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Proliferating Cell Nuclear Antigen Decreases in Normal Human Keratinocytes with Differentiation Stimuli But Not in an HPV Immortalised Cell Line

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Proliferating cell nuclear antigen (PCNA) is a co-factor for DNA polymerase δ, which replicates genomic DNA during cell growth and division. Using a monoclonal antibody to PCNA (PC10) and conventional immunofluorescent techniques, we have compared the effect of differentiation stimuli on PCNA expression in normal and HPV immortalised keratinocytes. Two positive nuclear staining patterns were observed, a strong speckled form characteristic of proliferating cells and a weaker diffuse form. Strong nuclear staining was present in 44 ± 4% (mean ± SEM) of normal keratinocytes proliferating as a monolayer in 70 μM calcium serum-free medium but decreased to 13 ± 3% after the differentiation stimulus of 2 mM calcium medium for 2 days. An even greater reduction was observed following other differentiation agents, 1,25 dihydroxyvitamin D_3, the phorbol ester TPA and the non-specific protein kinase C inhibitor staurosporine. Transforming growth factor-β, which slows keratinocyte growth without inducing differentiation, reduced strong staining to 17 ± 3% of cells, but with an increase in the diffuse pattern of staining from 39 ± 4 to 57 ± 3%. HPV immortalised cells were resistant to the above agents except staurosporine, which inhibited growth and reduced the strong nuclear staining from 44 ± 5% to 15 ± 2%. *Key words: PCNA; vitamin D; calcium; TPA; cell cycle.*

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The synthesis of proliferating cell nuclear antigen (PCNA), a nuclear protein of molecular weight 36 kDa, has been shown to correlate with the proliferative state of cells. PCNA is a co-factor for DNA polymerase δ (pol δ) and increases the processivity of the enzyme along the leading strand during DNA replication (1). Although there are variations in the intensity and pattern of PCNA staining throughout the cell cycle, the staining intensity correlates well with the incorporation of tritiated thymidine into nuclei during DNA synthesis (2). Two distinct populations of PCNA are detected by immunofluorescence and may be distinguished by different fixation techniques. Only a diffuse nuclear staining is seen with formaldehyde fixation but, using methanol/acetone fixation, Bravo et al. demonstrated that 20–30% of PCNA remained tightly associated with replicon clusters, indicating an important role in DNA synthesis (3). This close association between replicating DNA and PCNA has recently been confirmed by simultaneous immunofluorescent labelling and confocal microscopy (4).

Studies of normal human epidermis have shown that only a few keratinocyte nuclei stain positively for PCNA, in contrast to the marked staining in the hyperproliferative epidermis of psoriasis (5). In common with other cell types, for synchronised keratinocyte cultures the nuclear staining with PCNA correlates well with labelling of keratinocyte nuclei (6). In a primary culture of human keratinocytes 20–30% of cells express PCNA when grown as a proliferative monolayer in low calcium culture, indicative of the increased proliferation in vitro, but this decreases with the induction of differentiation by high calcium medium (6, 7). Using immunofluorescence techniques we have investigated whether changes in PCNA expression occur in cultured human keratinocytes following the other differentiation stimuli 1,25 dihydroxyvitamin D_3 (8) and the phorbol ester TPA (9). We have also investigated the action of staurosporine which, although a protein kinase C (PKC) inhibitor whilst TPA is a PKC activator, induces both mouse and human keratinocyte differentiation (10, 11). For comparison the effect of transforming growth factor beta (TGF-β) on PCNA was studied, since it inhibits keratinocyte growth without inducing differentiation (12).

Keratinocyte transformation by the SV40 sarcoma virus has been shown to increase PCNA nuclear staining to approximately 40%, consistent with the high growth rate of transformed lines (6). Increased PCNA expression has also been demonstrated in human papillomavirus (HPV) induced warts (13) and HPV immortalised keratinocyte lines (14). The change in expression of PCNA in normal keratinocytes following differentiation stimuli has been compared with a differentiation-resistant HPV type 16 immortalised keratinocyte line.

MATERIALS AND METHODS

Cell culture

Human keratinocytes were grown from normal foreskins or retro-aural skin removed during plastic surgery procedures. The epidermis was separated by incubation overnight at 4°C in phosphate-buffered saline (PBS) containing dispase (2 mg/ml; Boehringer Mannheim). Basal cells were disaggregated by incubation for 5 min at 37°C with trypsin (0.05%, ICN-Flow) and EDTA (0.02%). After washing, the cells were grown in the serum-free medium MCDB153 with an extracellular calcium concentration of 70 μM and supplemented with hydrocortisone (5×10^{-7} M), transferrin (5 mg/ml), insulin (5 mg/ml) and epidermal growth factor (10 ng/ml) (15). Bovine hypothalamic extract was used for cell passage to enhance attachment to fresh flasks but was not routinely present in the culture medium. Keratinocyte lines were used up to the fourth passage. The HPV cell line, which was cultured under the same conditions, had been immortalised by transfection of an open reading frame for the genes E6 and E7 from HPV 16 (16). Chemicals were purchased from Sigma unless otherwise stated. TGF-β was obtained from ICN-Flow and 1,25 dihydroxyvitamin D_3 was a gift from Dr. L. Binderup, Leo Pharmaceutical Products, Denmark.

