Synthetic epidermal pentapeptide and related growth regulatory peptides inhibit proliferation and enhance differentiation in primary and regenerating cultures of human epidermal keratinocytes

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Summary

A pentapeptide that inhibits proliferation of mouse epidermal keratinocytes *in vivo* and *in vitro* has been purified from mouse skin extracts. In the present study the effect of a synthetic analog of the epidermal pentapeptide on proliferation and differentiation of cultured human epidermal keratinocytes was investigated. In young, rapidly growing primary cultures the pentapeptide caused a dramatic decrease in mitotic activity and also induced pronounced changes in the balance between kinetically defined subpopulations of proliferating cells. A dipeptide derived from the pentapeptide was found to be at least as potent. A serine derivative of a hemoregulatory peptide also seemed to be active. When tested in epidermal cultures regenerating after removal of the suprabasal cell layers, both the pentapeptide and the dipeptide were shown to cause a delay in the proliferative response. Both peptides were also able to stimulate early (increase in cell size) and late (cornified envelope formation) events in the differentiation pathway of the keratinocyte. The apparent stimulatory effect on differentiation was most clearly seen in regenerating cultures, whereas the effect on primary cultures varied with the experimental set-up. It is suggested that homologous epidermal peptide(s) may play a major role in the regulation of human epidermal homeostasis.

Key words: epidermal peptides, human keratinocytes, cell culture, regeneration, differentiation.

Introduction

The epidermis is a large renewing cell system, where tissue homeostasis is maintained through coordinated control of keratinocyte proliferation and differentiation (Watt, 1988). Thus, under physiological conditions the epidermal thickness remains constant in spite of continual cell turnover. The mechanisms ensuring the normal balance between cell production rate in the basal layer and the rate of loss of terminally differentiated cells from the stratum corneum are largely unknown. Although keratinocytes are able to respond to a vast range of growth stimulators and inhibitors (Rheinwald and Green, 1977; Green, 1978; Boyce and Ham, 1983; Wille *et al.* 1984; Shipley *et al.* 1986; Barrandon and Green, 1987*a*; Coffey *et al.* 1987; Reiss and Dibble, 1988; Rubin *et al.* 1989) there is evidence that proliferation in the epidermis is also governed by heterogeneity within the proliferating cell population (Barrandon and Green, 1985; Barrandon and Green, 1987*b*). This indicates that regulation of proliferation and differentiation in the epidermis is a complex and highly integrated process. *Endogeneous* growth inhibitors may play an important role in this respect. In several tissues, including the epidermis, homeostasis has been assumed to be mediated by tissue-specific mitotic inhibitors ('chalones') acting as signals in a negative feed-back regulation (Iversen, 1981).

Human epidermal cultures provide a useful *in vitro* model for analyzing how proliferation and differentiation of the keratinocyte are regulated. In a previous study (Jensen and Bolund, 1986) we have shown that a partially purified pentapeptide extracted from mouse epidermis reduced the mitotic activity in human keratinocyte cultures and induced pronounced changes in the balance between kinetically defined subpopulations of proliferating cells. These studies are extended in the present report where the effects of synthetic epidermal pentapeptide and related peptides are investigated in primary and regenerating cultures of human keratinocytes. It is shown that the pentapeptide is able to influence proliferation as well as morphological differentiation *in vitro*.

Materials and methods

Cell culture

Experiments were performed on primary as well as regenerating cultures of adult human epidermal keratinocytes. Skin samples (mainly whole skin) were obtained from plastic surgery operations. Before cultivation, as much as possible of the subepidermal tissue was removed by gentle scraping. Primary

cultures were established from small explants (8 explants per 25 cm^2 tissue culture flasks) (Jensen and Therkelsen, 1981; Jensen *et al.* 1983). The cultures were grown in Dulbecco's Modified Eagle's medium (DMEM, GIBCO) plus 20 % (v/v) fetal calf serum (Gibco), penicillin (250 units ml^{-1}), streptomycin (25 $\mu g\ ml^{-1}$), hydrocortisone (0.4 $\mu g\ ml^{-1}$), and epidermal growth factor (EGF, Collaborative Research Inc., Lexington, MA), 10 $ng\ ml^{-1}$. The cultures were maintained in an atmosphere of 5 % CO_2 in air at 34–35 °C. Medium was changed twice a week.

Regenerating cultures were established from one-month-old primary cultures by incubation in low-calcium medium (Jensen and Bolund, 1988). At that age most primary cultures were subconfluent or confluent and showed extensive keratinization with desquamation. After incubation for 72 h at 37 °C in calcium-free minimal essential medium (SMEM, Gibco) without additives, all suprabasal layers were detached as flakes that could easily be removed after gentle shaking of the culture flask. This left a monolayer of basal cells. When the stripped cultures were re-fed medium with normal calcium concentration (DMEM with additives as described above) the basal cell monolayer gradually re-established a multilayered, keratinized tissue (Jensen and Bolund, 1988).

