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ORIGINAL PAPER

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# Immunohistochemical staining of proliferating cell nuclear antigen (PCNA) in malignant and nonmalignant skin diseases

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*Abstract* **Immunohistochemical staining of proliferating cell nuclear antigen (PCNA) was performed in skin from patients with various malignant and nonmalignant skin diseases using anti-PCNA monoclonal antibodies. The malignant diseases included squamous cell carcinoma (SCC), adult T lymphotrophic leukemia (ATL), mycosis fungoides, malignant melanoma and malignant lymphoma, and the nonmalignant diseases included severe treatment-resistant atopic dermatitis (AD), psoriasis vulgaris, verruca vulgaris, and others. The percentage of PCNA-positive cells (the labeling index, LI) was highest for the malignant diseases (56.5 ± 7.1%). The LIs for severe treatment-resistant AD, psoriasis, and verruca vulgaris were also significantly higher than those for the normal control or nonlesional skin of the patients. The PCNA LIs were, however, not significantly elevated in eczema and contact dermatitis. The high PCNA LIs in severe AD and psoriasis vulgaris were considerably lower in the skin improved by treatment. Labeling with Ki67, a nuclear protein expressed in cycling cells, was also performed in skin from subsets of each patient group. The results were very similar to those found with PCNA labeling. PCNA-positive cells were found throughout the dermis as well as the basal layer in the malignant diseases, whereas they were found only in the basal layer in the nonmalignant diseases. The results suggest that in human skin diseases, the extent of staining for PCNA, which is a cofactor of DNA polymerase-delta and is essential for cell proliferation, correlates with the extent to which the disease is treatment-resistant. In addition, our findings suggest that the PCNA LI and distribution of PCNA-positive cells in the skin may be helpful in the early diagnosis of skin malignancies.**

**Key words** PCNA-positive cells · Skin malignancies · Severe atopic dermatitis · Psoriasis vulgaris · Verruca vulgaris

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## Introduction

It has been found that proliferating cell nuclear antigen (PCNA) is a cofactor of DNA polymerase-delta and an index of the rate of cell turnover or cell proliferation (Galand and Degraef 1989; Prelich et al. 1987). PCNA has also been reported to be increased in malignancies (Garcia et al. 1989; Prelich et al. 1987; Suzuki et al. 1992), to fluctuate in relation to the normal hair cycle (Kitano et al. 1996), and to be increased in verrucae vulgaris (common warts) for which only physical treatments are available (Noel et al. 1995).

Recent developments in immunohistochemical methods for detecting PCNA using the monoclonal anti-PCNA antibody have enabled PCNA-positive cells in a variety of malignancies to be determined (Garcia et al. 1989; Noel et al. 1995; Prelich et al. 1987; Suzuki et al. 1992). We have previously reported that the percentage of PCNA-positive cells (labeling index, LI) as determined using the anti-PCNA antibody is high in skin malignancies and varies depending on treatment resistance in nonmalignant skin diseases (Niwa and Kawahira 1996; Niwa and Kawahira 1997).

In this study, we determined the PCNA LIs in skin samples from patients with malignant skin diseases and also in lesional samples from patients with severe atopic dermatitis (AD), psoriasis vulgaris (diseases known to be refractory to treatment) and common warts, which are typically treated with electrocautery or cryotherapy with liquid nitrogen. As controls, we also determined the PCNA LIs in normal-appearing skin from the same patients, and in skin from normal healthy individuals and from patients with contact dermatitis and mild AD, both

of which are highly responsive to treatment. The results showed that the PCNA LI was significantly elevated in the malignant skin diseases and the benign diseases refractory to treatment.

