Keratinocyte Proliferation in Epidermal Keratinocyte Disorders Evaluated through PCNA/Cyclin Immunolabelling and AgNOR Counting

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The assessment of cell proliferation is important to our understanding of hyperproliferative disorders. In this work we evaluated the proliferation characteristics of epidermal keratinocytes in diseases with abnormal keratinization by two different methods (immunostaining for the proliferating cell nuclear antigen – PCNA and histochemical staining for nucleolar organizer region – associated argyrophilic proteins – AgNORs). Twenty-seven specimens from diseases with an abnormal keratinization were studied and compared with specimens of normal human skin. As compared with the latter, the numbers of PCNA-positive epidermal keratinocytes were increased in psoriasis, congenital non-bullous ichthyosiform erythroderma, epidermolytic hyperkeratosis and chronic dermatitis and decreased in ichthyosis vulgaris, X-linked ichthyosis and pityriasis rubra pilaris. In most cases a parallel modification of AgNORs was found. We conclude that although PCNA immunolabelling and AgNOR staining do not provide strictly correlated values, both appear as useful markers for the assessment of keratinocyte proliferation in epidermal disorders. *Key word: Epidermal diseases.*

Cell proliferation is one of the most fundamental biological processes, the evaluation of which has many implications both for the pathophysiological understanding and the treatment of hyperproliferative, neoplastic or hyperplastic diseases. Several methods have been proposed for evaluating cell kinetics on histological material and some of them have been applied to the study of epidermal disorders. Two techniques recently introduced are histochemical staining for nucleolar organizer region-associated argyrophilic proteins (AgNORs) and immunohistochemical labelling for the proliferating cell nuclear antigen/cyclin (PCNA). These have been applied mostly to the study of several types of cutaneous tumours. On the other hand, epidermal disorders exhibiting an abnormal keratinization usually also comprise altered proliferation characteristics. In the present work we assessed for the first time the usefulness of PCNA immunolabelling and AgNOR histochemical staining in the study of epidermal diseases with an altered keratinization.

MATERIAL AND METHODS

Tissue samples

These included twenty-seven biopsies from several diseases characterized by an abnormal keratinization (Table I) and 6 specimens of normal human skin obtained through plastic surgery. The specimens had been collected in our dermatopathology laboratory, fixed in formalin and embedded in paraffin. The diagnosis had been established by examination of hematoxylin-eosin-stained sections using well-established criteria (1), taking also into account clinical data.

PCNA immunolabelling and counting

This was performed on 5 μm-thick deparaffinized tissue sections using a monoclonal antibody to PCNA/cyclin (clone PC10, Dako, Copenhagen, Denmark) (2) and a labelled streptavidin-biotin-peroxidase method (kit LSAB, Dako, Copenhagen, Denmark) with aminoethylcarbazole as chromogen. Counting of positive nuclei was performed under direct microscopic examination. Results were expressed as number of positive nuclei per mm² surface of epidermal section; the latter was evaluated with the aid of a semi-automatic image analyzer (Videoplan, Kontron, Munich). The mean number of PCNA-positive cell nuclei ($\pm$ SD) was then calculated for each disease group studied.

AgNOR histochemical staining and counting

This was performed according to the method of Ploton et al. (3). Briefly, after deparaffinization the sections were washed in deionized

Table I. *Results of PCNA and AgNOR counting*

Diagnosis	n°	PCNA[a]	AgNOR[b]
Psoriasis	6	1513 $\pm$ 185**	2.30 $\pm$ .25***
Epidermolytic hyperkeratosis	3	977 $\pm$ 968*	2.35 $\pm$ .40*
Congenital non-bullous ichth. erythroderma	3	974 $\pm$ 537*	2.16 $\pm$ .0.12*
Chronic dermatitis	6	715 $\pm$ 256*	2.77 $\pm$ .53***
Normal skin	6	555 $\pm$ 531	1.93 $\pm$ .14
Congenital palmoplantar keratoderma	2	552 $\pm$ 458*	2.36 $\pm$ .19*
Pityriasis rubra pilaris	3	444 $\pm$ 164*	1.89 $\pm$ .32*
X-linked ichthyosis	1	322*	2.65*
Ichthyosis vulgaris	3	113 $\pm$ 143*	1.66 $\pm$ .07**

a: mean ($\pm$ SD) number of labelled cells per mm² of epidermal section surface; b: mean ($\pm$ SD) number per nucleus.
Statistical significance (compared with normal skin by the Mann-Whitney U-test): * non-significant, ** $p < 0.05$, *** $p < 0.01$.