Growth inhibitors

The main epidermal growth inhibitor used in the present study was a synthetic pentapeptide originally characterized after purification from mouse skin extracts (Elgjo and Reichelt, 1984; Elgjo *et al.* 1986*a*; Reichelt *et al.* 1987). The pentapeptide has the structure: pyroGlu-Glu-Asp-Ser-GlyOH. A synthetic derivative composed of the two terminal amino acids of the pentapeptide (pyroGlu-GlyOH) has been found to have effects like the pentapeptide (Elgjo *et al.* 1986*a*) and was thus included in the present study. The dipeptide too, has been found in water extracts of mouse skin (Elgjo *et al.* 1986*a*).

The structure of the epidermal pentapeptide is very similar to a hemoregulatory pentapeptide purified and characterized by Laerum and Paukovits (Paukovits and Laerum, 1982; Laerum and Paukovits, 1984; Laerum and Paukovits, 1987). The hemoregulatory peptide was purified from granulocytes and shown to inhibit granulopoiesis of bone marrow cells *in vivo* and *in vitro*. The hemoregulatory peptide has the amino acid sequence pyroGlu-Glu-Asp-Cys-LysOH and may exist in a monomeric or an oxidized dimeric form with apparently opposite effects on granulopoiesis (Laerum and Paukovits, 1984, 1987). Both forms and a serine derivative of the monomer (cysteine exchanged with serine at position 4) were tested in the present study.

In the majority of experiments the peptides were added eight times over a period of 24 h or six times over a period of 48 h (mitotic activity measurements in primary cultures) at a concentration of 0.05 nmol ml^{-1} (50 μl of a 5 μM stock solution in 0.02 M acetic acid added to 5 ml of culture medium; for details, see Results). The small amount of acetic acid added did not change the pH of the growth medium and was without effect on growth and differentiation in control cultures. Repeated administration was considered necessary for long-term incubation because of the known or assumed instability of the peptides. In initial experiments the peptides were added every second hour; in later experiments, however, similar results were obtained with less-frequent administration of the peptides. In some experiments the effect of 2-h incubation with a single dose of 0.05 nmol ml^{-1} was also studied. To obtain a dose–response curve incubations were performed for 2 h with various concentrations of peptide (10^{-10} M to 10^{-6} M).

Measurement of mitotic activity

Measurements of the mitotic activity were performed on 2- to 3-week-old primary cultures that had been treated with peptide or vehicle for the last 48 h (see above). The measurements were performed with an inverted phase-contrast microscope as previously described (Jensen and Bolund, 1986). Two- to three-weeks old primary cultures are in a state of rapid lateral expansion with only slight differentiation. As the rate of lateral expansion may vary from culture batch to culture batch (i.e. cultures derived from different biopsies) all explants compared in each experiment were derived from the same biopsy. Different experiments were performed with skin samples from different individuals.

The mitotic activity was measured by counting the number of mitotic figures in the marginal 0.9 mm rim of the monolayer area of the outgrowth zone (equal to half a visual field, using objective ×10 and ocular ×12.5), excluding early prophases that are difficult to identify with certainty. Explants of about equal size (15–20 mm in diameter) were selected for analysis and the total circumference of each outgrowth zone was scanned. The numbers of mitotic figures thus represent a constant surface area (the peripheral approx. 0.5 cm^2 of the zone).

[^{3}H]thymidine incorporation, cell sorting and autoradiography

Both primary and regenerating cultures were labelled with [^{3}H]thymidine ([^{3}H]TdR) for combined analysis by cell sorting and autoradiography. All labelling experiments were performed with [*methyl*-^{3}H]thymidine (New England Nuclear, sp.act. 20 Ci $mmol^{-1}$) at a concentration of 10 μCi ml^{-1} for 30 min (Jensen *et al.* 1985). Primary cultures were labelled after 2–3 weeks when extensive lateral growth prevails, whereas regenerating cultures were labelled at selected times after refeeding normal calcium medium (up to 7 days post-stripping). After labelling, a single cell suspension was obtained by trypsinization and the cells were fixed in absolute ethanol (Jensen *et al.* 1983, 1985). The cells were then stained with a combination of mithramycin and ethidium bromide for flow cytometry of cellular DNA contents. The DNA frequency distributions were obtained with a fluorescence-activated cell sorter (FACS II) (Beckton Dickinson, Mountain View, CA). Cells were sorted from a window of 10 channels in the middle of the S-phase DNA distribution and from a window representing the left (lower) half of the G_1 peak. The sorted cells were collected on glass slides and covered with an Ilford K2 emulsion for autoradiography. The autoradiograms were exposed for 8 days, developed, and finally stained with Giemsa.

The numbers of labelled and unlabelled S-phase cells were counted in the light microscope. About 500 cells were counted in each experimental group. The labelled cells were further characterized by counting the number of grains per nucleus to obtain a grain count distribution (Jensen *et al.* 1983, 1985). The autoradiographic background was always ≤3 grains per nucleus. On the basis of the grain count distribution the S-phase cells were divided into three subpopulations: strongly labelled cells (≥25 grains per nucleus); weakly labelled cells (4–24 grains per nucleus); and unlabelled cells (0–3 grains per nucleus). In all experiments more than 98 % of cells sorted from the G_1 peak were classified as unlabelled.