## Materials and methods

### Tissues

Skin samples were obtained from both inpatients and outpatients attending Tosashimizu Hospital, Kochi, Japan, between April 1995 and March 1999. The samples were obtained from a total of 129 patients, of whom 16 had malignant skin diseases and 113 nonmalignant skin diseases. The malignant diseases included squamous cell carcinoma (SCC) ($n$ = 5), adult T lymphotrophic leukemia (ATL) ($n$ = 4), mycosis fungoides ($n$ = 3), malignant melanoma ($n$ = 2), and malignant lymphoma ($n$ = 2), and the nonmalignant diseases included severe treatment-resistant AD ($n$ = 38), psoriasis vulgaris ($n$ = 25), verruca vulgaris ($n$ = 10), mild to moderate AD ($n$ = 20), and eczema and contact dermatitis ($n$ = 20). Six healthy volunteers served as normal-skin controls. We defined severe and treatment-resistant AD as AD with systemic lichenification and thickening of skin or extensive prurigo nodularis very resistant to high-potency topical corticosteroids, as shown in Figs. 1–3.

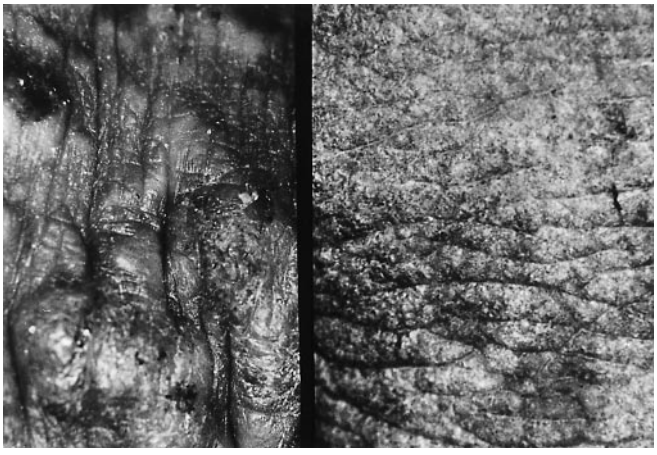

**Fig. 1** Diffuse lichenification and thickening of the skin in atopic dermatitis refractory to high-potency topical corticosteroids (26-year-old male)

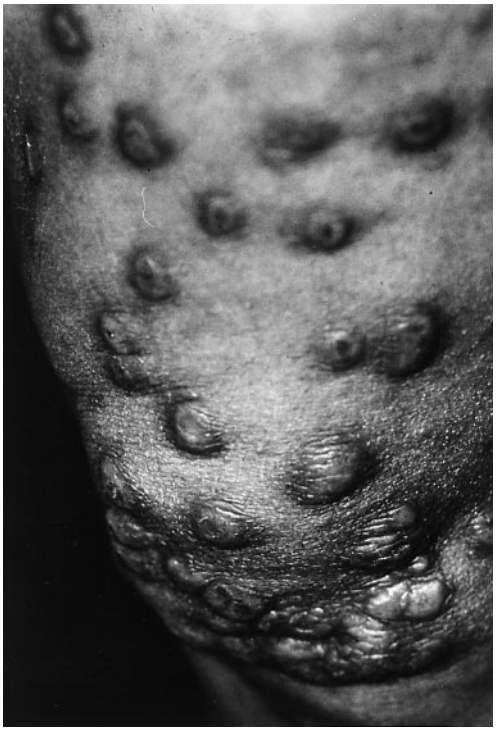

**Fig. 2** Marked prurigo nodularis in atopic dermatitis refractory to high-potency topical corticosteroids (46-year-old female)

