0022-202X/80/7406-0433$02.00/0
THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 74:433-436 1980

Vol. 74, No. 6
Printed in U.S.A.

Perturbation of Cell Cycle Progression in Mouse Epidermis Prior to the Regenerative Response

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Cell kinetic perturbations that resulted in a wave of increased cell division during the 6-8 hr lag period prior to regenerative DNA replication in mouse epidermis were examined. The epidermis was stimulated to proliferate by adhesive tape stripping, and flow cytometric DNA measurements of isolated epidermal basal cells, counting of mitoses, of Colcemid arrested metaphases and of labeled mitoses among basal cells in histologic sections were made. The results showed that mitotic peaks that occur in the pre-replicative period subsequent to tape stripping can be explained by a delay in cell progression through the S phase, followed by subsequent release and partial synchrony in further cell cycle progression. Early peaks of mitoses in epidermis stimulated to proliferate should therefore not, without further evidence, be assumed to originate from cells triggered into division from a resting G_2 compartment. The results also indicate an initial delayed cell progression through the G_2 phase, whereas the mitotic duration seemed to be initially reduced, indicating that the DNA synthesis phase and the G_2 phase are parts of the epidermal cell cycle that may be most vulnerable to various types of influences.

In epidermis a variety of external influences can induce regenerative cell proliferation that is preceded by a lag period of 6-12 hr. During the time interval before the wave of replicative DNA synthesis starts, peaks of mitoses have been observed [1-3] that were assumed to originate from cells resting indefinitely in G_2 phase [4] before triggered directly into cell division [1]. In mouse epidermis stimulated to proliferate by adhesive tape stripping an increasing hyperplasia can be seen from 6 hr after stripping [3], reaching a maximum at about 2-3 days [3,5]. In the present study we wanted to examine in more detail cell kinetic perturbations in the lag period of 6-8 hr [3,5] prior to the wave of increased epidermal DNA synthesis. Many cell kinetic parameters were simultaneously studied and correlated. Flow cytometric DNA measurements of isolated epidermal basal cells and counting of mitoses among basal cells in histologic sections revealed changes in the proportion of cells in S-phase, G_2-phase and mitosis. Colcemid arrested metaphases were scored in sections and showed changes in cell division activity, and ^{3}H-TdR incorporation studies showed changes in cell progression of labeled cells.

MATERIALS AND METHODS

Animals

Hairless mice of the hr/hr Oslo strain, 60-90 days old and weighing about 25 gm were used in all experiments. Eight animals were kept in each cage and supplied with water and food *ad libitum* under standard conditions.

Tape Stripping

Two successive and gentle adhesive tape strippings (Permacel) were applied to the same area of the back skin of the animals at different times of the day from 15 min to 6 hr before killing. Four animals from each cage were stripped and the other 4 served as controls. Differences in the values of cell kinetic parameters can be observed between control groups. Our experience indicates that these variations are mainly due to differences between cages in physical activity of the animals. Sex and age differences, however, may also result in intergroup variations of the measured values. To reduce the influence of such variations, control animals were always taken from the same cage as treated ones. The animals were killed in groups of 8 and always at 1200 to reduce the influence of circadian rhythms [6,7].

Cell Separation and Flow Cytometric DNA Measurements

Pieces of skin were removed from the animals' backs with an electrokeratotome [8]. The basal cells were separated from the differentiating cells by means of trypsin digestion [9] and shaken off the basement membrane into suspension. The basal cells were fixed in absolute ethanol, RNAse-treated and stained with ethidium bromide [10] and measured with an ICP 11 pulse cytophotometer (Phywe AG, Göttingen, West Germany). The DNA frequency distributions were obtained as histograms, each histogram representing 10-20,000 cells (Fig 1), and the proportion of cells in G_1, S and G_2 phase calculated by a planimetric method [11,12] and expressed as a percentage of controls (control value = 100%).