The proportion of cells in the different phases of the cell cycle was estimated for regenerating cultures by planimetry of the DNA frequency distributions as described by Clausen *et al.* (1976).

Morohological differentiation

The morphological differentiation was assessed by quantitation of cornified envelope formation and measurement of the size of unlabelled S-phase cells.

Cornified envelope formation was quantitated after incubation in stabilized suspension culture. The cultured cells were brought into suspension by trypsinization (Jensen *et al.* 1983) and then incubated in culture medium (MC) stabilized with methyl-cellulose (Methocel) using the protocol published by Green (1977). When keratinocytes are maintained in this medium they loose the ability to multiply, but are still able to undergo terminal differentiation with premature cross-linking of envelope precursors (Green, 1977; Green, 1980; Green *et al.* 1982).

Samples of the suspension culture were withdrawn at various times after the start of incubation and the cells were recovered by diluting tenfold with 10 mM Tris–HCl buffer (pH 7.4). To determine the proportion of cells that had formed a cornified envelope, counted cells were heated for 5 min in 10 mM Tris–HCl (pH 7.4) with 1 % sodium dodecyl sulfate and 20 mM dithiothreitol (modified from Sun and Green, 1976). The number of cornified envelopes was scored microscopically (phase-contrast) by counting in a hemocytometer and expressed as a percentage of the total number of cells.

In some experiments incubation in methyl-cellulose-stabilized medium was replaced by a 2 h incubation with the calcium ionophore X537A (50 $\mu g\,ml^{-1}$) after the cells had been brought into suspension (Rice and Green, 1979).

The projected cell diameter of sorted S-phase cells unlabelled after [^{3}H]TdR incorporation was determined with the aid of a measure introduced into the eyepiece of the microscope. The measure was calibrated using a micrometer slide. Only cells from regenerating cultures were measured. All measurements were performed at $\times 10^3$ magnification and 100–200 cells were analyzed on each slide.

Results

Proliferation in primary cultures

The effect of the regulatory peptides on proliferation in young primary cultures after 48 h treatment is shown in Table 1. Both the pentapeptide and the dipeptide had a marked inhibitory effect on the mitotic activity; the dipeptide being slightly more potent. On average, the dipeptide reduced the mitotic activity by 52 % whereas a 41 % reduction was observed with the pentapeptide. Control values varied somewhat from experiment to experiment. Several factors might contribute to the observed variation. Skin samples from different sites and individuals were used in different experiments (see Materials and methods). Furthermore, the timing (with respect to culture age and peak mitotic activity) of the analysis varied slightly from experiment to experiment. However, all comparisons were made between cultures from the same experiment.

When the labelling intensity of sorted S-phase cells after [^{3}H]TdR incorporation was investigated a dramatic heterogeneity as to the number of autoradiographic grains per cell nucleus was revealed; very strongly labelled (≥25 grains per nucleus) as well as weakly (4–24 grains per nucleus) and completely unlabelled (0–3 grains per nucleus) S-phase cells were found. This heterogeneity in labelling intensity of S-phase cells was similar to that observed in previous studies and most probably is a reflection of differences in DNA replication rate among subpopulations of cycling cells (Jensen *et al.* 1985). When primary cultures were treated with epidermal peptides for 48 h a clear reduction in the proportion of strongly labelled S-phase cells was observed. In contrast, the effect on the proportion of weakly labelled cells was only slight. In one of the experiments shown in Table 1 the percentage of strongly (and weakly) labelled S-phase cells was compared to the mitotic activity. As shown, the effect of both peptides on the labelling of the S-phase cells paralleled the effect on the mitotic activity.

As also shown in Table 1, the hemoregulatory peptides did not affect the mitotic activity in primary human keratinocyte cultures. However, a moderate reduction – statistically significant only at the 10 % level – of the mitotic activity could be observed after incubation with the serine derivative of the hemoregulatory monomer.

The effect on the labelling pattern of sorted S-phase cells after a 2 h incubation with a single dose of the epidermal pentapeptide is shown in Fig. 1. Only the fraction of