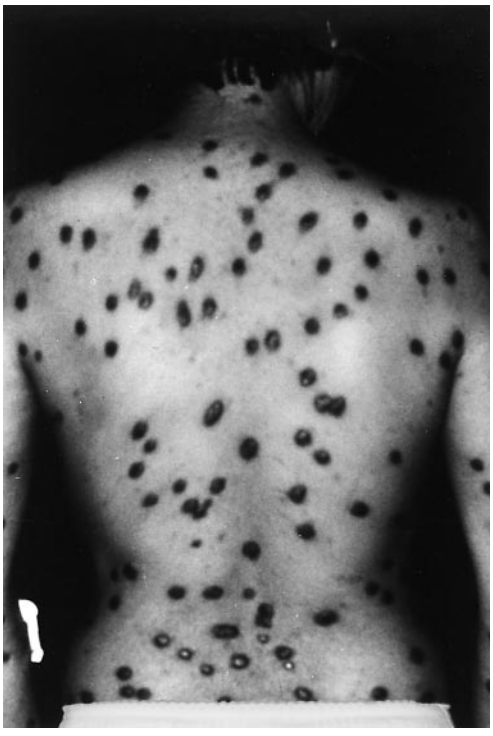

**Fig. 3** Systemically extensive prurigo nodularis in atopic dermatitis refractory to high-potency topical corticosteroids (36-year-old female)

### Immunohistochemical methods

The proportion of proliferating cells was determined by immunohistochemical staining for PCNA, as originally developed by Noel et al. (Noel et al. 1995), with some modifications. Briefly, after deparaffinization of the tissue sections, endogenous peroxidase was blocked by incubation in 3% $H_2O_2$-methanol for 3 min. After washing three times in phosphate-buffered saline (PBS), the sections were incubated with monoclonal mouse antihuman PCNA (clone PC10, IgG2a; Novocastra Laboratories, Newcastle upon Tyne, UK) for 30 min at room temperature at a dilution of 1:100 in 0.05 *M* Tris buffer, pH 7.6, with 0.1% $NaN_3$, then washed three times in PBS. The second antibody (biotinylated antimouse IgG; Boehringer, Mannheim) was applied at a dilution of 1:500, and incubated for 10 min at room temperature. Streptavidin-peroxidase (code no. KO680; DAKO LSAB Kit; DAKO Japan, Kyoto, Japan) was then applied to the sections in 0.05 *M* Tris buffer for 10 min at room temperature. Slides were developed, following washing in PBS three times, using 3.3% diaminobenzidine in 50 m*M* Tris buffer, pH 7.6. The sections were counterstained with Mayer's hematoxylin for 30 s at room temperature, and mounted in glycerin gel. Omission of the primary antibody (anti-PCNA antibody) served as a negative control.

**Table 1** The PCNA-$LI_s$ in skin biopsies from skin malignancies, severe AD, psoriasis vulgaris, common warts and control diseases

[1]$P < 0.05$, [2]$P < 0.01$, [3]$P < 0.001$, [4]$P < 0.0001$, *vs* nonlesional and normal skin
[a] Squamous cell carcinoma, adult T lymphotrophic leukemia, mycosis fungoides, melanoma, malignant lymphoma

| Disease | PCNA-positive cells (%) | | Distribution |
|---|---|---|---|
| | Lesional skin | Nonlesional skin | |
| Skin malignancies[a] | 56.5 ± 7.1[4] | 11.2 ± 1.9 | Epidermis and dermis |
| Severe AD | 34.1 ± 4.7[3] | 11.9 ± 1.5 | Basal layer |
| Psoriasis vulgaris | 24.2 ± 3.3[2] | 10.9 ± 1.6 | Basal layer |
| Common warts | 22.0 ± 3.3[2] | 9.9 ± 1.5 | Basal layer |
| Mild–moderate AD | 16.3 ± 2.8[1] | 10.3 ± 1.6 | Basal layer |
| Contact dermatitis, eczema | 13.1 ± 1.8 | 8.5 ± 1.5 | Basal layer |
| Normal skin | – | 8.6 ± 1.2 | Basal layer |

