then cut into about 20 pieces under a dissecting microscope, and was placed separately in a 35-mm dish with about 0.3 ml of culture medium. Two or 3 days later, 1 ml of fresh medium was added to each dish. Epidermal cell outgrowths appeared in 1 to 3 days of culture. The distance between the free edge of the outgrowth and the edge of the original skin sample was measured under a phase-contrast microscope at 10 days of culture. Fibroblasts appeared after 5 to 10 days of culture, and started to surround the epidermal outgrowths. When fibroblasts occupied the whole surface of the dish after approximately 1 month of culture, they were separated by treatment with 0.02% EDTA + 0.05% trypsin (1 : 1) for 1 to 3 min. Separate cells from each dish were washed by centrifugation, placed into four 35-mm culture dishes and cultured in 2 ml/dish of culture medium. Thereafter, subcultures were conducted with a 1 : 4 split. To examine the life span of fibroblasts, the maximum number of passages (MNP) of fibroblasts in vitro was determined. As normal controls, a 94-year-old female and an 18-year-old male were chosen.

Effects of FCS and FGF

Firstly, separated fibroblasts at the 2nd subculture were placed in a 24-well multiplate at the rate of about 1000 cells/well in 1 ml of DMEM + 20% FCS, and cultured for 2 days. Then the medium was replaced with 1 ml of DMEM with different concentrations of FCS or FGF; after 4 days of culture, the number of cells/400× field was determined under a phase-contrast microscope. In case of FGF, the culture medium was not supplemented with FCS.

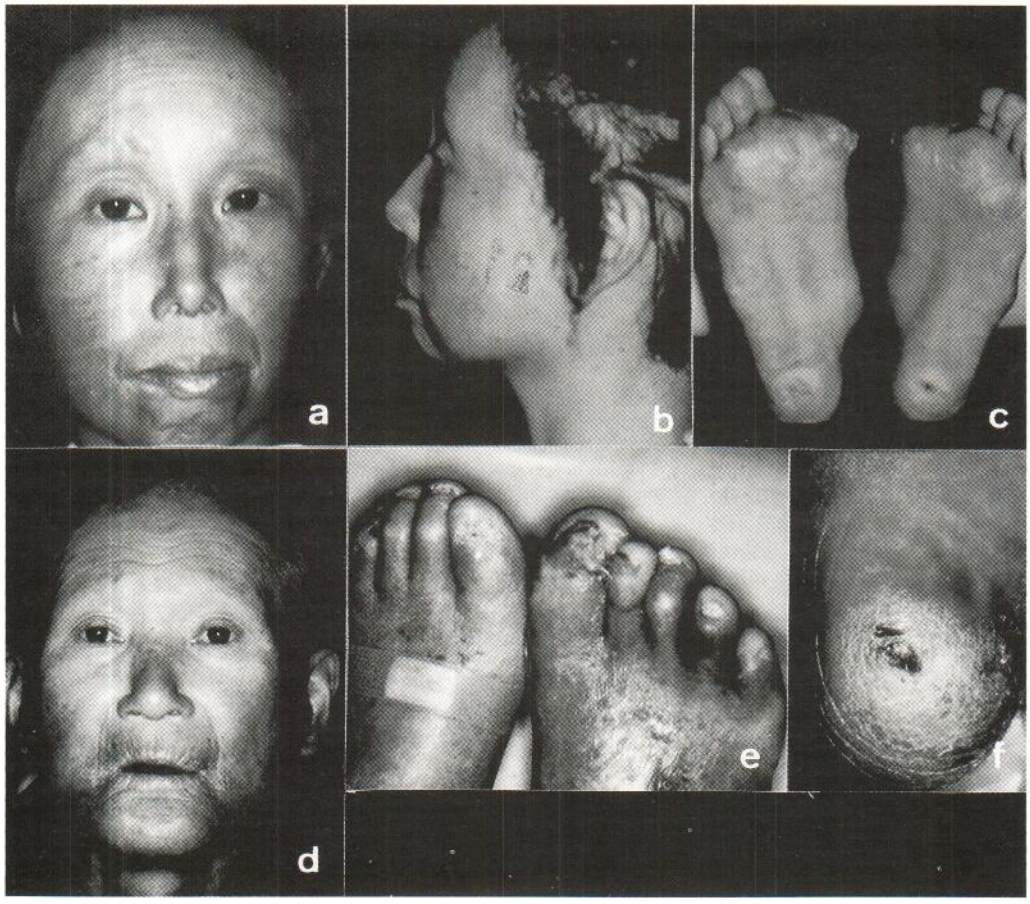

Fig. 1. Werner's syndrome. (*a–c*) Case 1. (*d–f*) Case 2. Beak-shaped noses (*a, b,* and *d*), alopecia (*b*), keratoses and ulcers on soles (*c* and *f*), and sclerodermoid skin on dorsal feet (*e*) are evident.

