Quantification of Cellular Proliferation in Acne Using the Monoclonal Antibody Ki-67

Helen E. Knaggs, Diana B. Holland, Chris Morris, Edward J. Wood,* and William J. Cunliffe
Department of Dermatology, The General Infirmary at Leeds; and *Department of Biochemistry and Molecular Biology, University of Leeds, Leeds, England

The mechanism by which ductal hypercornification occurs in acne is uncertain. We investigated proliferation in normal and acne follicles and in the interfollicular epidermis using the monoclonal antibody Ki-67, which reacts with a nuclear antigen expressed by cells in the G_1, S, M, and G_2 phases of the cell cycle.

Cryostat sections of biopsies from the interscapular region from acne patients and from normal volunteers were stained with Ki-67 antibody and counterstained with 2% methyl green. The number of Ki-67–positive nuclei in the basal layer were counted and expressed as a percentage of the total number of basal nuclei in the ductal or interfollicular epithelia. The data was expressed as mean percent ± SD. In normal follicles from acne-affected sites 17.40% ± 1.86% (n = 8) of the nuclei were Ki-67 positive. This was significantly higher ($p < 0.01$) than follicles from an area of skin unaffected by acne (11.01% ± 6.16%, n = 8). In the follicular epithelia of non-inflamed lesions, the percentage of Ki-67 positive nuclei was 23.44% ± 8.36% (n = 15). It was impossible to count the nuclei of follicular epithelium of inflamed lesions because little of this remained intact.

In normal interfollicular epidermis, Ki-67–positive nuclei represented 5.33% ± 3.36% (n = 8) of the total. This value was not significantly different from the value obtained for interfollicular epidermis near non-inflamed lesions (10.46% ± 4.45%, n = 15). However, the number of Ki-67–positive nuclei in the interfollicular epidermis near inflamed lesions was significantly higher than either of these two values: 25.26% ± 6.83%, n = 13, $p < 0.05$.

Our results with Ki-67 confirm that ductal hyperproliferation occurs in acne and shows that normal follicles from acne skin may be "acne-prone." *Key words: duct/follicular/keratinocyte/immunohistochemistry. J Invest Dermatol 102: 89–92,1994*

Acne vulgaris is a disease affecting the pilosebaceous unit. Although the aetiology of acne is unknown, four factors are known to contribute towards its pathogenesis: increased sebum excretion, hypercornification of the pilosebaceous duct, abnormal microbial action, and inflammation [1].

Ductal hypercornification is thought to result from hyperproliferation of basal keratinocytes and the subsequent retention of corneocytes within the follicular lumen [2,3]. This process contributes towards comedone formation, which represents one of the earliest clinical signs of acne. Comedones both those which are clinically evident and microcomedones, can develop into inflamed lesions.† Drugs that counteract hyperproliferation may therefore offer a therapeutic approach not just to comedonal acne but also to inflammatory lesions.

Evidence for the role of ductal hyperproliferation in acne was established in the early 1970s [2]. Tritiated thymidine incorporation into normal follicles and comedones revealed a higher labeling rate in comedones compared with normal follicles.

Recently questions have been raised concerning the validity of the earlier tritiated thymidine incorporation data. Keratinocytes are capable of catabolising thymidine [4–6], thus the radioactivity observed in dividing cells might result from thymidine catabolism rather than DNA synthesis. Also, addition of exogenous thymidine may stimulate DNA synthesis; for example, media designed for the optimal growth of keratinocytes contain substantial amounts of thymidine [7,8]. Moreover, exogenous thymidine only measures the amount of thymidine incorporated into DNA via the salvage pathway [9] and keratinocytes vary in their capacity to use this pathway [10]. Under some circumstances DNA synthesis may occur entirely *de novo* from free bases, sugars, and phosphate without passing through a step involving thymidine. Monoclonal antibodies have recently proved useful in studying the cellular events occurring in comedogenesis. The keratin profile of ductal keratinocytes in acne has been investigated by using antisera to a panel of keratins. The follicular epithelium was found to express the hyperproliferative keratins 6 and 16 rather than the differentiating keratins 1 and 10 throughout the suprabasal layers of the comedone wall. This further supports the idea that hyperproliferation is at least in part responsible for hypercornification.‡ However, levels of other major proteins such as involucrin appear to remain unchanged whereas involucrin is diminished in some conditions involving hyperproliferation [11].