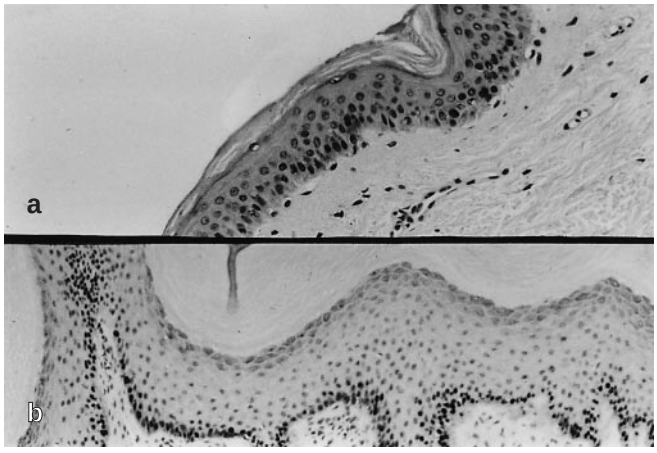


**Fig. 4 a** Normal skin: a few PCNA-positive cells are apparent in the basal layer of the epidermis (× 10). **b** Atopic dermatitis with prurigo nodularis: PCNA-positive cells are located mainly in the basal layer (× 40)

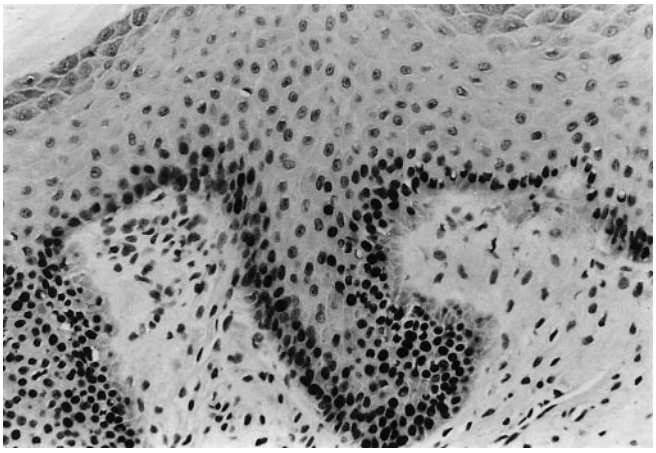

**Fig. 6** A lesion of severe refractory AD with prurigo nodularis: a marked increase in PCNA-positive cells is apparent in the basal layer (× 40)

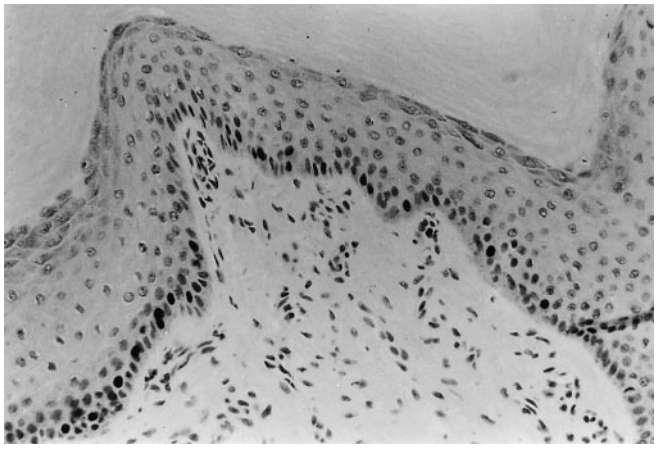

**Fig. 5** Normal skin: a few PCNA positive cell are apparent in the basal layer (× 40)

For the analysis of PCNA distribution, at least ten representative low-power (× 10) and high-power (× 40, × 80) fields were examined by two independent observers. A cell was scored as positive when there was distinct brown granular staining of the nucleus regardless of its intensity, and since PCNA staining, in general, differs within a lesion, the area with the highest rate of nuclear positivity was chosen for examination. The PCNA LIs in skin biopsies are expressed as the means ± SD of triplicate assays. Student's *t*-test was applied for statistical analysis. PCNA staining was first performed in skin samples from all the patients before treatment, and then in samples from subsets of the patients after treatment.

The specificity of PCNA labeling for cycling cells was confirmed by staining for the Ki-67 antigen (Ramires et al. 1997; Schluter et al. 1993), a large cell proliferation-associated ubiquitous nuclear protein with numerous repeated elements and one of a novel kind of cell cycle-maintaining proteins, in skin samples from subsets of each patient group.