Fig. 1. PCNA immunostaining of normal human epidermis: scattered basal and parabasal cells show nuclear staining (× 250).

water and incubated for 30–40 min in the dark with the staining solution; this was freshly prepared by mixing 1 volume of 2% gelatin solution in 1% formic acid with 2 volumes of a 50% aqueous solution of silver nitrate. For each specimen at least 150 basal cells were

Fig. 2. PCNA Immunostaining of psoriatic epidermis: there is an obvious increase in the number of labelled cells as compared with normal epidermis (× 250).

examined and the number of AgNORs counted by the same observer (EH) in order to avoid inter-observer variation. All dots of basal cell nuclei that could be separated after appropriate focusing were counted individually. The mean number of AgNORs (± SD) was then calculated for each disease group studied.

Statistical comparison
This was performed by the non-parametric U-test (Mann-Whitney).

RESULTS

PCNA/cyclin immunolabelling
This revealed in normal skin a variable number of positive nuclei, usually within the basal epidermal layer and occasionally also of the para-basal layer (Fig. 1). The labelling intensity was somewhat variable from one nucleus to another not only among the various specimens but also within the same section; however, every effort was made to count positive nuclei in a homogeneous manner, considering the overall staining intensity of each section. In the diseases studied the appearance of the labelling was generally similar to that obtained on normal skin; however, in some diseases an obvious increase in the number of labelled nuclei was seen; these were present not only within the basal cell layer but also within the malpighian layer. This was particularly the case in psoriasis lesions (Fig. 2). The results of PCNA counting are shown in Table I.

AgNOR histochemical staining in normal skin
This revealed a variable number of black dots within nuclei, of rather uniform size and shape. Basal cells as a rule contained more AgNORs than suprabasal ones. No major differences in the appearance of AgNORs between normal and diseased skin were detected (Figs. 3–4). The results of AgNOR counting are shown in Table I.
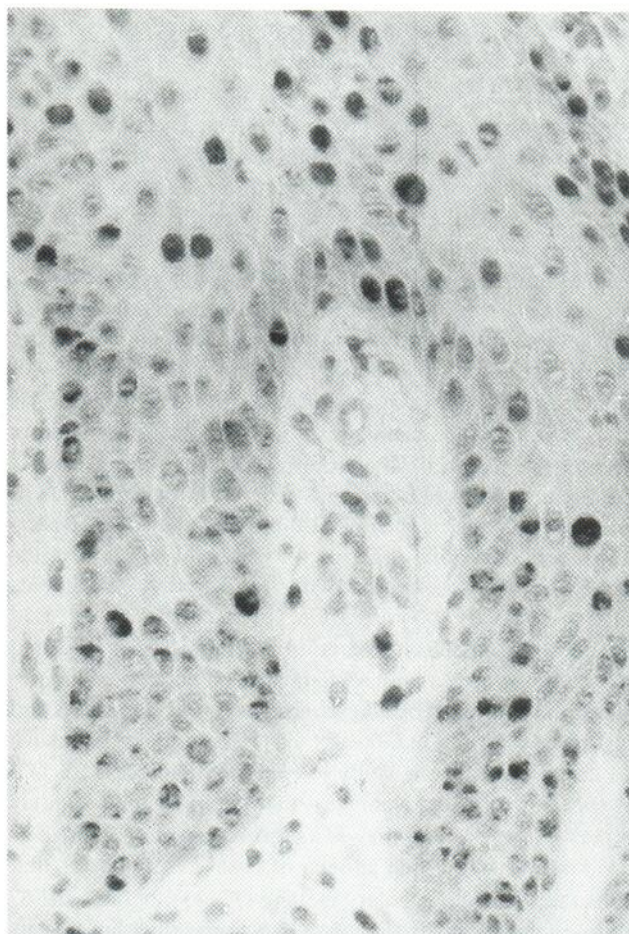
DISCUSSION