Therefore, we decided to re-evaluate the rate of cellular proliferation in acne using the monoclonal antibody Ki-67. The monoclonal antibody Ki-67 reacts with a nuclear antigen expressed by cells in the late G_1, S, M, and G_2 phases of the cell cycle [12–14]. The exact nature of the nuclear binding site of Ki-67 is unknown, although immunoblots have shown that Ki-67 binds to a large pro-

Manuscript received May 5, 1993; accepted for publication September 2, 1993.

Reprint requests to: Dr. HE Knaggs, Unilever Research, 45, River Road, Edgewater, NJ 07020.

† Blake J, Cunliffe WJ, Holland KT: The development and regression of individual acne lesions (abstr). *J Invest Dermatol* 87:130, 1986.

‡ Hughes B, Cunliffe WJ, Morris C, Leigh IM, Lane EB: Keratin profile of the pilosebaceous unit in sites prone to acne vulgaris—an *in situ* study (abstr). *J Invest Dermatol* 95:473, 1990.

0022-202X/94/$06.00

tein doublet of Mr 345 and 395 kDa. This protein is thought to interact with chromatin during the cell cycle. The use of this antibody allows actively dividing cells to be distinguished from quiescent cells. In practical terms Ki-67 has several advantages compared with tritiated thymidine. It offers a non-radioactive and more rapid method for quantifying epidermal proliferation. Furthermore, all actively cycling cells are labeled, not just those engaged in DNA synthesis, and there are no problems with catabolism of the label.

MATERIALS AND METHODS

Patients and Samples After informed consent, punch biopsies (3 mm wide, 4 mm deep) of follicles of normal-looking skin of acne subjects, non-inflamed lesions, and inflamed lesions were taken under local anesthetic from the interscapular region of acne patients with mild to moderate acne (total acne grades between 0.25 to 4 as assessed using the Leeds technique [15]). The patients had not received any treatment for their acne for a minimum of 6 weeks prior to taking the samples. The patients were between 16 and 33 years of age (the average age was 22 years). In total, eight normal acne follicles, eight open comedones, seven closed comedones and 13 inflamed lesions (six pustules, seven papules) were studied. Each follicle or lesion was obtained from a separate patient. Therefore, a total of 36 patients were studied. The biopsies were immediately snap-frozen and stored in liquid nitrogen until used. Strips of chest skin from non-acne patients undergoing open heart surgery were obtained, cut into biopsy sized pieces, and snap-frozen as described above. Chest skin was used to provide control follicles. Eight chest skin follicles were examined. These were obtained from different patients aged between 35 and 50 years (the average age was 41 years).

Serial sections were cut to a thickness of 6 μm on a cryostat (Anglia Scientific, Cambridge, UK) and thaw-mounted onto slides. The sections were air-dried for 4–24 hours, fixed for 10 min in acetone, and left until the acetone had evaporated.

Immunohistochemistry The staining method used is based upon the technique described by French *et al* [16]. Briefly, the sections were rehydrated in phosphate-buffered saline (PBS) and overlaid with the Ki-67 antibody (DAKO, Denmark), diluted 1:60 in PBS. Following incubation in a moist chamber for 45 min at room temperature, the slides were washed three times with PBS and incubated with rabbit anti-mouse horseradish-peroxidase–conjugated secondary immunoglobulin (DAKO, Denmark) (1:25 dilution in 1:20 human AB serum) for 35 min at room temperature and then washed. After staining with 3,3 diaminobenzidine tetrahydrochloride, the slides were washed well with water and counterstained with 2% methyl green. Negative controls were set up omitting the primary antibody. Sections of human tonsil obtained at tonsillectomy were used as positive controls. Tonsil contains over 40% positive nuclei for this antibody [17]. Stained sections were viewed using a light microscope (Leitz). Photographs were taken using Kodak professional black and white film.