Methods for Ki67 staining were similar to those for PCNA except for fixation and pretreatment, as follows. The biopsy specimens were fixed with 10% formalin instead of 100% alcohol which was used for PCNA staining. After deparaffinization, the sections were soaked in 10 m*M* citric acid buffer (pH 6.0), autoclaved at 121°C for 20 min, and then left to cool to room temperature for approximately 1 h. After washing, 3% $H_2O_2$-methanol was added to block endogenous peroxidase activity, and methodological procedures similar to those for PCNA staining were followed. The primary antibody for Ki67 was purchased from Immunotech (Marseille, France), and for PCNA from Novocastra Laboratories (Newcastle upon Tyne, UK) . Secondary antigens for both Ki67 and PCNA were obtained from Dako Corporation (Carpinteria, Calif.).

## Results

The PCNA LIs are shown in Table 1. The mean LI was 8.6 ± 1.2% and 11.9 ± 1.5% in normal-appearing skin from healthy individuals and patients with skin diseases, respectively. The LI was elevated in contact dermatitis and eczema (13.1 ± 1.8%), and in mild to moderate AD

was significantly elevated (16.3 ± 2.8%) compared with the two normal control groups ($0.01 < P < 0.05$, Table 1). The LI was also elevated in common warts (22.0 ± 3.3%) and in psoriasis vulgaris (24.2 ± 3.3%) and tended to correlate with the refractoriness to various therapies including corticosteroids. In AD patients with systemic lichenification and thickening of the lesions or extensive prurigo nodularis (as shown in Figs. 1–3), which were very resistant to high-potency topical corticosteroid, the LI was markedly elevated at 34.1 ± 4.7% ($P < 0.01$ compared with normal control). The LI was even higher at 56.5 ± 7.1% in the malignant diseases ($P < 0.0001$, Table 1). No staining was seen in the negative control samples, in which the primary antibody was omitted (data not shown).

PCNA-positive cells were restricted almost to the basal layer in normal skin and nonmalignant skin disease, and PCNA-positive cells were rarely found in the corneal or spinous layers or in the dermis (Figs. 4–9). In the malignant diseases, however, positive cells extended throughout the basal layer and the dermis (Figs. 10 and 11).

Similar results were obtained with Ki67, which is also a new kind of cell cycle-maintaining protein, as with PCNA. The Ki67 LI in malignant skin disease was 49.1 ± 6.5%, in severe AD 29.5 ± 4.2% and in mild AD 11.2 ± 1.9% (detailed data not shown).

A high PCNA LI (32.5 ± 5.2%) in skin from ten patients with severe AD before treatment was decreased to 21.2 ± 3.9% after treatment (detailed data not shown).