The assessment of cell proliferation in histological material can
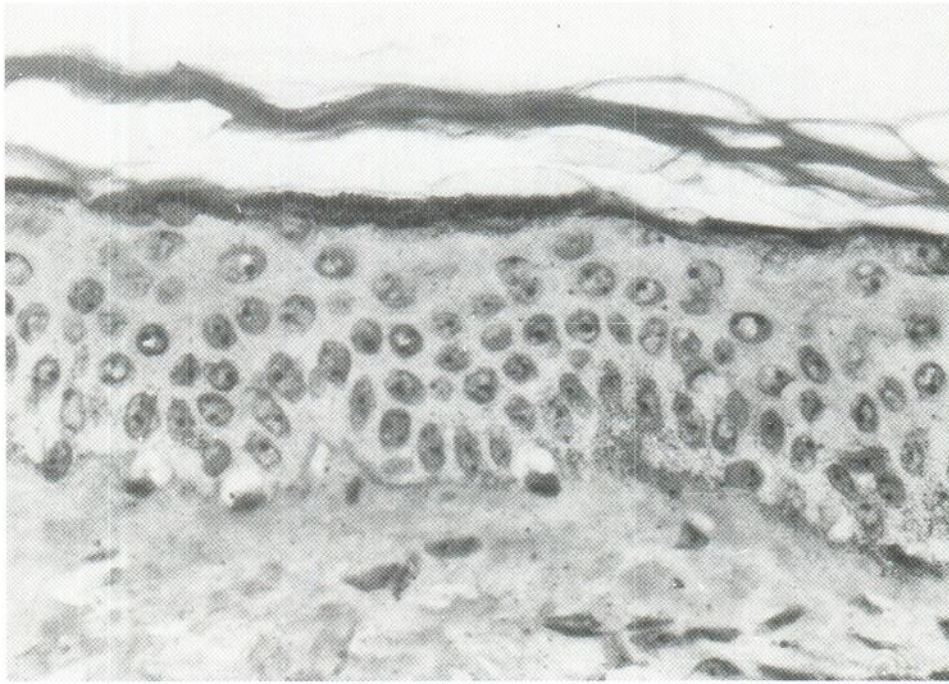
Fig. 3. AgNOR histochemical staining of normal human epidermis: AgNORs are visualized as black dots within the nucleus ($\times 400$).
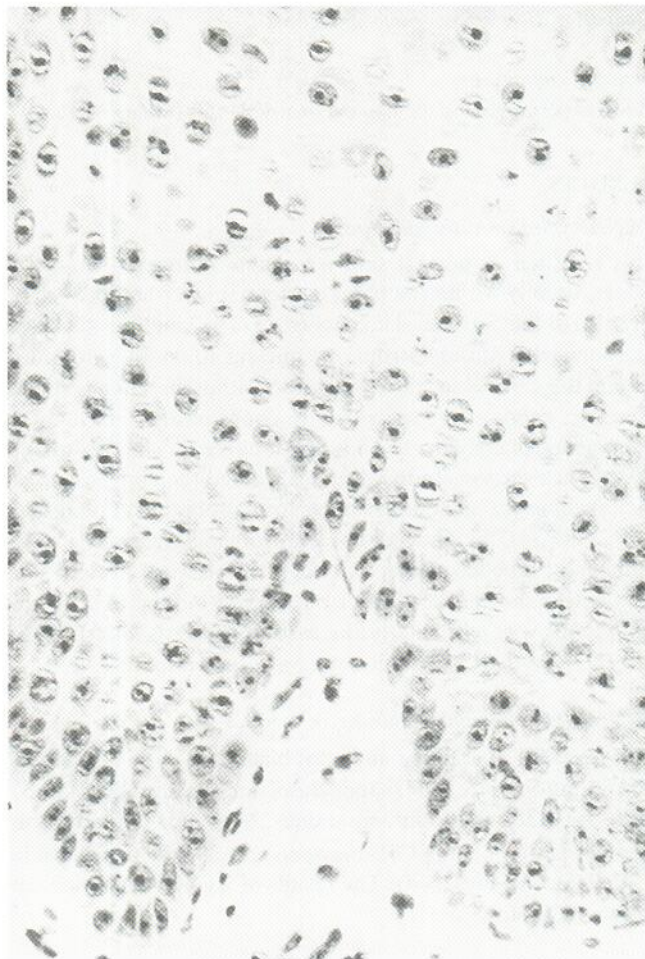