^{3}H-TdR Studies

Groups of animals were injected i.p. with 30 μCi ^{3}H-TdR (Sp act 6.7 mCi/mMol, 1 mCi/ml, New England Nuclear, Boston) immediately before stripping, and killed at intervals from 15 min to 3 hr after injection. The back skins of the animals were immediately flayed off, fixed in Bouin's solution for 4 hr, dehydrated, embedded in paraffin, cut at 5 μm, dipped in Kodak NTB 2 film emulsion diluted (1:1) with distilled water, exposed for 2 weeks, developed and stained with hematoxylin. The numbers of labeled and unlabeled mitoses were counted and the proportion of labelled mitoses calculated. A total of 30 mitoses from each animal were counted, and based on the background activity; cells with 3 grains or more over the nucleus were considered as labelled.

One group of animals was injected with 30 μCi ^{3}H-TdR i.p. immediately before stripping. The proportion of labeled cells (LI) and the mean grain count (MGC) were scored in sections prepared for autoradiography as described above. In 2 separate experiments groups of animals were injected with 30 μCi of ^{3}H-TdR i.p. 30 min or 60 min, respectively, after stripping. The animals were killed 30 min after injection and suspensions of basal cells prepared as described above. Smears were made from each suspension, air-dried, fixed for 20 min in absolute methanol and prepared for autoradiography as described, and the LI and the MGC calculated.

Mitotic Parameters

At various time intervals after stripping groups of animals were killed, the back skins were immediately flayed off and histologic sections processed as described earlier. The number of mitoses was counted among 1,000 basal cells in interfollicular epidermis and expressed as a percentage of controls (control = 100%).

Separate groups of animals were injected with 0.15 mg Colcemid, (Ciba, Basel, Switzerland) in 0.15 ml solution at the time of stripping. The groups were sacrificed from 45 min to 4 hr after stripping and histologic sections were prepared as described above. The number of

Manuscript received August 30, 1979; accepted for publication December 18, 1979.

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Abbreviations:
LI: Labeled cells
MGC: mean grain count
MI: mitotic index

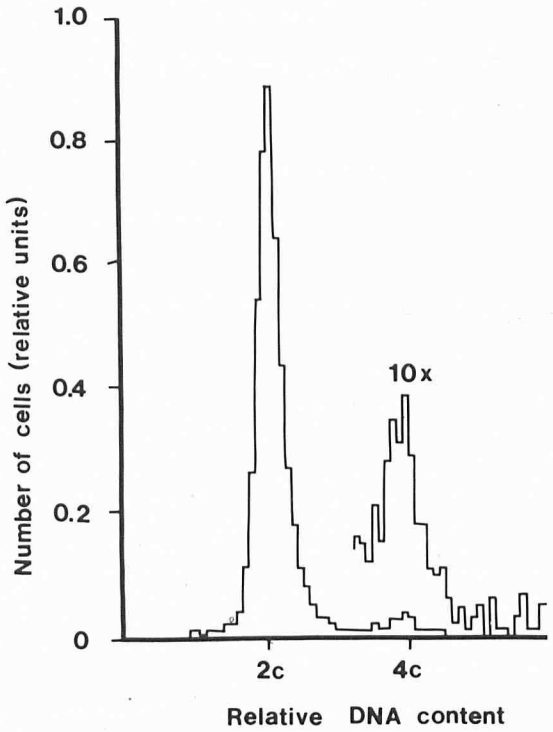

FIG 1. Example of DNA frequency distribution from isolated epidermal basal cells, measured by flow cytometry. A magnification of the pulses in the G_2 (*4C*) region shows that there is essentially no cell clumping.

Colcemid arrested metaphases were counted among 1,000 basal cells in interfollicular epidermis and expressed as a percentage of controls (control = 100%).

RESULTS

For statistical analysis a 2-tailed Students *t*-test was used. When the *p* value was <0.05, this is indicated in the text.

Flow cytometric DNA measurements (Fig 2)

All treatment values are given as a percentage of control values (100%) derived from untreated animals from the same cage, in order to avoid the influence of intergroup variations (see Materials and Methods; Tape stripping).