The results were quantitated by projecting the slides using a projecting microscope onto a white screen and counting Ki-67–positive nuclei (magnification × 500). Growth fractions were obtained by expressing the number of Ki-67–positive nuclei as a percentage of the total number of basal cell nuclei counterstained with methyl green. Several microscopic anatomical sites were counted: at least three areas of the epidermis, two areas remote from the follicle and one perifollicular area, and the entire duct of the normal follicle or non-inflamed lesion. The follicular epithelium was divided into the acroinfundibulum (the upper fifth of the duct) and the infrainfundibulum (the lower four-fifths of the duct). These areas were counted separately. All of the sites counted consisted of at least 200 cells. The data was expressed as mean percentage ± SD and analysed using the Mann-Whitney U test.

RESULTS

The monoclonal antibody Ki-67 stained a proportion of the basal nuclei of keratinocytes in interfollicular and intrafollicular epithelia. It was therefore possible to count the number of positive nuclei and express them as a percentage of the total number of basal nuclei, for a given length of skin.

The results for Ki-67 staining of interfollicular epidermis are shown in Fig 1. Ki-67–positive nuclei represented 5.33% ± 3.36%† of the total normal interfollicular epidermis. This value was not significantly different from that associated with interfollicular epidermis from acne patients, 6.50% ± 3.00% (Fig 2*a*). In the interfollicular epidermis associated with non-inflamed lesions, the

† Mean ± SD.

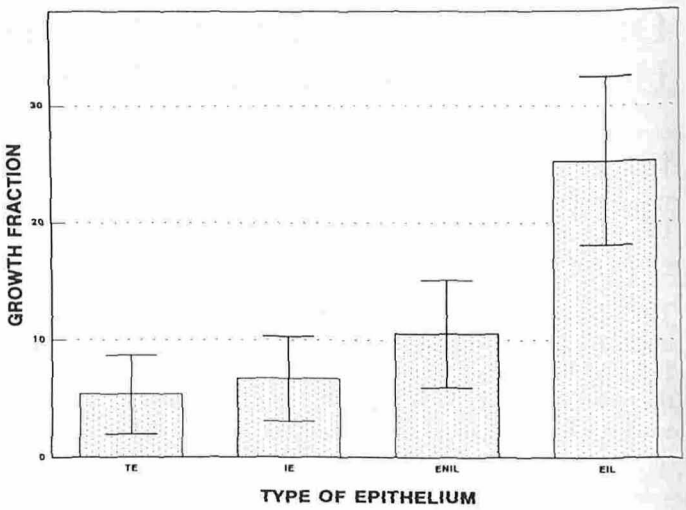

Figure 1. Growth fractions for different types of interfollicular epidermis stained with Ki-67 as described in *Materials and Methods*. Values represent the number of basal nuclei positive for Ki-67 expressed as a percentage of the total number of basal nuclei counterstained with methyl green. Values were calculated as mean percentage ± SD. TE, thoracic epidermis from a control patient; IE, interscapular epidermis from an acne patient; ENIL, interscapular epidermis around a non-inflamed lesion; EIL, interscapular epidermis around an inflamed lesion.

growth fraction for basal keratinocytes was 10.46% ± 4.45%. This value was significantly different, $p < 0.02$, from that for normal epidermis. On these sections, two distinct regions with different labeling patterns could be identified depending on their distance from the follicular pore: the perifollicular area (i.e., the epidermis adjacent to the follicular orifice) and an area remote from the follicular orifice. When counted separately, these regions were found to have significantly different proportions of cycling cells, 17.49% ± 5.09%and 8.09% ± 5.31%, respectively ($p < 0.01$). The growth fraction for the perifollicular area was also significantly different from that for the normal epidermis, $p < 0.01$, but the growth fraction for the area distal to the follicle was not. No differences were found between the interfollicular epidermis of open and closed comedones.