Fig. 4. AgNOR histochemical staining of psoriatic epidermis: AgNORs have the same appearance as those seen in normal epidermis but are more numerous ($\times 400$).

be achieved by a variety of methods, each one of which has advantages and disadvantages (4, 5). The simplest one is the direct microscopic counting of cells in mitosis, expressed as a percentage of mitotic cells ("mitotic index") or as number of mitoses per 10 high-power fields ("mitotic count"). This method can be performed in routinely-stained sections but has the disadvantage of considerable inter-observer variations. A refinement of the evaluation of cell proliferation was achieved by the study of ³H-thymidine incorporation. ³H-thymidine is a pyrimidine analogue incorporated in DNA-synthesizing cells, thus providing a good estimation of the fraction of cells in S phase of the cell cycle (but not of the length of S phase). The disadvantages of this technique, that has been applied to the study of epidermal disorders (6), include the necessity of preincubating the freshly-excised tissue with ³H-thymidine, as well as the potential hazards resulting from the manipulation of radioactive material. The latter problem has more recently been circumvented thanks to the generation of antibodies to pyrimidine analogues, such as 5'-bromodeoxyuridine (BrdU) or 5'-iododeoxyuridine (7). BrdU labelling yields results equivalent to those obtained with ³H-thymidine (8) and its handling is simple and rapid. One of the most widely used tools for studying cell proliferation is Ki-67; this monoclonal antibody recognizes a poorly-characterized antigen of the nuclear matrix expressed by all cycling cells (i.e. in late G1, S and G2/M phases but not in G0/early G1) (9). Some authors have used this reagent for the study of epidermal diseases (10, 11). However, a major disadvantage – that in our opinion severely hampers its use on the skin – is its (cross)reactivity with an as yet unknown cytoplasmic antigen of basal keratinocytes that occults the specific nucleolar labelling (12, 13); besides, Ki-67 is only applicable to frozen tissue sections, thus limiting its utility in diagnostic pathology.

More recently, antibodies to other antigens reflecting cell

proliferation have been generated. PCNA/cyclins belong to a family of such cell-cycle regulatory proteins. PCNA was first detected in proliferating but not resting cells of human tissues by autoantibodies from patients with systemic lupus erythematosus (14). Cyclin was discovered through two-dimensional gel electrophoretic studies of proliferating and quiescent cells (15). Later the identity of these two proteins (PCNA & cyclin) was shown (16). PCNA is a 36 kDa nuclear acidic non-histone protein identified as an auxiliary protein of DNA polymerase-d (17, 18). Although differences could exist between various cell lines, the expression of PCNA/cyclin seems to be highly increased during the late G1 and early S phases of the cell cycle (19–21); this also appears to be the case in cultured human keratinocytes where PCNA expression is correlated with DNA synthesis (22). Several anti-PCNA/cyclin monoclonal antibodies are currently available (PC10, TOB7, 19A2, 19F4) and some of them can be used on formalin-fixed tissue sections. The number of PCNA-positive cells seems to correlate with Ki-67 counts in nodal lymphoid neoplasms (non-Hodgkin leukaemias/lymphomas) (23, 24) and with ^{3}H-thymidine counts in cultured epidermal keratinocytes (23).

Up to now, only limited data have been made available concerning the expression of PCNA in normal and diseased epidermis. It appears that PCNA is expressed in normal skin by a small percentage of basal keratinocytes (25); an increased number of PCNA-positive cells has been reported in cases of psoriasis (26), keratoacanthoma, Bowen's disease, melanoma, verrucous and squamous cell carcinoma (27–29).

