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Two Binding Sites for Ki67 Related to Quiescent and Cycling Cells in Human Epidermis

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The monoclonal antibody Ki67 (Ki67) binds to a nuclear antigen expressed by cycling cells of several human tissues and to the cytoplasm of the basal layer cells of squamous epithelia. We have used an immunohistochemical method to visualize the binding sites of Ki67 in normal and hyperproliferative epidermis. Cytoplasmic staining was present in the basal layer cells of normal epidermis, but was decreased in psoriatic and post-tapestripping epidermis. In sections of normal epidermis only a small minority of nuclei were positive, but sections of psoriatic epidermis and epidermis 40 and 48 h after tapestripping showed large numbers of positive nuclei in the basal and suprabasal layers. Since recent reports strongly suggest that the cell production rate is regulated by changes in the number of cycling cells, the hypothesis that Ki67 binds also in human epidermis to the nuclei of cycling cells is supported by the present findings. *Key words: Monoclonal antibody Ki67.*

(Accepted April 6, 1989.)

Acta Derm Venereol (Stockh) 1989; 69: 512–515.

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The monoclonal antibody Ki67 was originally raised against a nuclear substance of Hodgkin lymphoma cells, which is also expressed by actively cycling cells of several other human tissues (1, 2). The hypothesis that the nuclear binding of Ki67 in epidermis is associated with actively cycling cells is based on analogy with these tissues. At least in human leukocytes, the nuclei of S, G_2, M and G_1 cells were positive for Ki67, but those of G_0 cells negative. Cells entering the cell cycle from a G_0 state became positive before entering the S-phase but after increased RNA amounts, typical for G_1, were detected; this indicates that after quiescence the S-phase is not preceded by a common G_1 phase (3). The discrimination between actively cycling and quiescent (G_0) cells is of very great general interest because it allows the quantification of growth fractions. It is especially important for human epidermis because the existence of quiescent (G_0) cells is still not generally accepted and the relative number of cycling cells has never been directly determined.

However, this is only one aspect of Ki67. According to the product information supplied by the manufacturer (Dakopatts), Ki67 also binds to a cytoplasmic structure in the basal layer of squamous epithelia such as epidermis. This binding site also appears to be related to proliferation. We have developed an immunohistochemical procedure for human epidermis and applied it to biopsies of normal, psoriatic and tapestripped skin, in order to determine the proportion of Ki67-positive cells and to investigate the expression of the cytoplasmic binding site under these circumstances.

MATERIALS AND METHODS

Sample procedure

Shave biopsies were taken using a standard technique from normal skin of healthy volunteers, from the borders of previously tapestripped areas (16, 32, 40 and 48 h after stripping) on the backs of healthy volunteers and from the border of an untreated lesion of a psoriatic patient. Within 15 min,

these were frozen to −20°C. All samples were processed within 48 h of biopsy. Sections (8 μm) were cut using the cryostat.

Immunohistochemical procedure

Sections were first air-dried for 60 min, cold, under a fan. When stored, they were kept at −20°C for up to 2 days and, after defrosting, again air-dried for 15 min. They were then fixed in acetone for 15 min followed by air-drying for 2 min and rehydration in phosphate-buffered saline (PBS) with 0.01% Tween 80 for 1 min.

The sections were incubated for 30 min with the monoclonal antibody Ki67 (mouse-anti-human proliferative cells; Dakopatts, Denmark), 1:20 in PBS in a moist chamber followed by washing three times for 10 min in PBS. They were then incubated for 30 min with a rat–anti-mouse peroxidase (Dako-immunoglobulins, Denmark), 1:25 in PBS with 5% human AB serum in a moist chamber. After washing three times for 10 min in PBS, the sections were incubated for 10 min in a peroxidase substrate solution: 50 ml Na-acetate buffer (pH 4.85) with 10 mg aminoethylcarbazole in 2.5 ml dimethylformamide solvent; this was filtered and 20 μl H_2O_2 30% was added just prior to incubation. The procedure was completed by a 1 min wash in deionised water and inclusion in fluorostab.

RESULTS

Fig. 1 *a* illustrates a section of normal unstimulated epidermis. On average, 12.8 ± 1.5 (SD) nuclei per mm were Ki 67-positive ($n=5$). Using 1.31 times the basal cells per mm as a measure of the germinative population (5) and assuming Ki67 positive nuclei per mm to represent the cycling pool, the average growth fraction (percentage of the germinative population which are actively cycling) was calculated as 5.2 ± 1.0% (SD). The cytoplasm of the basal layer cells is stained, unstained nuclei appearing as light areas in stained cytoplasm.

In Fig. 1 *b*, at the border of a psoriatic lesion, the cytoplasmic staining of the basal cells disappears from uninvolved (right) to lesional (left), while nuclear staining becomes visible in many of the basal and suprabasal nuclei of the lesional part (left). In the chronic psoriatic plaque, 340.3 ± 112.9 (SD) nuclei per mm were stained ($n=6$). Using the measure for the germinative population of Weinstein et al. (6), a growth fraction of 56.1 ± 18.6% (SD) was yielded.