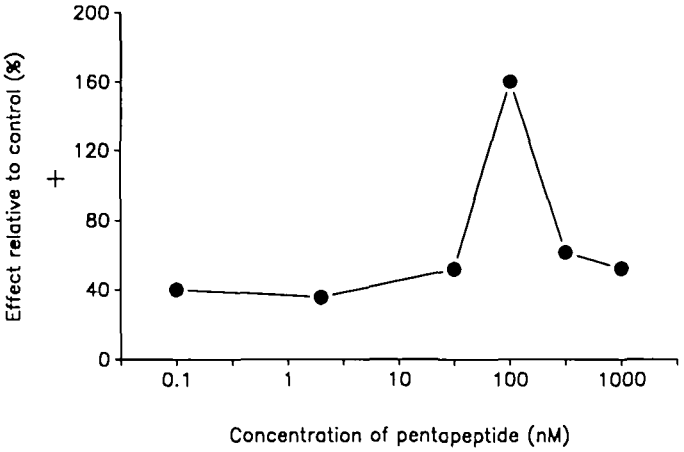

Fig. 1. Dose–response curve showing the proportion of strongly labelled (≥25 grains per nucleus) mid S-phase cells in primary cultures incubated for 2 h with different concentrations of epidermal pentapeptide. Each point represents data on cells pooled from six cultures (the cellular outgrowth from 48 explants). Skin samples were obtained from two different patients. The values are expressed as percentage increase in relation to the corresponding fraction in cultures only treated with vehicle (*x*-axis corresponds to control levels). The cultures were labelled with 10 $\mu Ci\,ml^{-1}$ [^{3}H]TdR for the last 30 min of the incubation period.

Table 1. *The effect of epidermal growth inhibitors on the mitotic activity and on the percentage of S-phase cells strongly and weakly labelled with [^{3}H]thymidine in primary human epidermal outgrowths* in vitro

Experiment	*n**	No. of mitoses per outgrowth zone (mean±S.E.M.)		Percentage of S-phase cells that were labelled	
				Strongly	Weakly
Experiment I					
Control	12	151±4.9	$2P<0.001$§		
Pentapeptide†	12	92±10.8	$2P<0.001$§		
Dipeptide†	12	64±7.3	$2P=0.05$§		
Experiment II					
Control	18	395±15.6	$2P<0.001$§	33 0	57.8
Pentapeptide†	18	230±5.4	$2P<0.001$§	16.5	51.0
Dipeptide†	18	216±6.4	$0.1<2P<0.2$§	10.3	50.1
Experiment III					
Control	6	143±15.5			
Hemoreg. peptide‡ (monomer)	6	159±11 7	$0.05<2P<0.10$§		
Hemoreg. peptide‡ (dimer)	6	151±4.9			
Hemoreg. peptide‡ (serine derivative)	6	99±14.8			

* Number of outgrowth zones per experimental group.
† 0.05 nmol added per ml of culture medium every 8 h for 48 h.
‡ 0.05 nmol per ml of culture medium added repeatedly (16 times) over 48 h.
§ Student's *t*-test.

strongly labelled S-phase cells is shown. In the whole dose range investigated, short-term incubation with the pentapeptide resulted in an *increased* proportion of strongly labelled S-phase cells with an apparent maximum at 100 nM. These results were similar to those obtained earlier with the crude peptide where a small maximum also was apparent at 100 nM (see Fig. 3 of Jensen and Bolund (1986)).

Proliferation in regenerating cultures

The suprabasal layers were stripped off one-month-old differentiated primary cultures by a 72 h incubation in low-calcium medium. After stripping, the regaining monolayer of basal cells were re-fed medium with a normal calcium concentration with or without addition of epidermal peptides. The peptides were added repeatedly (8 times) during the first 24 h followed by two additions daily. At selected times post-stripping the cultures were labelled with [^{3}H]TdR followed by analysis in the cell sorter. Proliferation was assessed by determination of the percentage of cells in the S-phase of the cell cycle (obtained from the DNA frequency distributions) and by analysis of the grain count distribution of sorted S-phase cells after autoradiography. The results of representative experiments are presented in Figs 2 and 3. In normal cultures the stripping process induces a regenerative response in the remaining monolayer of basal cells similar to that observed after tape stripping *in vivo* (Jensen and Bolund, 1988). *In vitro* the regenerative reaction seems to involve the recruitment to the cycling population of cells with a high rate of DNA replication, which are destined for rapid differentiation. This is indicated by a dramatic increase in the percentage of cells in S-phase and a corresponding increase in the proportion of strongly labelled S-phase cells. Both parameters peak at 72–96 h post-stripping, which coincides with a peak in mitotic activity (Jensen and Bolund, 1988). Morphologically, this results in a rapid re-establishment of the suprabasal layers, whereas lateral expansion is suppressed (Jensen and Bolund, 1988).

Incubation of regenerating cultures with the epidermal pentapeptide delayed the increase in the proportion of

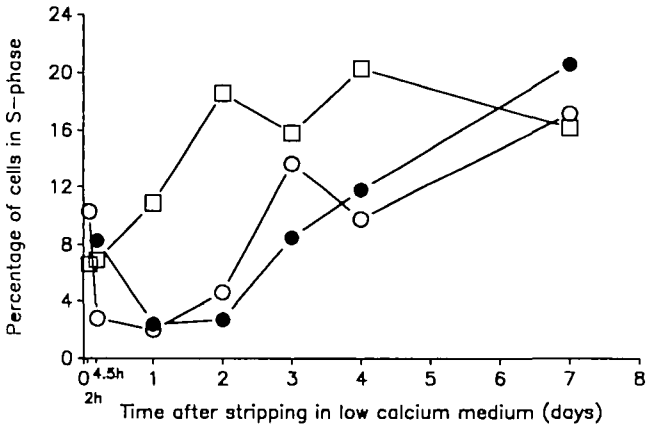

Fig. 2. Variation in the percentage of cells in S-phase with time after low-calcium stripping of primary cultures. (□——□) Control cultures; (●——●) cultures recieving a high dose of epidermal pentapeptide within the first 24 h after stripping (0.05 nmol ml^{-1}, ×8) followed by maintenance dosage (0.05 nmol ml^{-1}, ×2 daily); (○——○) cultures treated with epidermal pentapeptide as above except that the high dosage was repeated from 72 h to 96 h post-stripping. Each point represents data on cells pooled from five cultures.

