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Proliferating Activity of Dermal Fibroblasts in Keloids and Hypertrophic Scars

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Monoclonal antibodies were used to demonstrate proliferating cell nuclear antigen (PCNA) and Ki-67 antigen of dermal fibroblasts in formalin-fixed, paraffin-embedded tissue sections of keloids, hypertrophic scars and normal skin. PCNA-stained fibroblasts were more pronounced than Ki-67, which showed only scanty Ki-67-positive fibroblasts. The mean density of dermal fibroblasts was significantly higher in keloids and hypertrophic scars than in normal skin ($p<0.01$). The proliferating activity of fibroblasts detected by PCNA was significantly higher in keloids than in hypertrophic scars or normal skin ($p<0.01$). The mean densities and the proliferating activities were apparently not correlated with the age of the patient. This indicates that keloids, with higher density and proliferating activity of dermal fibroblasts, continue to increase their volume and invade the surrounding tissue, while hypertrophic scars, with higher density and lower proliferating activity, show a tendency towards spontaneous regression. ***Key words: proliferating cell nuclear antigen; Ki-67.***

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Keloids and hypertrophic scars have a similar clinical appearance: they are raised, red and firm with a smooth, shining surface, consisting of fibroblastic proliferation within the dermis causing cosmetic problems. They are often considered to represent different stages of the same process (1, 2). However, keloids show a tendency to grow beyond the sites of their original injury with no apparent tendency towards spontaneous regression, and are included among the benign dermal tumors. In contrast, hypertrophic scars never grow beyond their original boundaries and have a tendency to regress after an extended period of time. There has been no clinical clear-cut explanation for the difference of the two conditions, although studies using tissue culture or biochemical methods have been reported (3).

In this study, the proliferating activities of fibroblasts were evaluated among keloids, hypertrophic scars, and normal skin, with the use of monoclonal antibodies to proliferating cell nuclear antigen (PCNA)/cyclin (4) and to Ki-67 antigen (5), widely used to detect proliferating activities in normal and tumor tissues (6–10).

MATERIALS AND METHODS

Ten Japanese patients with keloids (7 females and 3 males; ages 5 to 69 years, mean 30.5 years), 10 hypertrophic scars (6 females and 4 males; ages 4 to 44 years, mean 16.7 years), and 10 normal skin specimens obtained from the edge of the excised benign lesions (5 females and 5 males; ages 5 to 70 years, mean 36.0 years) were studied. Keloids were of 2 to 30 years' duration (mean 17.9) and had originated from minute injuries such as acne or vaccination in the presternal, deltoid or scapular regions. Hypertrophic scars were of 0.2 to 6 years' duration (mean 1.4) and had originated from traumatic, burn or surgical scars in the upper and lower extremities, trunk or face. Normal skin was from the upper extremity, trunk or face.

Paraffin sections were made from 10% formalin-fixed tissues. Sections were taken from the center of each lesion (4 to 6 μm in thickness, perpendicular to the skin surface), placed on glass slides, dried overnight at 45°C, deparaffinized in xylene, and then rehydrated through graded concentrations of alcohols. Sections for Ki-67 staining were placed on silane-coated glass slides (silanized slides; DAKO, Denmark) to prevent the tissue from detaching during pretreatments (11, 12) and placed in a thermoresistant plastic box filled with 10 mM, pH 6.0, citrate buffer. The tissue sections in the box were pretreated in a microwave oven 7 times for 3 min each at 500W (11) or in an autoclave for 15 min at 121°C (12) and then cooled at room temperature for 1 h.

Immunohistochemical staining was performed by a biotin-streptavidin-peroxidase technique using 3-amino-9-ethyl carbazole as a substrate. After the endogenous peroxidase activity had been blocked, sections were incubated in mouse IgG2a monoclonal antibody against PCNA (PC1O; DAKO), diluted 1:20 with phosphate-buffered saline or in mouse IgGl monoclonal antibody against Ki-67 antigen (MIB-1; IMMUNOTECH S.A., France), diluted 1:50 for 60 min at 37°C. They were then stained with biotinylated rabbit anti-mouse IgG (ready to use; Nichirei, Japan) for 10 min at room temperature, followed by peroxidase-conjugated streptavidin (ready to use; Nichirei) for 5 min at room temperature. The sections were rinsed for 5 min at room temperature in phosphate-buffered saline between incubations. The coloration was terminated with the reactivity of the epidermal basal cells as the marker. After immunostaining, sections were counterstained with Mayer's hematoxylin and mounted in Glycergel (DAKO).