Epidermis surrounding inflamed lesions showed a strikingly greater percentage of Ki-67–positive nuclei. This value, 25.26% ± 6.83%, was significantly higher than all other values obtained for the interfollicular epidermis (Fig 2*b*). Growth fractions for intrafollicular epidermis are shown in Fig 3. Ki-67 labeling of epithelium lining normal follicles from chest skin was 11.01% ± 6.16%, which was significantly different compared with the value obtained for normal epidermis (5.33% ± 4.20%), $p < 0.05$. When the areas of the infundibulum were counted separately, the value obtained for the acroinfundibulum of normal ducts was lower, 5.48% ± 4.20%, compared with the value for the infrainfundibulum, 11.24% ± 7.26%, but these values were not significantly different from each other. In the ductal epithelium lining normal follicles from acne patients 15.48% ± 2.93% of the basal keratinocytes were Ki-67 positive (Fig 4*a*). This value was significantly higher, $p < 0.01$, compared with follicles from patients with no acne. The value for the Ki-67 growth fraction for the acroinfundibulum of the duct was 8.80% ± 2.36% and for the infrainfundibulum the growth fraction was 17.40% ± 1.86%. These values were significantly different from each other, $p < 0.01$.

In non-inflamed lesions the percentage of Ki-67–positive nuclei was 23.44% ± 8.36% (Fig 4*b*). This value was significantly different from that obtained for normal follicles from acne skin, $p < 0.01$. The growth fractions obtained for open and closed comedones were 24.46% ± 7.50% and 23.53% ± 10.37%, respectively. In contrast to normal follicles, there was little difference between the number of cycling cells labeled in the ductal keratinocytes in the acroin-

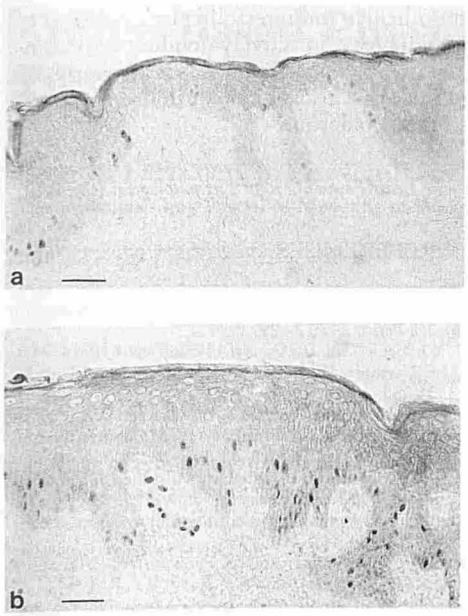

Figure 2. *a*) Normal epidermis from the chest of a non-acne patient immunoperoxidase stained with Ki-67. *Bar*, 50 μm. *b*) Epidermis adjacent to an inflamed lesion immunoperoxidase stained with Ki-67. More keratinocytes stained positive for Ki-67 in epidermis around inflamed lesions compared with normal epidermis *(a)*. *Bar*, 100 μm.

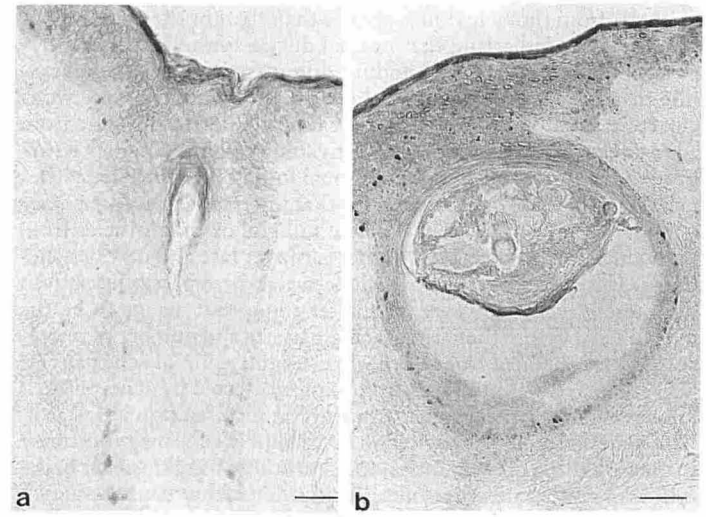

Figure 4. *a*) A normal follicle from the back of an acne patient stained with Ki-67. *Bar*, 100 μm. *b*) A non-inflamed lesion from the back of an acne patient stained with Ki-67. More keratinocytes are labeled with Ki-67 in the non-inflamed lesion compared with the normal follicle *(a)*. *Bar*, 50 μm.

fundibulum, 25.01% ± 9.92%, and in the infrainfundibulum, 19.47% ± 7.61%.

It was not possible to obtain a value for ducts in inflamed lesions because little of the follicular epithelium remained intact.