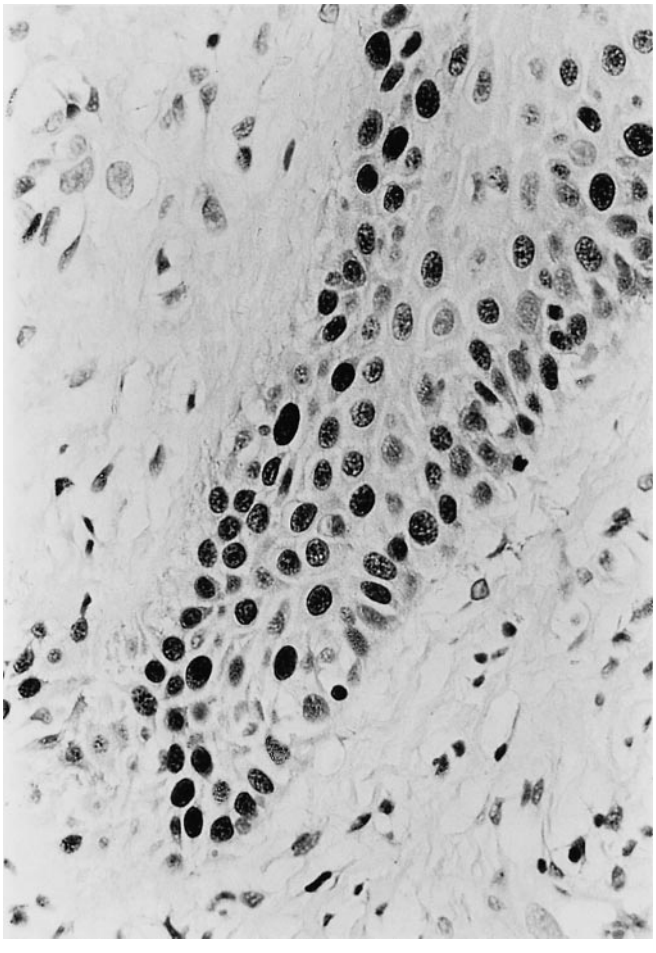

**Fig. 7** A lesion of severe AD with thickening and lichenification: an increase in PCNA-positive cells is apparent in the basal layer (× 80)

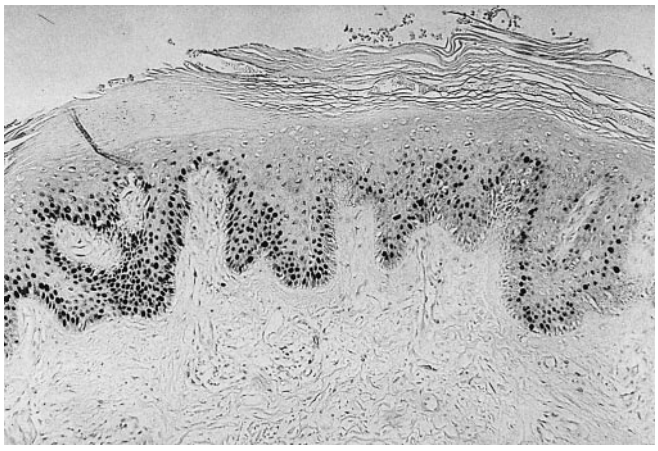

**Fig. 8** A lesion of psoriasis vulgaris: PCNA-positive cells are apparent in the basal layer (× 10)

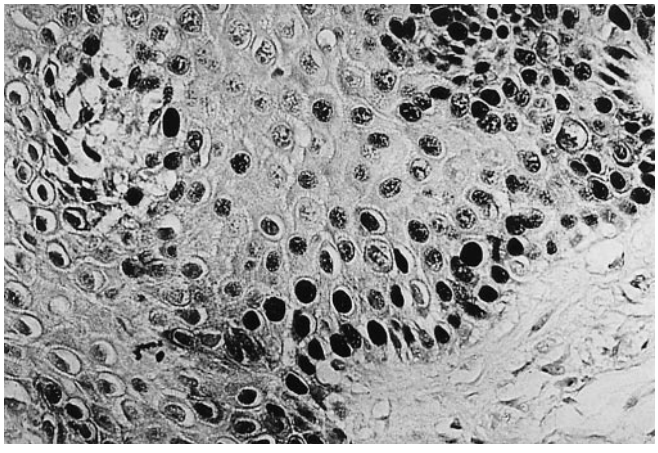

**Fig. 9** A lesion of psoriasis vulgaris: cells showing nuclear staining with anti-PCNA antibody are scattered in the basal layer (× 40)

## Discussion

In skin malignancies, PCNA-positive cells were found in abnormally high abundance throughout the dermis as well as the basal layer. Increased numbers of PCNA-positive cells were also observed in severe treatment-resistant AD, in severe psoriasis vulgaris, and in common warts. Our findings also suggest that the PCNA LI may be an indicator of resistance to treatment of a skin disease and provide prognostic information at the initial visit.