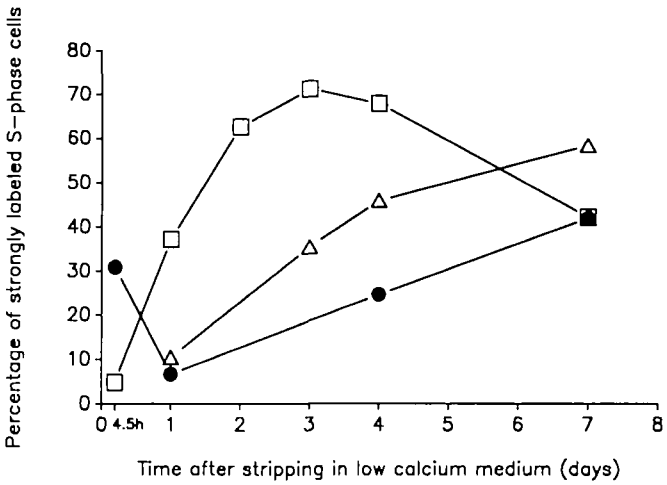

Fig. 3. Variation in the proportion of strongly labelled (≥25 grains/nucleus) S-phase cells with time after low-calcium stripping of primary cultures. At the times indicated, cultures were labelled with 10 μCi ml^{-1} [^{3}H]TdR for 30 min. (□——□) Control cultures; (●——●) cultures treated with epidermal pentapeptide; (△——△) cultures treated with dipeptide. Same dosage regime as in Fig. 2 (high dosage within the first 24 h followed by maintenance). Each point represents data on cells pooled from five cultures.

cells in S-phase (Fig. 2). No clear maximum was discernable; instead, a strong reduction in the proportion of cells in S-phase was followed by a gradual recovery after 72 h. Seven days post-stripping the S-phase fraction in cultures treated with pentapeptide was actually increased compared to control cultures. In one experiment the high dosage of pentapeptide applied within the first 24 h (see above) was repeated after 72 h; this, however, only resulted in a minor temporary second decrease in the proportion of cells in S-phase (Fig. 2). It is also apparent from Fig. 2 that the S-phase fraction shortly after stripping (2 h) is *larger* in cultures treated with pentapeptide than in control cultures.

The effect of treatment with epidermal peptides on the labelling intensity of sorted S-phase cells closely paralleled the effect on the proportion of cells in S-phase (Fig. 3). Shortly after stripping (4½ h) the proportion of strongly labelled S-phase cells was *larger* in treated than in control cultures. This was followed by a strong reduction, but later the proportion of strongly labelled S-phase cells increased again. Increasing the dosage of pentapeptide at 72 h did not significantly affect the S-phase labelling (data not shown). The long-term labelling pattern of the S-phase cells in treated cultures varied somewhat in different experiments. However, in most experiments, there was a clear trend towards a higher proportion of strongly labelled S-phase cells in the treated cultures at day 7 post-stripping. This was paralleled by a similar trend towards a higher mitotic activity at day 7 in treated cultures compared to control cultures (data not shown).

Morphological differentiation in primary cultures

The stage of differentiation in primary cultures treated with epidermal peptides was estimated by quantitation of cells with a cornified envelope. In preliminary experiments, a direct determination of the proportion of cornified cells *in situ* (i.e. without incubations as described below) was attempted. However, because very few cells

Table 2. *The effect of epidermal growth inhibitors on the percentage of cells with a cornified envelope in primary human epidermal outgrowths* in vitro

Experiment	Percentage of cells with a cornified envelope*	
	Incubation in MC for 11 days	Incubation with X537A for 2 h
Experiment I†		
Control	30.2	
Pentapeptide	16.7	
Dipeptide	14.2	
Experiment II‡		
Control	48.8	
Pentapeptide	39.9	
Dipeptide	24.2	
Experiment III†		
Control		9.3
Pentapeptide		11.7
Dipeptide		15.5

MC, methyl-cellulose-stabilized medium; X537A, ionophore.
*Each value represents the mean of two determinations.
†Penta/dipeptide, 0.05 nmol per ml medium added every third hour for 24 h.
‡Penta/dipeptide, 0.05 nmol per ml medium added every 8 h for 48 h.

had cornified spontaneously in these young, rapidly proliferating cultures a comparison of treated and untreated cultures was impossible. After a short incubation with the calcium ionophore X537A about 10 % of the cells formed a cornified envelope in control cultures; as shown in Table 2, treatment with pentapeptide or dipeptide increased the proportion of cells with a cornified envelope, with the dipeptide being the most potent of the two. In contrast, when the production of cornified envelopes was determined after incubation in methyl-cellulose-stabilized medium (MC-medium) for 11 days a clear inhibition was observed after treatment with epidermal peptides. Again, the dipeptide was apparently more potent than the pentapeptide (Table 2; see also Discussion). To ensure that this inhibitory effect on cornified envelope formation did not represent a cytotoxic action, cell viability was determined after staining with ethidium bromide and acridine orange (Pards *et al.* 1979). No difference in cell viability was observed between peptide-treated and untreated cultures (data not shown).