RESULTS

Life span of fibroblasts in culture

For an indication of the life span of fibroblasts, the MNP of fibroblasts in vitro was determined. In Case 1 (Fig. 2, left), the MNP of fibroblasts in skin from different body areas ranged from 8 to 12 (average 9.6): the proximal and distal portions did not differ significantly in MNP ($p > 0.1$). In Case 2 (Fig. 2, middle), the MNP of fibroblasts in skin from different body areas ranged from 11 to 16 (average 13.5): the head portions, the proximal portions, the upper extremities, and the lower extemities were also not significantly different ($p > 0.1$ for all combinations). In a 94-year-old female normal control (Fig. 2, right), the MNP of fibroblasts in vitro ranged from 15 to 22 (average 18.5), showing no significant difference in MNP between proximal and distal portions of the body. In another control, an 18-year-old male, the MNP ranged from 29 to 42 (average 34.6): the face 15, the chest 16, the back of the hand 21 and the dorsum of the foot 22, also showing no significant difference in MNP between proximal and distal portions of the body (figures not shown).

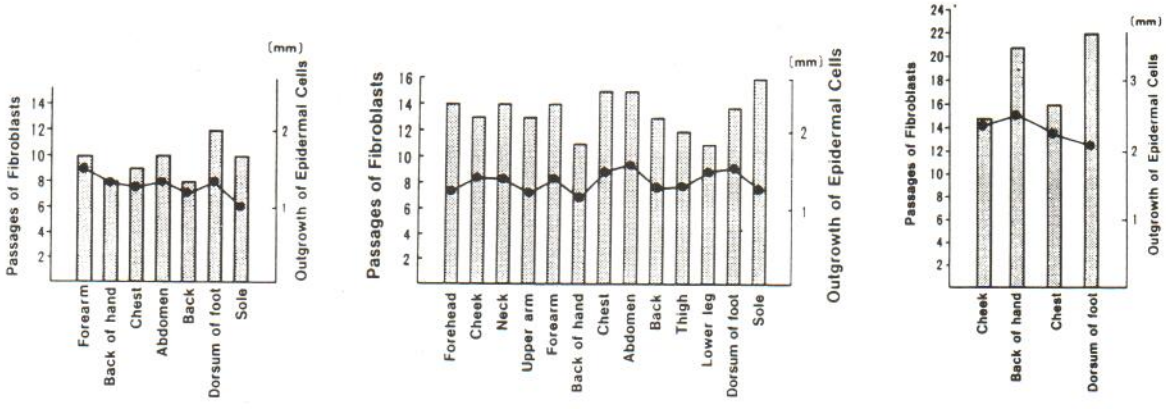

Fig. 2. The maximum number of passages (MNP) of cultured fibroblasts, and width of epidermal outgrowth in initial outgrowth culture. *Left:* Case 1. *Middle:* Case 2. *Right:* Normal control. Bars indicate MNP for various parts of the body. (●) Width of epidermal outgrowth for various parts of the body at 10 days of culture.

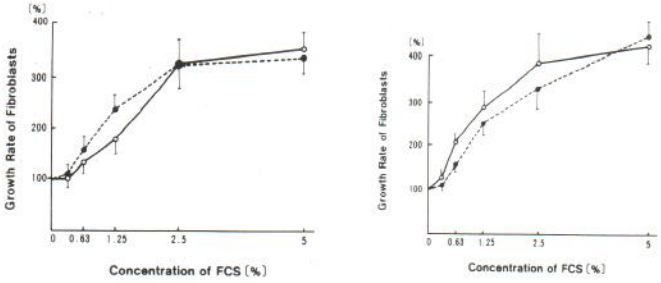

Fig. 3. Effect of FCS on growth of fibroblasts in vitro. *Left:* Case 1. *Right:* Case 2. Fibroblasts from the back (○), and from the dorsal foot (●).

Outgrowth of epidermis

In Cases 1 and 2, and one normal control (Fig. 2, left, middle and right) there was no significant difference in growth rates of epidermal outgrowths between specimens from proximal and acral areas.

Effect of FCS on fibroblast growth

In Cases 1 and 2 (Fig. 3, left and right), fibroblasts from proximal (back) and distal (dorsum of foot) areas of the body responded well to addition of FCS, showing similar patterns of growth stimulation. Cells responded well at a minimum concentration of FCS (0.63%). The growth rate began to slow down at 2.5% of FCS, approaching a plateau at around 5%.