The same phenomena as in the border of a psoriatic lesions are evident in Fig. 1 *c* on the border of an area that was tapestripped 48 h before. From normal (left) to tapestripped (right), the cytoplasmic staining decreases and numbers of positive nuclei increase. On the extreme right and in the detail (Fig. 1 *d*) the damage to the stratum corneum is visible. Fig. 1 *e* shows that 16 h after tapestripping, the decrease in the cytoplasmic staining is already present, but increased numbers of positive nuclei are not yet seen. After 32 h the picture was the same. After the tapestripping and before large numbers of nuclei are becoming Ki67-positive, we see a period which persists for at least 16 h (from 16 to 32 h post-tapestripping) with only weak cytoplasmic and incidental nuclear staining. The first increased nuclear staining was detected 40 h following tapestripping (Fig. 1 *f*). At this time the average Ki67-positive nuclei were 252.1 ± 35.1 (SD) per mm ($n=4$), yielding a growth fraction of 80.9 ± 11.3% (SD).

DISCUSSION

Prior to the observations of Gelfant in 1976 (9), it was generally believed that cell production was essentially regulated by variation of the cell cycle time (from 36 to 311 h) and that the majority of the germinative cells were actively cycling (6–8). However, Gelfant found that a large proportion of the basal cells in normal epidermis were not labelled with radioactive thymidine after an exposure time of 14 days. This was the first observation supporting the existence of a large G_0 cell population. More recently, other arguments in favour for the G_0 concept were presented in a flow cytometric analysis of proliferative activity following tapestripping epidermis (10, 11). The validity of the hypothesis that, in analogy with other tissues, Ki67 binds to the nuclei of actively cycling cells in epidermis is confirmed by the present observation of large numbers of Ki67-positive nuclei in psoriatic (1 *b*) and post-tapestripping epidermis (1 *d*, 1 *f*), both hyperproliferative conditions with necessarily a large proportion of cycling cells. The present data indicate a growth fraction of about 5% and a G_0 cell pool of more than 90% for normal epidermis.

In addition to the nuclear staining, cytoplasmic staining of the basal layer was observed. It is dramatically reduced in the hyperproliferative psoriatic lesion (Fig. 1 *b*) and in tapestripped epidermis (Fig. 1 *c*) compared with normal epidermis (Fig. 1 *a*). Thus it seems that there is a negative relation between cytoplasmic staining and proliferation. When germinative cells have entered the S-phase 40 h after tapestripping (10), the numbers of positive nuclei are increased. Preceding this, as previously mentioned, there is a period with only weak cytoplasmic and incidental nuclear staining (from 16 to 32 h). This interval might be related to the synchronized transition of numerous