The hemoregulatory dimer, monomer or its serine derivative were all without a significant effect on the proportion of cornified envelope-containing cells determined after 2 h incubation with ionophore (data not shown).

Morphological differentiation in regenerating cultures

The morphological differentiation in regenerating cultures was assessed by quantitation of cornified envelope formation after incubation in MC-medium and by measurement of mean cell diameter of unlabelled S-phase cells. Fig. 4 presents the combined results of four different experiments where the proportion of cornified cells was determined in control cultures and in cultures treated with the epidermal peptides. All four experiments gave similar results. In each experiment the peptides (0.05 nmol ml^{-1}) were added every second hour within the first 28 h post-stripping. At the end of the treatment period cells were harvested by trypsinization and incubated in MC-medium. Samples for cornified envelope quantitation were withdrawn at different times as indicated in Fig. 4. It is shown that both peptides stimulated cornified envelope formation in regenerating cultures with the effect of the dipeptide exceeding that of the pentapeptide. The effect of both peptides was statistically significant after incubation in MC-medium for 14 days ($2P<0.05$; Student's *t*-test).

Further evidence for increased differentiation in the presence of epidermal peptides was sought from cell size measurements, since the size and the state of differentiation of the keratinocyte has been shown to be positively correlated both *in vivo* and *in vitro* (Green, 1980; Barrandon and Green, 1985; Jensen *et al.* 1987). The mean cell diameter of sorted S-phase cells was determined at different times after stripping. Only the diameter of unlabelled S-phase cells was measured, since there are indications that among these cells there are both the most undifferentiated, small, basal cells and the most differentiated, large, cycling cells (Jensen *et al.* 1987). The results are shown in Table 3, where it can be seen that the mean cell diameter of unlabelled S-phase cells increased with time in control cultures as well as in cultures treated with the epidermal peptides. However, in peptide-treated cultures this increase was significantly larger than in control

Table 3. *Mean cell diameter of unlabelled S-phase cells maintained in medium with or without epidermal peptides for different periods after stripping in low calcium medium*

Experimental group	Mean cell diameter (μm) ±S.E.M. at indicated time post-stripping			
	4.5 h	24 h	7 days	
Control	14.2±0.29	14.6±0.46	16.1±0.25	$2P<0.001$†
+ Pentapeptide*	14.6±0.23	15.9±0.22	20.0±0.35	
+ Dipeptide*	–	16.7±0.23	19.6±0.30	$2P<0.001$†

*0.05 nmol ml^{-1} of culture medium added every third hour during the first 24 h followed by 0.05 nmol ml^{-1} of culture medium twice daily.
†Student's *t*-test.

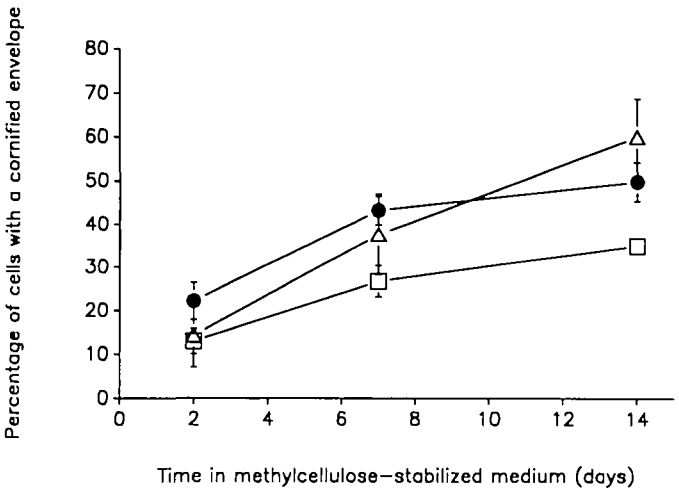

Fig. 4. Variation in the percentage of cells with a cornified envelope in low-calcium stripped, regenerating cultures. The cultures were treated repeatedly (8 times) with peptide (0.05 nmol ml^{-1}) or vehicle during the first 28 h post-stripping, after which the cells were harvested and incubated in methylcellulose-stabilized medium. Data from 4 different experiments are given as mean values±S.E.M. (□——□) Control cultures; (●——●) cultures treated with epidermal pentapeptide; (△——△) cultures treated with dipeptide.

cultures. As noted above, the smallest (≤10 μm) unlabelled S-phase cells are thought to be the most immature of all S-phase cells (Jensen *et al.* 1987); during the early phases of regeneration after low-calcium stripping they usually amount to about 10% of the S-phase population. As regeneration proceeds through the following 7 days their proportion is gradually reduced to about half the initial value in normal cultures whereas in peptide-treated cultures they were almost absent at day 7 (data not shown).