Effect of FGF on fibroblast growth

In Cases 1 and 2, and one normal control (Fig. 4, left, middle and right), both proximal and distal skin fibroblasts responded similarly well at the minimum dose of about 6 ng/ml, reaching a plateau at 50 ng/ml.

DISCUSSION

Both patients in this report exhibited the principal features of Werner's syndrome as defined by Thannhauser (4), i.e., short stature with thin extremities and a stocky trunk, premature greying of the hair, premature baldness, patches of stiffened skin (especially in the face and lower extremities), trophic ulcers on the legs, incipient cataracts, and a tendency towards diabetes. In addition to these, several traits considered by Salk (1) to be part of the phenotype of Werner's syndrome were also observed, including a thin, high-pitched voice and hyperkeratosis over bony prominences and on the soles of the feet. One of the striking findings in this disease is the consistent appearance of areas of cutaneous induration resembling scleroderma. The nature of this connective tissue lesion remains unknown. It has been hypothesized that Werner's syndrome represents a hereditary systemic mesenchymal disease, and this is supported by the fact that collagen synthesis, and collagenase activity examined in fibroblasts in vitro, have yielded values differing from controls (5, 6). The ages of the patients fell between those of the 2 normal controls, and it was surprising that the MNP of the fibroblasts in vitro was smaller in both patients than in one of the normal controls, a 94-year-old female. There seemed to be no difference between the MNP of fibroblasts in vitro in the proximal and distal portions of the body in the controls.

If the life spans of fibroblasts derived from proximal and distal body parts in Werner's syndrome differ, the scleroderma-like skin in acral areas may be related to changes in fibroblast life span. Our present report showed no significant difference between the life span of fibroblasts derived from proximal and distal parts of the body. We have already reported (7) that in case 1 the epidermal cells and fibroblasts grew more poorly and slowly than in the normal controls. In a previous report (8), the efficiency of human keratinocyte colony formation in vitro was observed to decrease as age advanced. We have previously obtained almost the same findings as Bauer et al. (3), i.e., hardly any fibroblasts obtained from the skin of the extremities of the present patients could be cultured in outgrowth cultures or cell cultures after trypsin digestion of skin slices (unpublished data). At first, we explanted relatively large specimens of skin (about 1.5-mm punched-out pieces of skin), and obtained epidermal outgrowths, but it was difficult to obtain fibroblasts growth in the culture; the migration and multiplication of fibroblasts seemed to be blocked by relatively large and hyperkeratotic epidermal outgrowth (unpublished data).

The skin of the distal parts of the extremities in Werner's syndrome is scleroderma-like and relatively hyperkeratotic. For that reason, we later managed to have the 1.5-mm punched-out skin specimens dissected into much smaller pieces; under these conditions, fibroblasts developed and grew well. It seemed that it was much more difficult for fibroblasts to develop in the outgrowth cultures from patients than from normal controls. Explant cultures require more time than cell cultures when using trypsin treatment to yield fibroblast growth in primary culture, but the former may be a surer method of obtaining intact fibroblasts. FGF is known to stimulate the growth of fibroblasts (9, 10, 11), and fibroblasts are known to possess FGF receptors (12). According to a previous

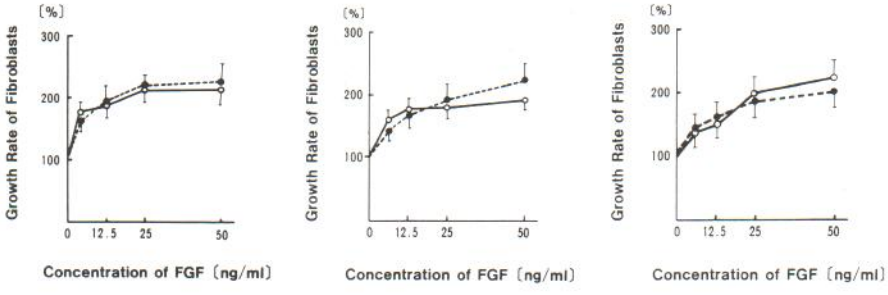

Fig. 4. Effect of FGF on growth of fibroblasts in vitro. *Left:* Case 1. *Middle:* Case 2. *Right:* A 55-year-old normal male control. Fibroblasts from the back (○), and from the dorsal foot (●).

report (13), FGF stimulation was weaker in Werner's syndrome than in controls. In that study, the authors used fibroblasts from skin from just one part of the patient's body. In the present study, the effect of FCS and FGF was observed on fibroblasts from different parts of the body, and no differences were observed in the degree of stimulation by these substances.

This investigation appears to show that there is no difference in degree of ageing in various parts of the body in Werner's syndrome, as far as epidermal and dermal fibroblasts are concerned.

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