PCNA exists in the nucleus of all cells. It is a 36-kDa acidic nuclear protein, and a cofactor of DNA polymerase-delta that is essential for DNA synthesis. It is activated during the late G1 phase through early S phase (Galand and Degraef 1989; Prelich et al. 1987). It is there-

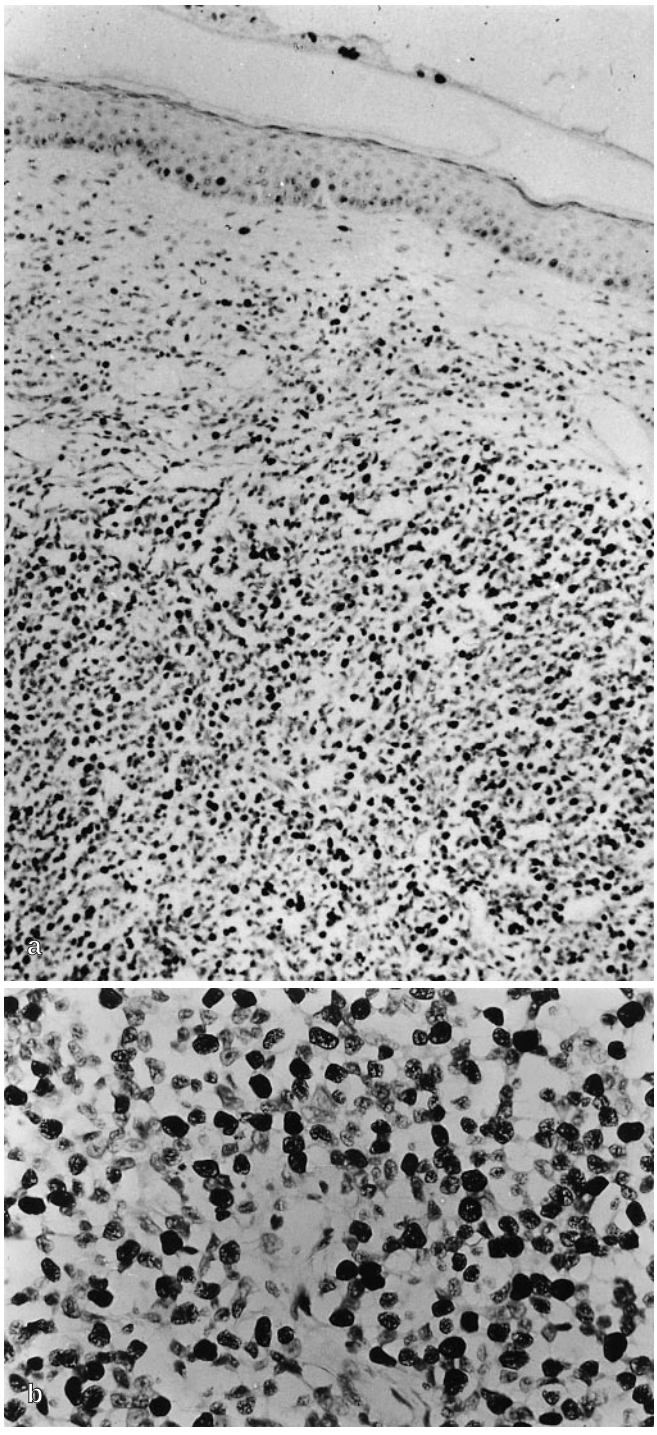


**Fig. 10** **a** A malignant lymphoma: many PCNA-positive cells are apparent extending into the dermis as well as in the basal layer (× 10). **b** A malignant lymphoma: numerous PCNA-positive cells are apparent throughout the dermis as well as in the basal layer (× 40)

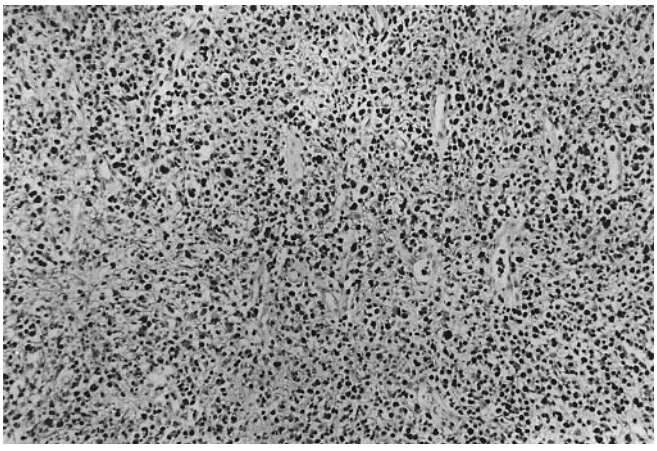

**Fig. 11** Adult T lymphotrophic leukemia: abundant PCNA-positive cells are apparent in the dermis as well as in the basal layer