The proportion of cells with S-phase DNA content increased to 125% and 130% of controls at 1 hr and 1 hr 30 min after stripping, respectively ($0.05 > p > 0.01$, pooled values). Control values were again reached at 2 hr 30 min. The proportion of cells with G_2 phase DNA content showed an initial peak of 125% of controls at 30 min after stripping. This was followed by a second and longer lasting increase of about 150% of controls at 2 hr and 2 hr 30 min ($0.05 > p > 0.01$, pooled values). Control values were again reached at 3 hr after stripping.

^{3}H-TdR Incorporation and Mitotic Parameters (Tables I to IV)

Changes in the proportions of cells in mitosis are shown in Fig 2, *lower panel*. A gradual decrease in the mitotic index occurred after stripping, with a minimum of about 70% of control values at 1 hr and 1 hr 30 min ($0.05 > p > 0.01$, pooled values). At 2 hr 30 min a peak of about 150% of control values ($0.05 > p > 0.01$) occurred, before control values again were reached at 4 hr.

Changes in the proportion of Colcemid arrested metaphases after stripping are shown as a percentage of control values in Table I. At about 1 hr 15 min after stripping a minimum of about 50% of control values was reached, followed by a peak of about 160% at 2 hr ($p < 0.01$). Control values were reached at 4 hr after stripping. Hence, during the initial 4 hr period after stripping the number of total cell divisions was the same in the stripped as in the nonstripped epidermis.

The mitotic duration (T_M) can be calculated when the mitotic index (MI) and the mitotic rate (R_M) are known: $T_M = MI/R_M$. A linear increase of Colcemid arrested metaphases in hairless mouse epidermis starts after a delay of about 1 hr [13]. The T_M was therefore calculated for the time intervals 1–2 hr and 1–4