Immunofluorescence staining

Cells were cultured on glass coverslips and fixed with methanol and acetone (1:1 v/v) at room temperature for 10 min. Cells were incubated

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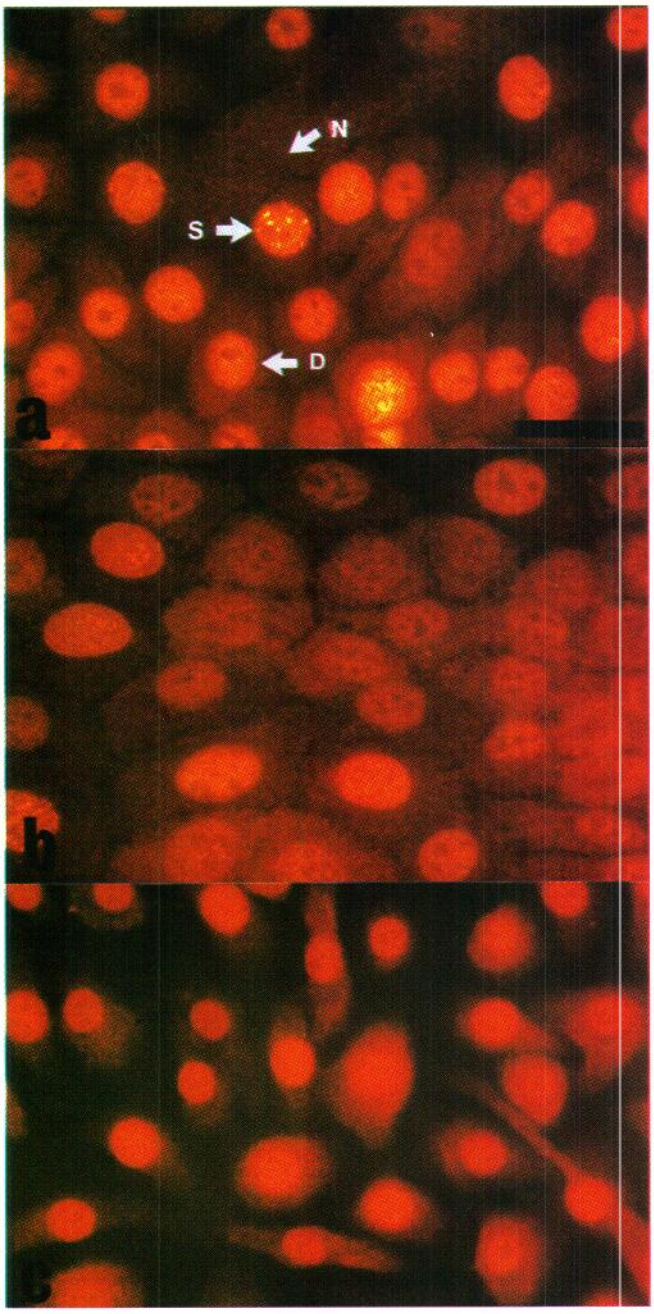

Fig. 1. PCNA staining of normal keratinocytes with monoclonal PC10 and rhodamine conjugated secondary antibody: (*a*) control, (*b*) 2 mM calcium for 48 h and (*c*) 5 ng/ml TGF-β for 48 h. In (*a*) the cells indicated by labelled arrows demonstrate strong speckled (*S*), diffuse (*D*) and negative (*N*) staining patterns. The bars represent 25 μm.

with a 10% normal rabbit serum in PBS for 10 min to block non-specific binding, followed by a 1:50 dilution of mouse monoclonal antibody to PCNA (PC10, Novacastra) for 30 min at room temperature (17). Fluorescein or rhodamine-conjugated rabbit anti-mouse immunoglobulin 1:50 (Dakopatts Ltd.) was used to visualise staining. For each condition 4 coverslips of cells were studied, and for each of these a number of fields were examined to give a count of between 100 and 200 cells per coverslip. A minimum of 500 cells were studied for each condition and for normal keratinocytes cells from at least two donors were used. Results were expressed as a percentage of total cell number with the mean percentage and standard error of the mean for each staining pattern (diffuse, or strong granular). A cell was classed as strong granular staining if at least three bright nuclear spots were present and diffuse if the nucleus was clearly visible against the background, but there were no more than two bright nuclear spots.