fore known to promote cell cycling and to be an index of cell proliferation. It is present to some degree in normal cells. There have been a few studies examining PCNA-positive cells in normal skin. Kitano et al. (Kitano et al. 1996) examined cell proliferation in the anagen, catagen, and telogen stages of the hair cycle and in follicles of normal scalp, and found that PCNA is increased during anagen and decreased during catagen. In malignancies, the increase in PCNA is presumably associated with uncontrolled DNA synthesis and cell cycling.

It is well known that loss of tumor suppressor genes such as p 53 and Rb protein is implicated in carcinogenesis and is associated with dysregulation of cell proliferation (Levine et al. 1991; Nevins 1992; Vogelstein and Kinzler 1992). We did not examine these tumor suppressor genes in this study, but the increase in PCNA-positive cells throughout the skin in the malignant diseases suggests that this is a surrogate marker for these genetic defects. As expected, we found some PCNA labeling in normal skin (around 10%). The correlation between PCNA-positive cells in inflammatory skin diseases and in skin diseases refractory to treatment suggests that PCNA positivity can be used as a measure of treatment resistance in benign skin diseases as well as in early diagnosis of skin malignancies.

It is also interesting that the PCNA LI correlated with disease activity: the LI was higher in severe stages and was decreased after treatment. PCNA seems also to be an indicator of disease activity.

Noel et al. (Noel et al. 1995) have also demonstrated that in common warts and verrucous carcinoma, which is a rare variety of squamous cell carcinoma (SCC) with a low metastatic potential, the PCNA-positive cells are principally localized in the basal layer but in SCC, which has a high metastatic potential, PCNA-positive cells are randomly scattered throughout the skin of the tumor. Their findings support those of this study on the distribution pattern of PCNA-positive cells in the skin in nonmalignant and malignant skin diseases (Figs. 4–9, 10, 11).

The LIs for Ki-67, which is also a cell proliferation-associated antigen and a new kind of cell cycle-maintaining protein, were evaluated in skin samples from subsets of each patient group simultaneously with the determination of the PCNA LIs, and similar findings were obtained. This supports the assertion that PCNA promotes cell cy-

cling and is an index of cell proliferation, and even seems to confirm the hypotheses presented here that the PCNA LI can be used in the early diagnosis of skin malignancies and that its magnitude can be used as a measure of treatment resistance in benign skin diseases.

Niwa (Niwa 1995) has more recently reported finding oncogenic papillomavirus-associated DNA together with a marked increase in PCNA-positive cells in a few percent of patients with severe treatment-resistant AD, and this seems to be an indicator of increased susceptibility to skin cancer among these patients.

Kawahira and Kanzaki (Kawahira and Kanzaki 1997) reported that PCNA is useful in the early diagnosis of malignant lymphoma. However, their examination was limited to the detection of PCNA-positive lymphocytes in the skin. In the present study, we determined the PCNA LIs in the different cell lineages throughout the skin in a variety of skin diseases including treatment-responsive and treatment-resistant benign and malignant diseases. On the basis of our findings, a very high PCNA LI (more than 50%) would be highly suggestive of malignancy. An elevated LI but less than 50% is suggestive of an actively cycling lesion that would be relatively resistant to topical corticosteroids, even if not malignant. Thus, examination of skin for PCNA-positive cells is a relatively simple procedure that may be useful in clinical practice for the early diagnosis of malignancy and as a prognostic indicator in nonmalignant conditions.

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