A total of 1000 nuclei of dermal fibroblasts were counted in keloids and hypertrophic scars, except for areas showing hyaline degeneration, and 500 nuclei in normal skin at a magnification of ×400. The mean density and the PCNA-positive rate of dermal fibroblasts were compared among keloids, hypertrophic scars and normal skin using the Wilcoxon rank-sum test and Welch-*t*-test. The mean density and the PCNA-positive rate were also compared with the age of the patients.

RESULTS

The epidermal keratinocytes showed satisfactory stainings both for PCNA and Ki-67, and their densities were almost similar (data not shown). However, the dermal fibroblasts showed much less and/or variable staining for Ki-67 than PCNA (Fig. 1); i.e. the Ki-67-positive rate of fibroblasts was 4.5% in a keloid in which the PCNA-positive rate was 63.6%, and 4.4% in a hypertrophic scar in which the PCNA-positive rate was 31.0%. There was no apparent difference between specimens pretreated in the microwave oven and those in the autoclave. Therefore, only PCNA-stained sections were subjected to statistical evaluation of the proliferative activity of dermal fibroblasts (Fig. 2).

The mean density of dermal fibroblasts varied from 40 to 71 in keloids, 43 to 100 in hypertrophic scars, and 18 to 36 in normal skin per high-power field (/HPF) at ×400 (a 0.25-mm^2 area). There were statistically significant differences between keloids and normal skin and between hypertrophic scars and normal skin, using the Welch-*t*-test or Wilcoxon rank-sum test ($p<0.01$). No statistically significant difference was found between keloids and hypertrophic scars (Table I). There was no

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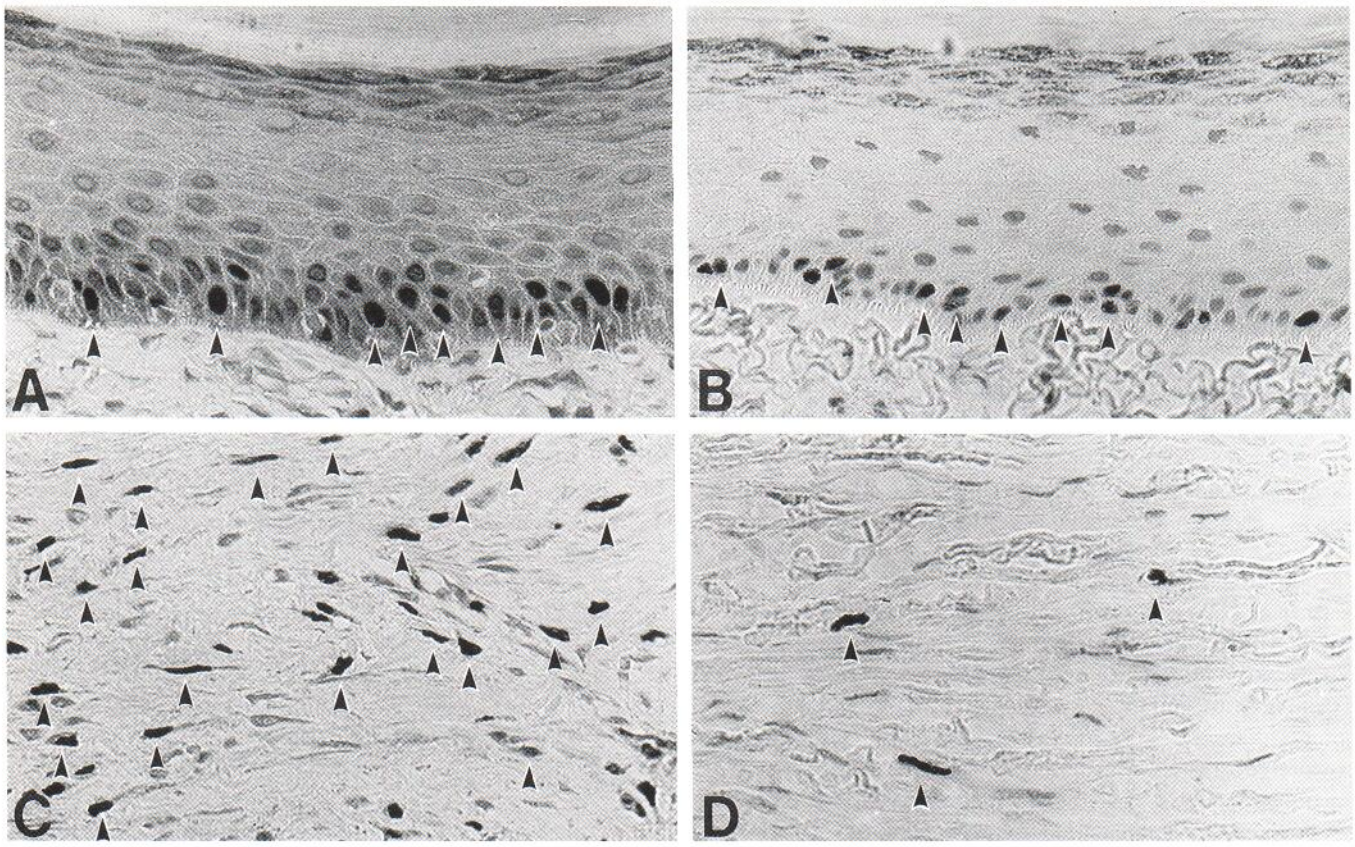