On the other hand, nucleolar organizer regions (NORs) are loops of DNA occurring within nucleoli that encode for ribosomal RNA; these are located on human acrocentric chromosomes (n°s 13, 14, 15, 21 & 22) and are associated with AgNORs that control their transcription. AgNORs comprise the nucleolar proteins C23 or nucleolin, B23 and a subunit of RNA-polymerase I; they can be visualized and counted on tissue sections by a simple histochemical technique using a solution of silver nitrate. The number of NORs (and therefore of AgNORs) increases with an increased transcriptional or proliferative activity of the cell (30, 31). AgNOR counts have been reported to correlate with Ki-67 immunoreactivity in lymphomas (32) and breast cancer (33, 34). In dermatopathology, AgNOR counting has so far been applied to the study of benign and malignant neoplasms of melanocytic (reviewed in 35), fibrohistiocytic (36) and epithelial (37–39) origin.

Previous investigations have addressed the question of the proliferative profile of epidermal diseases, by studying mitotic counts, ^{3}H-thymidine incorporation and Ki-67 immunostaining. The present study was prompted by the fact that within the group of epidermal diseases very few data have been published regarding PCNA expression and virtually no data at all regarding AgNOR counts. In our experience, PCNA immunolabelling proved to be a rapid procedure giving generally reproducible results. The slight variations in staining intensity observed among the different specimens are presumably due to the (inevitably) variable duration of fixation. The results we obtained with PCNA are on the whole in keeping with those obtained in previous works using mitotic counts, ^{3}H-thymidine/BrdU incorporation or Ki-67 immunolabelling. Psoriasis,

the most extensively studied disease, exhibits and increased mitotic count (40, 41), increased ^{3}H-thymidine (6, 42) and BrdU uptake (43, 44) and Ki-67 immunolabelling (10, 11). In accordance with these results, psoriasis showed in our study the highest increase of PCNA expression and AgNOR numbers ($p < 0.05$ and < 0.01, respectively). Chronic dermatitis ("lichen simplex") has been reported to show an increased ^{3}H-thymidine uptake (45) and Ki-67 immunolabelling (10); in keeping with this, we found an increase both of PCNA and AgNOR counts that could be related to the epidermal hyperplasia seen histologically in this disease. Congenital nonbullous ichthyosiform erythroderma and epidermolytic hyperkeratosis, characterized by an increased ^{3}H-thymidine uptake (6, 46) and increased mitotic counts (41), also showed increased PCNA and AgNOR counts. Conversely, ichthyosis vulgaris and X-linked ichthyosis, considered to comprise a normal or reduced epidermal turnover – evaluated through ^{3}H-thymidine uptake (6), ki-67 immunolabelling (9), mitotic counts (41) or by their transit time (47) – showed reduced PCNA expression. Interestingly, we found that both PCNA and AgNOR values of PRP were significantly decreased as compared with psoriasis ($p < 0.05$). This finding suggests that the two diseases, despite clinical similarity, bear distinct cell kinetic characteristics; this is consistent with a different histological picture (1). Remarkably, an increased ^{3}H-thymidine uptake has been reported in PRP (47); the cause for this discrepancy is presently unknown but the heterogeneity of PRP (48) provides a possible explanation.

On the other hand, we found AgNOR counting somewhat more problematic than PCNA counting. However, AgNORs were usually readily visualized, being uniformly sized, shaped and stained; this fact, along with the ease in observing basal cells and distinguishing them from suprabasal or inflammatory cells rendered AgNOR counting easier in epidermal diseases as compared with tumoural proliferations, disorders where AgNOR counting has up to now been applied. When the values of PCNA and AgNOR counting were compared, it was found that in most diseases these varied in a similar way; thus, when compared with normal skin, AgNOR counts were increased in the case of psoriasis ($p < 0.01$), congenital nonbullous ichthyosiform erythroderma, chronic dermatitis ($p < 0.01$) and epidermolytic hyperkeratosis and decreased in the case of PRP and ichthyosis vulgaris ($p < 0.05$). The diseases in which AgNOR counts were increased despite reduced PCNA counts included X-linked ichthyosis and congenital palmoplantar keratoderma. The unique case of X-linked ichthyosis studied does not allow conclusions to be drawn. With respect to congenital palmoplantar keratoderma we were unable to find in the literature data concerning epidermal kinetics. It is relevant to remember here that the number of AgNORs is not correlated solely to the proliferative activity but seems also to reflect the transcriptional (metabolic) activity of the cell; therefore a discrepancy between AgNOR and PCNA counts as observed in the present study could be due to the fact that epidermal keratinocytes may show an increased metabolic activity unrelated to their proliferative capacity.

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