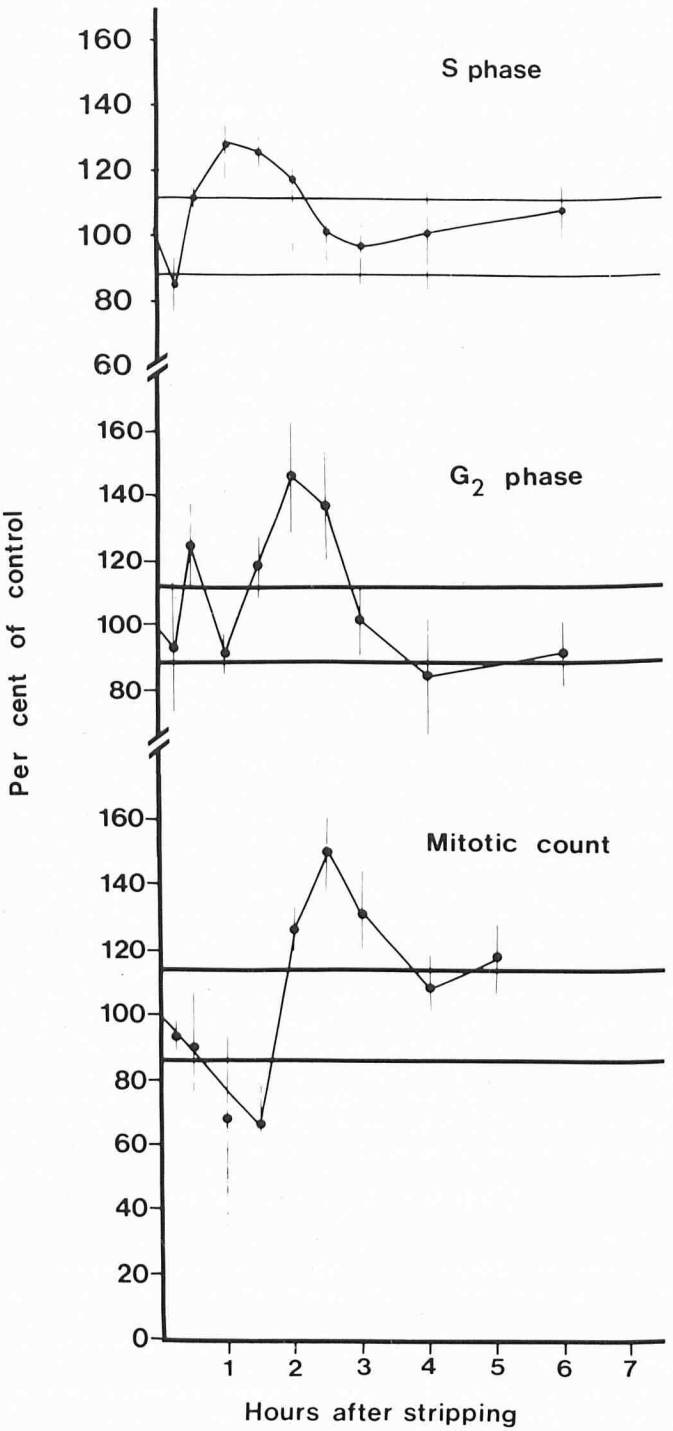

FIG 2. Changes in the proportion of epidermal basal cells with S phase DNA content (*upper panel*), G_2 phase DNA content (*mid-panel*) and changes in mitoses after gentle adhesive tape stripping of mouse epidermis. The changes are relative and expressed as a percentage of controls (control value 100%).

hr after stripping. The proportion of Colcemid arrested metaphases after 2 and 4 hr, respectively (Table I), and the mean of the representative mitotic indices (Fig 2, *lower panel*) were used in the calculations. The results showed that the T_M seems to be reduced compared with controls shortly after stripping (Table II).

Changes in the percent labeled mitoses (PLM) are seen in Table III. From 1 hr 30 min after stripping, the proportion of labeled epidermal mitoses in the stripped animals was decreased compared with that of controls (at 3 hr, $0.05 > p > 0.01$). More than 50% of all mitoses, however, were labeled at 2 hr and 3 hr after stripping. The LI of epidermal basal cells and the MGC over labeled nuclei of animals injected with ^{3}H-TdR at the time of stripping, 30 min later, or 60 min later are shown in Table IV. The results show that the LI ($0.05 > p > 0.01$) and the MGC were increased compared with controls at 45 min after stripping, both parameters were reduced at 1 hr after stripping (MGC: $0.05 > p > 0.01$), whereas control values were reached at 1 hr 30 min after stripping.

TABLE I. *Changes in the proportion of Colcemid arrested epidermal metaphases after adhesive tape stripping*[a]

Time after stripping	Arrested metaphases per 1000 cells		
	Stripped	Control	Percent of control
45 min	21.8 ± 1.8[b]	23.8 ± 4.4	92 ± 8
1 hr	19.3 ± 1.7	19.7 ± 1.8	98 ± 9
1 hr 15 min	19.1 ± 1.9	36.0 ± 8.4	53 ± 5
1 hr 30 min	35.3 ± 4.8	27.8 ± 5.1	127 ± 17
2 hr	63.5 ± 2.5	39.3 ± 4.4	162 ± 6
3 hr	107.5 ± 17.6	77.5 ± 12.4	139 ± 23
4 hr	69.8 ± 8.0	62.3 ± 5.3	112 ± 13

[a] Stripping was performed immediately after i.p. injection of 0.15 mg of Colcemid, and stripped and control groups were killed at various intervals after stripping. Metaphases were counted per 1000 basal cells. Treatment values are given as a percentage of controls in the last column (controls = 100%).
[b] SEM.

TABLE II. *The mean mitotic duration (hr) calculated for the intervals 1–2 hr and 1–4 hr, respectively, after adhesive tape stripping of mouse epidermis*

Time interval after stripping	Mitotic duration (T_M)	
	Stripping	Control
1–2 hr	0.9 hr	1.6 hr
1–4 hr	1.7 hr	1.8 hr

TABLE III. *Percent labeled mitoses after adhesive tape stripping of mouse epidermis*[a]

Time after stripping	Percent labeled mitoses	
	Stripped	Control
15 min	6.6 ± 2.0[b]	4.4 ± 3.0
45 min	13.0 ± 9.0	7.8 ± 2.0
1 hr	11.0 ± 4.0	8.0 ± 4.0
1 hr 30 min	11.0 ± 5.0	19.2 ± 4.0
2 hr	54.0 ± 2.0	67.0 ± 9.0
3 hr	63.0 ± 4.0	78.0 ± 1.0

[a] 30 μCi ^{3}H-TdR was given i.p. immediately before stripping.
[b] SEM.