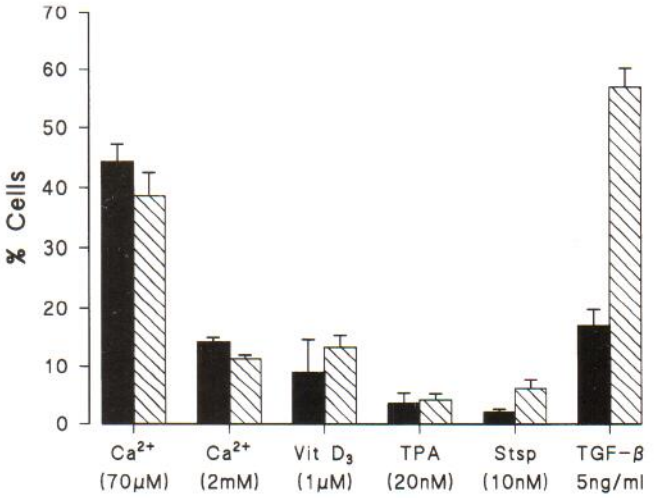

Fig. 2. PCNA staining of normal human keratinocytes following differentiation stimuli for 2 days. Results are expressed as a percentage of total cell number with the SEM shown by the error bar. Strong speckled nuclear staining is shown by the solid bars and diffuse nuclear staining by the hatched bars.

RESULTS

PCNA staining of keratinocytes was confined to the nucleus and examples of the two patterns observed, strong granular and weak diffuse staining, are shown in Fig. 1a. Compared to controls the degree of staining was much less following the differentiation stimulus of high extracellular calcium (Fig. 1b). The number of cells staining and the proportion in each staining category was quantified by cell counting, and the effect of the differentiation stimuli on PCNA staining in normal human keratinocytes is shown in Fig. 2. At 48 h the differentiation stimuli of 2 mM calcium medium, 1 μM 1,25 dihydroxyvitamin

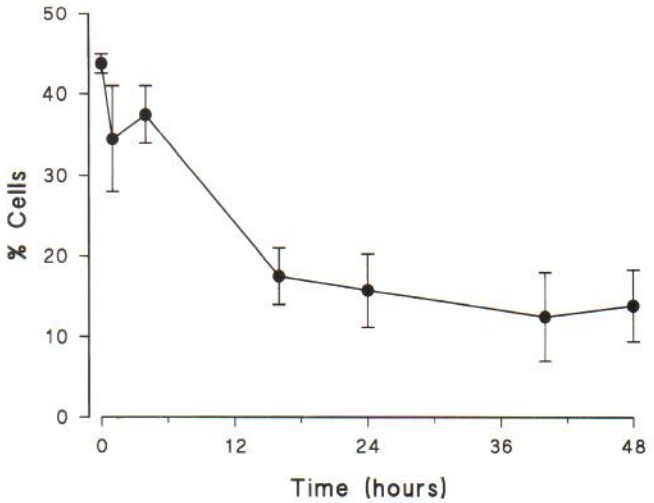

Fig. 3. Time course of the reduction in strong nuclear staining of normal keratinocytes following an increase in extracellular calcium from 70 μM to 2 mM. Results are expressed as mean percentage of cells staining taken from 3–8 coverslips and with the SEM shown by the error bars.

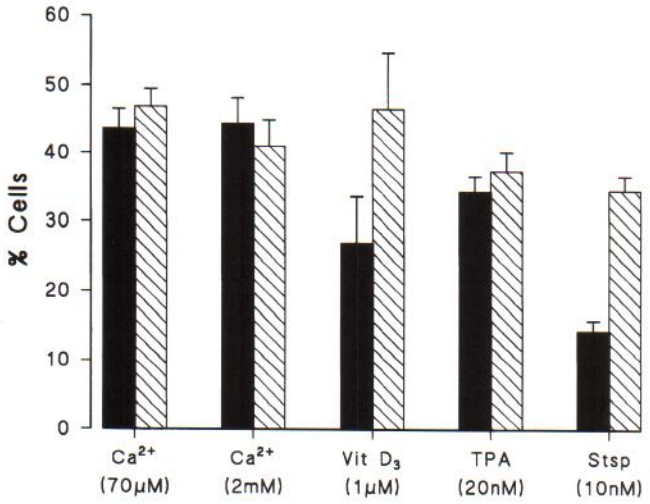

Fig. 4. PCNA staining of HPV transformed keratinocytes following differentiation stimuli for 2 days. Results are expressed as a percentage number of total number of cells with the SEM shown by the error bar. Strong speckled nuclear staining is shown by the solid bars and diffuse nuclear staining by the hatched bars.