Fig. 1. PCNA and Ki-67 expression in a hypertrophic scar. *Arrowheads* show PCNA-positive keratinocytes (A), Ki-67-positive keratinocytes (B), PCNA-positive fibroblasts (C) and Ki-67-positive fibroblasts (D) (x 280).

apparent correlation between the densities of fibroblasts and age in each group of lesions.

PCNA-positive fibroblasts were relatively evenly distributed in non-hyalinized areas of keloids and hypertrophic scars. In normal skin, fibroblasts were more densely observed in the papillary and subpapillary dermis, where PCNA-positive cells were scattered. The PCNA-positive rates varied from 63.6 to 89.1% in keloids, 29.3 to 64.1% in hypertrophic scars, and 27.2 to 48.6% in normal skin. Statistically significant differences were present between keloids and hypertrophic scars, and between keloids and normal skin, using the Welch-*t*-test or Wilcoxon rank-sum test ($p<0.01$). No statistically significant difference was found between hypertrophic scars and normal skin (Table I). Fibroblasts in keloids showed higher PCNA-positive rates than hypertrophic scars and normal skin in all ages. There was no apparent correlation between PCNA-positive rates and age in each group of lesions.

DISCUSSION

A familial or racial predisposition is occasionally observed in keloids, and hormonal effects on its pathogenesis have been