DISCUSSION

The present study shows that perturbations in cell cycle progression occur during the so called lag period prior to increased DNA replication in regenerating epidermis. The sequence of increasing cell numbers in the various cell cycle compartments during the first 4 hr after stripping (Fig 2) strongly indicates a partial block in S phase followed by a synchrony in cell cycle progression from S-phase through G_2 phase to mitosis. We assume, however, that the strength and type of influence will modify both the amplitudes and the timing of the cell kinetic alterations. A more severe stripping might therefore induce a longer lasting block that is followed by a peak of mitotic activity somewhat later [3]. The peak also in the proportion of Colcemid arrested metaphases shows that the changes in cell cycle distributions are really resulting in changes in cell flux between the compartments. That the 4 hr value of arrested metaphases is similar in both stripped and nonstripped epidermis indicates that any initial arrest in cell cycle propression is subsequently counterbalanced by an accellerated cycle progression. The time sequence of the peaks of cells in the S-phase, G_2 phase and mitosis suggests that the cells are traversing from S-phase through G_2 phase and into mitosis within 1 hr 30 min. Although the major part of the released cells are assumed to start at the S/G_2 transition, the passage is more rapid than to be expected from the mean duration of the G_2 phase (2 hr) and mitosis (45 min) in the hairless mouse epidermis at the time before noon [7]. This may be explained by a preferential arrest of a subpopulation of cells cycling more rapidly through G_2 phase than the majority of cells.*

The accumulation of cells with S-phase DNA content as observed initially in the present study, may either be induced by increased flux at the G_1/S transition or delayed flux through and out of S-phase, or by a combination. The decreased LI and MGC at 1 hr and the normalized values at 1 hr 30 min after stripping, however, indicates that the cells are delayed or arrested in the S-phase and thereafter released as a partially synchronized cohort. A similar reduction in ^{3}H-TdR uptake was also observed by Hennings and Elgjo shortly after adhesive tape stripping [5]. The reason why these authors did not observe any prereplicative increased mitotic activity is probably due to their infrequent sampling of data at this time.

The reduced proportion of mitotic figures and Colcemid arrested metaphases at 1 hr 30 min after stripping clearly confirms a short-lasting block in cell cycle progression prior to cell division as indicated by the S phase parameters. The increase in the fraction of cells with S phase DNA content to about 125% after 1 hr 30 min is consistent with a normal influx to S phase [7] combined with blocked flux from S phase to G_2 phase. Since there is no significant decrease in the size of the G_2 compartment during the same time interval (on the contrary, there is a peak at 30 min), this indicates that the cells are also

* Clausen OPF, Thorud E, Aarnes E: Evidence of rapid and slow progress of G_2 cells in mouse epidermis, in press.

TABLE IV. *Epidermal ^{3}H-TdR labeling index (LI) (%) and mean grain count (MGC) over labeled nuclei in groups of stripped and control mice at 45 min, 1 hr and 1 hr 30 min after stripping*

Time of stripping	Time of ^{3}HTdR injection	Time of sacrifice	Labeling index		Mean grain count	
			Stripped	Controls	Stripped	Controls
1130	1130	1215	7.6 ± 0.6[a]	5.7 ± 0.5	20.4 ± 3.2	15.1 ± 0.5
1100	1130	1200	4.7 ± 0.6	5.7 ± 0.3	28.1 ± 2.0	37.3 ± 1.1
1030	1130	1200	4.4 ± 0.5	4.8 ± 0.3	33.8 ± 6.3	33.9 ± 3.5

[a] SEM.

initially delayed through the G_2 phase. Without an initial delay and subsequent release of cells with G_2 phase DNA content one should, because of the increased initial labeling, (Table IV), expect an increased fraction of labeled mitoses when the mitotic peaks occur at around 2 hr after stripping (Fig 2, Table I). The changes in percent labeled mitoses therefore also suggest an initial G_2 delay. The rapid initial traverse through mitosis, as indicated by the T_M calculations (Table II), is thus in contrast to the delayed traverse through S and G_2 phase immediately after stripping. However, only slightly more than 50% of the cells in S-phase are labeled in normal hairless mouse epidermis under the labeling conditions used [14], indicating that many unlabeled mitoses in both the stripped and control groups at the time when the mitotic peaks occur, might have been in the S phase at the time of stripping.