D_3, 20 nM TPA and 10 nM staurosporine were all associated with a highly significant reduction in both the strong speckled and diffuse positive PCNA staining patterns. In control cultures grown in 70 μM calcium, the strong, speckled staining was present in 44% of nuclei but this decreased to 13% with 2 mM calcium and less than 10% with the other differentiation stimuli ($p < 0.025$, Kruskal-Wallis test). In contrast, 5 ng/ml TGF-β reduced the number of cells with a strong, speckled pattern but increased the diffuse positive staining (Fig. 1c, Fig. 2). The time course of the reduction in strong PCNA staining following addition of 2 mM calcium is shown in Fig. 3. There was no significant change during the first 4 h but a gradual reduction over 48 h. The reduction was significant by 16 h ($p < 0.05$, Mann-Whitney U test) and the further reduction highly significant ($p < 0.0003$, Cuzick's test for trend). PCNA staining was not observed in the suprabasal layers of stratified cultures following differentiation stimuli. HPV transformed cells were much less sensitive to the differentiation stimuli and, except for staurosporine, showed little or no reduction in cell growth. Likewise, there was no significant reduction in PCNA staining in the HPV transformed cells following incubation with the differentiation stimuli, except for staurosporine which was associated with a marked reduction in PCNA staining ($p < 0.026$, Kruskal-Wallis test) (Fig. 4).

DISCUSSION

We have shown that growth inhibition with TGF-β and the induction of differentiation with a variety of stimuli in normal human keratinocytes are associated with a highly significant reduction in nuclear PCNA staining. The strong speckled staining pattern, characteristic of the PCNA staining during DNA replication, was reduced with all stimuli, but only for TGF-β was this accompanied by an increase in diffuse staining, indicating persistence of PCNA within the nucleus. In contrast, the HPV immortalised line showed little reduction in PCNA staining, except following treatment with staurosporine.

Our results confirm earlier studies that cultured keratinocytes express high levels of PCNA (5, 6). In keratinocytes TGF-β has been shown to slow cell growth without inducing differentiation, and cells accumulate at the G1/S boundary (12). Following TGF-β it is probable that whilst PCNA is not bound to pol δ at replicon clusters, it persists in the nucleus to give the diffuse staining pattern. Differentiation stimuli cause a reduction in thymidine labelling and withdrawal of keratinocytes from the cell cycle (8, 9). The reduction in PCNA expression by 2 mM extracellular calcium was similar to that reported by Miyagawa et al. (5), but we have shown that the other differentiation stimuli of 1,25 dihydroxyvitamin D_3, TPA and staurosporine are more potent in this respect. TPA inhibits the thymidine labelling of keratinocytes more rapidly than 1,25 dihydroxyvitamin D_3 (8, 9) and is also a more potent inhibitor of expression of PCNA. Those stimuli which induce differentiation and withdrawal from the cell cycle are associated with a reduction in total PCNA staining, not only a redistribution within the nucleus. The half-life of PCNA has been reported to be 20 h (2) and this relatively long half-life of the protein explains the gradual fall in PCNA levels after addition of 2 mM calcium. In stratified cultures PCNA expression has been shown to be associated with the smaller basal cells and correlated with DNA synthesis (6); this is consistent with the absence of staining we found in suprabasal differentiating cells.

PCNA expression in the HPV immortalised cells was insensitive to the differentiation stimuli apart from staurosporine. These HPV cells are a high passage line selected for resistance to calcium-induced differentiation, and 2 mM calcium did not reduce the PCNA staining from the control level of 44% strong speckled staining. The protein products of the HPV E6 and E7 genes cooperate to immortalise human keratinocytes. Transfection with E6 alone has no effect whilst E7 alone induces hyperproliferation, but the cells eventually senesce (16). In addition it has recently been shown in keratinocytes that the degradation of one of the cell cycle regulatory proteins, p53, is enhanced by transfection of the HPV E6 gene (18). Thus the E6 gene product reduces the levels of the growth inhibitory "anti-oncogene" protein p53, making the cells resistant to differentiation agents. The E7 protein product binds to the retinoblastoma tumour suppressor gene product pRB105 and inactivates it (19). The HPV immortalised keratinocytes line has two HPV proteins which combine to promote cell growth, immortalisation and inconsequence persistence of PCNA. TGF-β inhibition of keratinocyte proliferation involves suppression of c-myc transcription, but this effect is blocked by the E7 gene product (20).

We have shown that all keratinocyte differentiation stimuli cause a reduction in PCNA expression, but this action is blocked in differentiation resistant HPV immortalised cells. TGF-β does not induce differentiation of normal keratinocytes and PCNA persists within the nucleus in the absence of cell growth. We conclude that strong speckled staining is associated with DNA replication and the weaker diffuse pattern observed occurs in cells accumulating in the G1 phase of the cell cycle. Withdrawal from the cell cycle and differentiation result in the gradual loss of PCNA expression over 48 h.

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