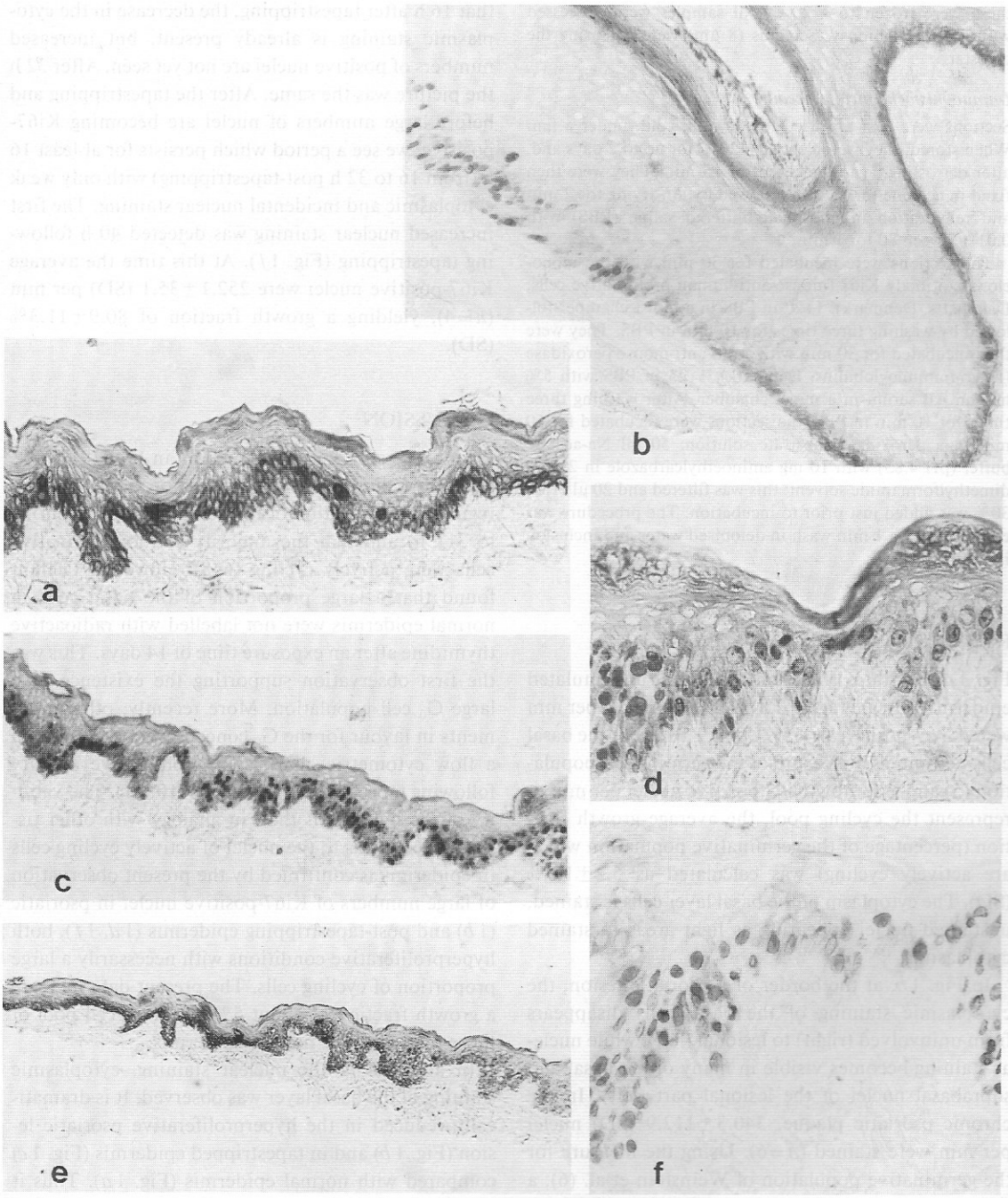

Fig. 1. Cryostat sections of human epidermis were processed as described in the text. (*a*) Normal epidermis. (*b*) Border of a psoriatic lesion. (*c*) Border of an area that was tapestripped 48 h before: (*left*) normal epidermis; (*right*) stripped epidermis. (*d*) Detail of an area 48 h after stripping: decreased cytoplasmic staining of the basal layer, many positive nuclei. (*e*) Border of an area that was tapestripped 16 h before; from normal (*left*) to stripped (*right*) decreasing cytoplasmic staining of the basal layer, incidental nuclear staining. (*f*) Detail of an area 40 h after stripping, similar to (*d*).

basal cells from the quiescent to the cycling state (11), the analogue of the specific Ki67-negative G_1 phase between G_0 and S phase, as has been suggested for leukocytes (3).

It should be noted that the cytoplasmic and the nuclear binding sites are simultaneously present in the few cells in unstimulated epidermis that are cycling (Fig. 1 *a*). This excludes the possibility of one binding site moving after stimulation, from the cytoplasm to the nucleus. The nature of the nuclear binding site is not known, but is very probably a protein, since its expression in lymphocytes can be inhibited by cycloheximide (3); the nature of the cytoplasmic binding site, however, remains obscure. Further research is required to clarify these points.

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Use of Sodium-chloride Separated Human Skin in Detection of Circulating Anti-basement Membrane Zone Antibodies

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The sensitivity of the indirect immunofluorescence (IIF) technique for detection of circulating basement membrane zone (BMZ) antibodies was evaluated, employing NaCl-separated human skin and intact skin as substrate. Consecutive serum samples from 12 patients with clinically, histologically and immunohistologically verified bullous pemphigoid (BP) were investigated in parallel on both substrates, in dilutions ranging from 1:10 to 1:1,280. All BP sera showed linear deposits of IgG at the BMZ on intact skin, with titres ranging from 10 to 160. On NaCl-separated skin, all BP sera produced a linear epidermal fluorescent band for IgG, with titres ranging from 80 to 1,280. None of the sera showed deposits of IgM anti-BMZ antibodies. Sera from 5 healthy donors (dilutions 1:10) produced no fluorescence, either on intact or on NaCl-separated skin. The serum-titres of circulating anti-BMZ IgG antibodies in 2 patients with corticosteroid-resistant BP were significantly reduced (from 160 to <10) during treatment with plasmapheresis, when using NaCl-separated skin as substrate for IIF, whereas the serum-titres showed insignificant reduction (from 20 to <10), when using intact skin as substrate. We conclude that the IIF method is more sensitive for detection of circulating anti-BMZ antibodies, when NaCl-separated skin as compared with intact human skin is employed as substrate. ***Key words: Immunofluorescence; NaCl-separated skin; Bullous pemphigoid.***

(Accepted April 24, 1989).

Acta Derm Venereol (Stockh) 1989; 69: 515–519.

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The direct (DIF) and indirect immunofluorescence (IIF) techniques are widely used in the diagnosis of bullous diseases of the skin. By DIF on skin from