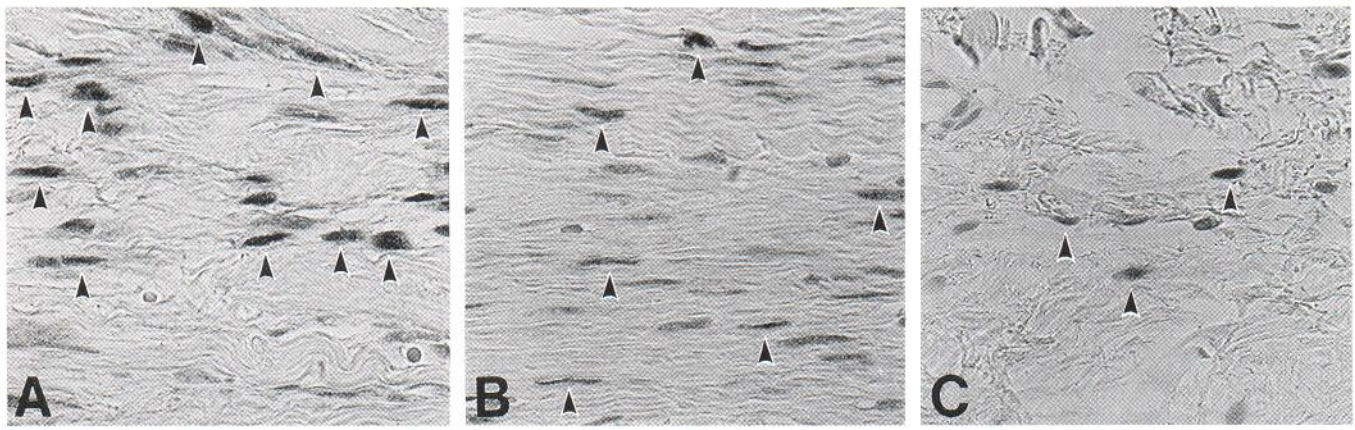

Fig. 2. PCNA expression of dermal fibroblasts. *Arrowheads* show PCNA-positive fibroblasts in a keloid (A), a hypertrophic scar (B), and normal skin (C) (x 280).

Table I. *The mean density and PCNA-positive rate of dermal fibroblasts in keloids, hypertrophic scars and normal skin. HPF:* high-power field.

	N	Density of fibroblasts	PCNA-positive rate of fibroblasts
		Range (mean±SD)(/HPF)	Range (mean±SD)(%)
Keloids	10	40–71 (57.6±9.4)	63.6–89.1 (75.1±8.1)
Hypertrophic scars	10	43–100 (62.0±17.5)	29.3–64.1 (45.6±10.6)
Normal skin	10	18–36 (25.7±5.5)	27.2–48.6 (38.8±6.3)

* Statistically significant difference ($p<0.01$; in both Welch-*t*-test and Wilcoxon rank-sum test)
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postulated (1). The functional activation of dermal fibroblasts has been considered to play an important role in the formation of both keloids and hypertrophic scars. However, there has been no conclusive data, explaining the proliferating activity of the lesional fibroblasts in vivo.

The epidermal keratinocytes were used as a marker for PCNA and Ki-67 reactivities to standardize the methods in the present experiment. Although the reactivities for PCNA and Ki-67 on the epidermal keratinocytes were similar, those on the dermal fibroblasts showed stronger PCNA reactivity. This might be explained by the following observations: 1) the epitopes of Ki67 antigen on fibroblasts were more strongly masked than those on keratinocytes by formalin-induced cross-linking of proteins (12), and 2) the long half-life of PCNA (13) showed much stronger effects on fibroblasts because of the difference of the cellcycle time between keratinocytes and fibroblasts.

The densities of dermal fibroblasts in keloids and hypertrophic scars were significantly higher than in normal skin, and the PCNA positive rate in keloids was also statistically significantly higher than that in hypertrophic scars and normal skin. This appears to correspond to the clinical features of keloids, which show a continuous increase of their volume and growth towards the surrounding skin. It is known that keloids have a tendency to develop in adolescence and early adulthood. However, there were no significant correlations between the density of fibroblasts or PCNA-positive rate and the age of the patients in the present study. In contrast, there was a statistically significant difference in the densities of fibroblasts, but not in PCNA-positive rates, between hypertrophic scars and normal skin. This may partly explain the clinical observation that hypertrophic scars never grow beyond their original boundaries and show a tendency towards spontaneous regression.

Recently it has been reported that the formation of keloids is prevented by the use of tranilast {N- (3, 4-dimethoxycinnamoyl) anthranilic acid (N-5')}, which selectively inhibits collagen accumulation (14) or fibroblast proliferation (15). The present data may be compatible with the pharmacological role of tranilast in keloids.

The PCNA-positive fibroblasts were relatively evenly distributed in the non-hyalinized areas of keloids and hypertrophic scars, though the differences of the proliferating activities between the center and the edge of the lesion could not be evaluated in the present study. Keloids are likely to have a regional heterogeneity of cell proliferation, since they have an irregularly shaped appearance and grow with increased skin tension. The high proliferation activity of the fibroblasts in keloids seems to be responsible for the continuous and excessive synthesis of collagen.

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