Discussion

The purified murine epidermal pentapeptide was originally tested on mouse epidermis. It was shown to inhibit the epidermal mitotic rate by 40–50% in hairless mice *in vivo* as well as in primary mouse epidermal cultures (Elgjo *et al.* 1986*b*). Similar results were obtained with the synthetic pentapeptide and a synthetic dipeptide composed of the N- and C-terminal amino acids of the pentapeptide (Elgjo *et al.* 1986*a*; Reichelt *et al.* 1987). Recently, the pentapeptide was shown to inhibit growth of normal human keratinocytes *in vitro* (Watt *et al.* 1989) and of mouse hair follicles *in vivo* and *in vitro* (Paul *et al.* 1989). In contrast, established keratinocyte cell lines turned out to be insensitive to the peptide (Watt *et al.* 1989). The present study shows that the effects of the synthetic pentapeptide on proliferation in primary human epidermal cultures are in line with previous observations. Both mitotic activity and [^{3}H]TdR labelling intensity of sorted S-phase cells were reduced in our primary cultures.

The effect of the synthetic pentapeptide on regenerating keratinocyte cultures was also investigated. The pentapeptide was shown to reduce strongly the labelling intensity of S-phase cells in regenerating cultures. Furthermore, the pentapeptide inhibited the increase in the proportion of cells in S-phase that is normally observed during the regenerative response (Jensen and Bolund, 1988). Together these effects resulted in a delayed regenerative response in cultures treated with pentapeptide. With time, however, a gradual recovery from the inhibition was observed, this recovery could only be slightly and temporarily inhibited by intensifying the dosage of pentapeptide. This finding suggests that – in consequence of continued treatment – the cells become refractory to the action of the pentapeptide.

The principal effect of the pentapeptide on the S-phase population seemed to be to reduce the proportion of strongly labelled (rapidly cycling) S-phase cells; there is evidence that the strongly labelled S-phase cells constitute a distinct sub-population responsible for rapid expansion of cell mass both during lateral growth and during regeneration (Jensen *et al.* 1987); these cells may thus correspond to the so-called transient amplifying cells described by others (Potten, 1981).

The effect on proliferation of the dipeptide was similar to that of the pentapeptide; in fact, in most experiments the dipeptide seemed to be even more potent than the pentapeptide. A hemoregulatory peptide, which inhibits granulopoiesis *in vivo* and *in vitro* (Paukovits and Laerum, 1982; Laerum and Paukovits, 1984, 1987), was without effect on proliferation of cultured human keratinocytes. However, a serine derivative of the hemoregulatory peptide, which is very similar in structure to the epidermal pentapeptide, seemed to inhibit mitotic activity in primary keratinocyte cultures although the effect was quantitatively less marked than that of the epidermal pentapeptide. The epidermal pentapeptide was without effect on the mitotic activity in cultured fibroblasts from human skin (unpublished observations). These results all indicate a high degree of molecular specificity in the peptide–cell interaction.

The epidermal pentapeptide has been reported to stimulate terminal differentiation in primary mouse epidermal cultures. Continous treatment for 24 h increased the number of cells forming a cornified envelope (Elgjo *et al.* 1986*b*). In contrast, Watt *et al.* (1989) were unable to demonstrate an effect on differentiation in cultures of human keratinocytes. The present study shows that both the pentapeptide and the dipeptide stimulated differentiation in regenerating human cultures, whereas the effect on primary cultures varied with the experimental set-up.

From the results of the present study it seems reasonable to conclude that the principal effect of the epidermal peptides on cultured human keratinocytes was to inhibit proliferation and to stimulate differentiation. However, the results also indicate that the effect of the epidermal peptides on human keratinocytes is more complex. Thus, in contrast to the effect of long-term incubation, short-term incubation with the pentapeptide resulted in an *increased* [^{3}H]TdR labelling intensity of the S-phase cells. This was seen in both primary (Fig. 1) and regenerating (Figs 2 and 3) cultures and similar results have previously been obtained with the crude, partially purified pentapeptide from mouse skin extract (Jensen and Bolund, 1986) and with the synthetic pentapeptide (Watt *et al.* 1989). Furthermore, the effect of the epidermal peptides on cornified envelope formation in our primary cultures also seemed to vary with the experimental set-up. When cornified envelope formation was determined after incubation with calcium ionophore the epidermal peptides were shown to increase the proportion of cells with a cornified envelope; in contrast, when the quantitation of cornified envelope formation was done after long-term incubation in MC-medium the epidermal peptides reduced

the proportion of envelope-containing cells. Short-term incubation with ionophore probably induces cross-linking only in that proportion of cells that have already accumulated enough envelope precursors at the time of incubation, whereas long-term incubation in MC-medium is likely to induce premature cross-linking of envelope precursors in all cells able to undergo terminal differentiation.