The present results show that a peak in the mitotic index or mitotic rate that occurs before the main regenerative response in epidermis stimulated to proliferate, can be explained by an arrest in S-phase followed by a release and subsequent partial synchrony in cell cycle traverse. The cells also seem to be initially delayed in G_2 phase, whereas a slight reduced T_M is observed. This indicates tnat in epidermal cells the DNA synthesis phase and the G_2 phase may be the most vulnerable ones to various types of influences. The results also show that any early peak in mitotic parameters in epidermis stimulated to proliferate and probably also in other tissues should not, without further evidence, be attributed to cells triggered into mitoses from a resting G_2 population [4].

Aasa Schjølberg and Eva Moen gave technical assistance.

REFERENCES

1. Krieg L, Kühlmann I, Marks F: Effect of tumor-promoting phorbol esters and of acetic acid on mechanisms controlling DNA synthesis and mitosis (chalones) and on the biosynthesis of histidine-rich protein in mouse epidermis. Cancer Res 34:3135–3146, 1974
2. Zil JS: Vitamin A acid effects on epidermal mitotic activity. J Invest Dermatol 59:228–232, 1972
3. Clausen OPF, Lindmo T: Regenerative proliferation of mouse epidermal cells following adhesive tape stripping. Cell Tissue Kinet 9:573–587, 1976
4. Gelfant S, Candelas GC: Regulation of epidermal mitoses. J Invest Dermatol 59:7–12, 1972
5. Hennings H, Elgjo K: Epidermal regeneration after adhesive tape stripping of hairless mouse skin. Cell Tissue Kinet 3:243–252, 1970
6. Tvermyr EMF: Circadian rhythms in hairless mouse epidermal DNA-synthesis as measured by double labelling with H3-thymidine (H3Tdr). Virchows Arch B Cell Pathol 11:43–54, 1972
7. Clausen OPF, Thorud E, Bjerknes R, Elgjo K: Circadian rhythms in mouse epidermal basal cell proliferation. Cell Tissue Kinet 12: 319–337, 1979
8. Skjaeggestad Ø: Experimental epidermal hyperplasia in mice; relation to carcinogenesis. Acta Pathol Microbiol Scand (suppl) 169:1–126, 1964
9. Laerum OD: Oxygen consumption of basal and differentiating cells from hairless mouse epidermis. J Invest Dermatol 52:204–211, 1969
10. Göhde W, Dittrich W: Impulsfluorometrie—ein neuartiges Durchflussverfahren zur ultraschnellen Mengenbestimmung von Zellinhaltstoffen. Acta Histochem (suppl) 10:429–437, 1971
11. Göhde W: Zellzyklusanalysen mit dem Impulscytophotometer. Der Einfluss chemischer und physikalisher Noxen auf die Proliferationskinetik von Tumorzellen. Thesis, Münster, West Germany, 1973
12. Clausen OPF, Lindmo T, Sandnes K, Thorud E: Separation of mouse epidermal basal and differentiating cells for microflow fluorometric measurements. Virchows Arch B Cell Pathol 20: 261–275, 1976
13. Thorud E, Clausen OPF, Bjerknes R: The stathmokinetic method in vivo: Time response with special reference to circadian variations of epidermal cell proliferation in the hairless mouse. Cell Tissue Kinet, in press
14. Clausen OPF, Thorud E, Bolund L: DNA synthesis in mouse epidermis: labelled and unlabelled basal cells in S phase after administration of tritiated thymidine, submitted for publication

Announcement

The Fifth Annual Postgraduate Course and Workshop in Medical Mycology-Dermatomycology will be held from July 29–August 1, 1980 at the Memorial Hospital Medical Center—University of California, Irvine Center for Health Education in Long Beach, California. For further information contact: Margaret Frederick (Program Representative), MHMC-UCI, Center for Health Education, 2801 Atlantic Avenue, Long Beach, California 90801.