Overall, no correlation was found between the growth fraction of the pilosebaceous ducts in the normal acne follicles and non-inflamed lesions with age, sex, or acne grade of the interscapular region of the acne patients studied (n = 36).

DISCUSSION

The monoclonal antibody Ki-67 offers a quick and convenient method for estimating the number of cells in the G_1, S, M, and G_2 phases of the cell cycle. Our results obtained for normal epidermal turnover using Ki-67 show that at any given moment 5–7% of the basal cells are proliferating. These results are comparable with previous findings for epidermis obtained using tritiated thymidine [18,19] and would indicate that either Ki-67 or tritiated thymidine can be used to obtain a valid measure of cell turnover in epidermis. Good correlation between the value obtained for the growth fraction using the monoclonal antibody Ki-67 and tritiated thymidine labeling indices has previously been reported [17,20].

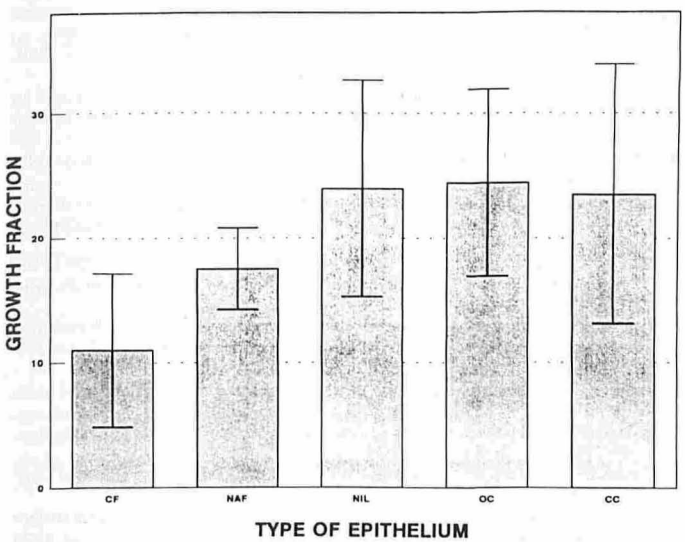

Figure 3. Growth fractions for intrafollicular epidermis stained with Ki-67 as described in *Materials and Methods*. Values represent mean percentage ± SD. CF, control follicle; NAF, follicle from the back of an acne patient; NIL, non-inflamed lesion from an acne patient; OC, open comedone; CC, closed comedone.

The results show the existence of a large population of non-cycling cells in normal epidermis. This is in agreement with the present theory that more than 90% of basal epidermal cells are in the G_0 phase of the cell cycle [21,22]. Prior to these observations it was generally believed that the majority of basal cells in epidermis were actively cycling [23,24]. The results presented here also reveal that a large quiescent population of basal keratinocytes exists in normal pilosebaceous ducts. Thus, in response to epidermal damage the follicles can become a major source of proliferating keratinocytes by recruiting the resting basal cells into the cell cycle. This supports the observation made by Eisen *et al* [25].

An unexpected finding was the increased proportion of Ki-67–positive basal nuclei in the interfollicular epidermis contiguous to acne lesions compared with normal epidermis. Keratin 6 and 16, markers for hyperproliferation have also been shown to be expressed adjacent to non-inflamed lesions (personal observation). Because the ductal epithelium has many features in common with the interfollicular epidermis [3], the perifollicular hyperproliferation associated with comedones may simply represent a response of the keratinocytes at this site to the same factors stimulating comedone formation. The dramatic Ki-67 labeling in the epidermis in inflamed lesions is likely to be due to inflammatory mediators. The inflammatory infiltrate in the dermis may liberate mitogenic substances that could diffuse away from the lesion and be responsible for stimulating hyperproliferation of keratinocytes in the epidermis distal to the lesion. Keratinocyte hyperproliferation of the epidermis around inflamed lesions in acne may be to aid the repair of the duct after rupture during inflammation. Hyperproliferation of the interfollicular epidermis may explain the reported change in the antigenicity of the cornified envelopes surrounding non-inflamed lesions in acne [26].