There is increasing evidence that growth-regulatory peptides, in general, may be considered multifunctional and that the effect of a given peptide on a cell may depend on several factors such as the culture conditions, stage of differentiation, and the presence or absence of other growth-regulatory peptides (Sporn and Roberts, 1988). In this context it should also be noted that the primary keratinocyte cultures used in the present study were characterized by rapid lateral expansion with minimal differentiation. This situation was quite different from that prevailing in the regenerating cultures: the regenerative response is thought to be effected by a recruitment to the dividing population of cells destined for rapid terminal differentiation rather than lateral growth, which is suppressed during regeneration (Jensen and Bolund, 1988).

The mechanism of action of the epidermal pentapeptide is at present unknown. The observations made in the present study could be explained by assuming an isolated, inhibitory effect of the peptide on the cell cycle; as cell growth is unaffected this might lead to increased differentiation as a secondary phenomenon. An argument against this way of action is the observation that short-term treatment with epidermal peptide apparently stimulated a cohort of strongly labelled (rapidly cycling) S-phase cells in primary as well as in early regenerating cultures. This cohort of strongly labelled S-phase cells may be destined for rapid differentiation (see below). Alternatively, the principal effect of the pentapeptide could be to stimulate differentiation leading to a diminished pool of proliferating cells. This mechanism of action may be supported by the observation that the number of small, unlabelled S-phase cells was strongly reduced in regenerating cultures treated with the pentapeptide; as noted above, the small, unlabelled S-phase cells may be the most immature of the cultured cells (Jensen *et al.* 1987). On the other hand, the fact that the pentapeptide inhibited cornification of cells in primary cultures after long-term incubation in methyl-cellulose-stabilized medium argues against this simple way of action.

Thus, the pentapeptide may have separate effects on proliferation and differentiation. It has been argued that, although proliferation and differentiation in the epidermis are tightly coordinated, they may be regulated independently (Watt, 1988). There is growing evidence of heterogeneity among the proliferating cells in epidermis, both *in vivo* (Potten *et al.* 1982; Clausen *et al.* 1983) and *in vitro* (Dover and Potten, 1983; Barrandon and Green, 1985; Jensen *et al.* 1985; Albers *et al.* 1986; Barrandon and Green, 1987*b*; Jensen *et al.* 1987), and the results of the present study may be indicative of the pentapeptide acting on more than one subpopulation of proliferating cells that may differ with respect to their degree of maturation (Jensen *et al.* 1987). In primary cultures, treatment with pentapeptide may stimulate a cohort of proliferating cells to pass through the last expansion cycle and subsequently initiate rapid terminal differentiation (stimulated cornification after short-term incubation with ionophore). The other proliferating cells may be inhibited (long-term depression of proliferation) leading to a decrease in the pool of cells that becomes committed to terminal differentiation (decreased fraction of cells able to form cornified envelopes after long-term incubation in MC-medium). Different subpopulations may also differ in responsiveness, both quantitatively and qualitatively, to growth regulatory substances and some studies have suggested that proliferating basal cells can be divided into inhibitor-sensitive and -insensitive subpopulations (Marks *et al.* 1978; Richter *et al.* 1984).

The identity of control mechanisms responsible for the development and maintenance of homeostasis in mammalian epidermis has been extensively sought. Continuous cell renewal is a prominent feature of epidermal homeostasis. Although a large number of epidermal growth stimulators and inhibitors have been described the major factors that regulate cell renewal in the epidermis are still largely unknown. Basic knowledge about how these factors may interact and contribute to the maintenance of tissue homeostasis in the epidermis is also lacking. One mechanism that has been proposed for epidermal homeostasis is a negative feed-back control. It assumes that the suprabasal, terminally differentiating cells produce a substance that normally suppresses the rate of cell renewal in the basal layer; if cells in the suprabasal layers are lost through injury the inhibition is relieved, resulting in increased proliferation until the suprabasal layers are regenerated and homeostasis restored (Bullough, 1962; Iversen, 1981). The experimental evidence supporting a negative feed-back mechanism in epidermis seems to be comparatively strong (Iversen, 1981).

Until recently, however, knowledge about the nature of the endogeneous growth inhibitor that is thought to be synthesized by the differentiating cells has been completely lacking. Although the site of synthesis is still unknown, an epidermal peptide like the one purified from mouse skin and investigated in the present study is a good candidate for such a role in the regulation of cell renewal and tissue homeostasis in the epidermis.

We are indebted to Dr N. C. Petersen, head of the department of Plastic Surgery, University Hospital of Aarhus, for supplying us with the skin samples, and to Christian Knudsen, Viggo Nielsen, and Søren Pedersen for excellent technical assistance. This work was supported by the Danish Medical Research Council, the Danish Cancer Society, and the Bioregulation Research Center at Aarhus University.

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(Received 19 March 1990 – Accepted 1 June 1990)