Only one report to date has attempted to quantify the cellular dynamics occurring in acne vulgaris [2]. Plewig *et al* used tritiated thymidine in contrast to Ki-67 used in this study. In addition, Plewig *et al* was only able to obtain samples from male prisoners with acne who ranged in age from 21 years to 42 years. The samples used in the study reported here were obtained from acne patients attending the out-patient clinic at Leeds General Infirmary and possibly provide a broader representative sample of typical acne sufferers as regards grade, age, and sex.

Our results show that the growth fraction of normal follicular

epithelia from thoracic skin is greater than that for the epidermis of thoracic skin, indicating that normal ductal epithelium is in a hyperproliferative state. This finding differs from Plewig's observation that there was no difference between the turnover of interfollicular and intrafollicular epidermis. Clinically normal follicles from acne-affected sites were found in this study to have a higher Ki-67 growth fraction compared with normal follicles obtained from thoracic skin. Possible explanations are that interscapular follicles have a higher turnover rate than thoracic follicles or that follicles from individuals with acne have a higher turnover rate and may be acne-prone. It is difficult to distinguish between these possibilities from the present data. However, the latter suggestion seems to be the most likely because there is an increase in size and number of microcomedones obtained from the normal-looking skin of acne patients [27] compared with follicles from skin unaffected by acne. Little is known about the mechanisms controlling proliferation in intrafollicular epithelium. The higher flux of sebum in acne may contribute to the sloughing off of corneocytes, thus stimulating turnover of the follicular epithelium. This idea is hypothetical but could be analogous to the effect of sellotape stripping stimulating epidermopoiesis [28]. Alternatively, the concentration of linoleic acid may be diluted in follicles with a high sebum flow and the follicular epithelia subjected to a localized linoleate deficiency. This may stimulate hyperproliferation in follicles from acne patients [29]. Evidence supports the fact that a deficiency of linoleic acid exists in acne [30]. There was a wide range in growth fractions for normal follicles from acne patients. It can be argued that follicles with the highest growth fractions may be in the early stages of comedogenesis and may evolve into acne lesions. At the present time, there are no ways of identifying and therefore of sampling follicles in the early stages of comedogenesis to test if these follicles do indeed have a high number of Ki-67–positive nuclei.

The values for the growth fractions of the acroinfundibulum and the infrainfundibulum of clinically normal follicles were significantly different from each other suggesting that cellular turnover in the infrainfundibulum may be somewhat higher than that in the acroinfundibulum. The higher number of cycling cells in the infrainfundibulum may help to explain why comedogenesis commences in this area in acne vulgaris [3]. Furthermore, hyperproliferative keratin 16 staining in this area in normal ducts but not in the acroinfundibulum has been reported by Hughes.‡ The growth fraction in non-inflamed lesions was higher than the growth fraction of clinically normal sebaceous follicles from areas affected by acne.

The basal cells of both the acroinfundibulum and infrainfundibulum were found to be in a state of hyperproliferation. This agreed with the results of Hughes showing that the suprabasal cells of comedones expressed keratins 6 and 16 throughout the follicular epithelium lining the comedone. As Plewig *et al* [2] stated, once comedones have become clinically visible there is continual recruitment of quiescent basal cells into the cell cycle despite the increasing pressure from the accumulating material in the lumen of the follicle. The additional keratinocytes produced presumably contribute further to the formation of comedones. It is possible that the mechanical pressure may also contribute to the increased turnover seen with lesional epithelia. Our results do not reveal any differences in cellular turnover between the epidermis of open and closed comedones, whereas previously reported data for comedones had indicated that open comedones have a lower labeling index compared with closed comedones. The reason for hyperproliferation in acne is uncertain. Attention has focused on a linoleate deficiency in sebum [29], androgen control of ductal proliferation [31],§ and cytokines produced by ductal corneocytes [32].

Using Ki-67, the growth fractions of the interfollicular and intrafollicular epidermis of acne have been quantitated and this confirms and extends our present understanding of ductal hyperproliferation, a characteristic feature of acne. The results clearly demonstrate that in intrafollicular and interfollicular epidermis of acne individuals there is an increase in actively dividing cells compared with skin from non-acne patients. Thus acne is a hyperproliferative disorder of the follicle that also directly or indirectly affects the interfollicular areas of the epidermis.

This work was supported by The Leeds Foundation for Dermatological Research.

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