Recruitment of Quiescent (G_0) Cells Following Epidermal Injury Is Initiated by Activation of the Phosphoinositol Cycle

Joris J. Rijzewijk, M.D., Franz W. Bauer, Ph.D., Jan B. M. Boezeman, M.Sc., Rudi Happle, M.D., and Paul D. Mier, Ph.D.

Department of Dermatology, University of Nijmegen, Nijmegen, The Netherlands

Under normal circumstances, the rate of production of new cells by the epidermis is rather low, but injury results in a burst of mitotic activity that continues until repair is complete. It is now recognized that most cells of the germinative population are in a resting (G_0) state, and "postinjury cell renewal" is the consequence of G_0 cells entering the mitotic cycle. The biochemical events triggering this process, however, are unknown. Here we show that phorbol myristate acetate (PMA) is able to induce G_0 mobilization in the epidermis of the nude mouse. Further, we demonstrate that amiloride (an inhibitor of the membrane Na^+-H^+ pump), applied topically to human skin, abolishes almost completely the regenerative response after experimental injury. We suggest that activation of the phosphoinositol cycle may initiate recruitment of G_0 cells in the epidermis. *J Invest Dermatol 90:44–47, 1988*

Injury to the epidermis (mechanical, chemical, or immunologic) results in a transient increase in the rate of production of new cells, the elevation persisting until the repair process is complete. It was formerly believed that this response resulted from a shortening of the cell-cycle time [1]. However, it is now clear that the cell-cycle time of the epidermal keratinocyte is rather constant, and that the regenerative response following injury is the consequence of the recruitment of a resting (G_0) pool of cells into the actively cycling population [2–5]. We have recently described experiments in which the outermost layer of human epidermis (stratum corneum) was removed by repeated application of adhesive tape ("tape stripping"), and the perturbations in the kinetics of the underlying keratinocytes monitored during the repair process. Our data indicated that the G_0 pool comprises about 80% of the total germinative population; these cells enter the cycle as a discrete cohort, appearing maximally in S and G_2M phases about 40 h after injury [3].

The biochemical events that initiate this process, however, remain quite unknown. Our attention has been drawn to reports suggesting that, in certain cell types, activation of the phosphoinositol cycle may lead to cell division. Hydrolysis of phosphatidylinositol 4,5-biphosphate yields diacylglycerol (DAG), an activator of protein kinase C [6–9]; this enzyme catalyses the phosphorylation of a membrane antiport exchanging cytosolic H^+ ions with extracellular Na^+ [10–12]. The resulting increase of intracellular pH appears to trigger further events culminating in mitosis [13–18]. An early observation that ultraviolet radiation-induced injury causes an increased phospholipid turnover in epidermis [19] supported the idea that a mechanism primarily involving the phosphoinositol cycle might operate in this tissue. We have investigated this possibility in two ways: first, by the use of phorbol 12-myristate 13-acetate (PMA), which is able to substitute for DAG in the activation of protein kinase C [20], and, second, by blocking the H^+-Na^+ antiport with a specific inhibitor, amiloride [10].

MATERIALS AND METHODS

PMA Experiments Nude mice (hr/hr), approximately 12 weeks old, were obtained from Olac (Bicester, England). They were maintained under normal laboratory conditions (room temperature about 22°C) and fed ad libitum.

Aliquots of 0.1 ml of a solution of 125 nM PMA (Sigma Chemical Co, St. Louis, MO) in acetone were applied to the backs and flanks of the animals using a pipette; this concentration had been established in preliminarly experiments to be the lowest that still gave a consistent hyperproliferative response. To remove the skin specimens as superficially as possible (approximately 0.2 mm thick and 3 mm diameter), we stiffened the biopsy sites with cyanolit glue. Samples were taken at 1-h intervals up to 24 hours, using a razor-blade in conjunction with a metal guard [21]. The biopsies were processed for flow cytometry as described below.

Amiloride Experiments These studies were carried out using healthy volunteers (ages 20–30) who gave informed consent to this experiment. Prior approval of the ethical committee of this university was obtained. Sites on the back were subjected to repeated "stripping" with adhesive tape until a moist, glistening surface indicated total removal of the stratum corneum; this usually required about 25 applications. The base cream (cremor cetomacrogolis without acidum sorbicum), containing 0, 0.06, 0.2, or 0.6% amiloride (Sigma), was applied immediately after stripping and replenished after 20 h. Biopsies were taken and processed further as described below.

It was not possible to establish control data for the effects of amiloride on normal (unstripped) skin since this substance would not be expected to penetrate the stratum corneum.

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Reprint requests to: J. J. Rijzewijk, M.D., Dept. of Dermatology, University of Nijmegen, Javastraat 104, 6524 MJ NIJMEGEN, The Netherlands.

Abbreviations:

DAG: diacylglycerol
DMSO: dimethyl sulfoxide
G_0 cells: resting cells
PMA: phorbol 12-myristate 13-acetate
SG_2M cells: cells in the S, G_2, and M phases of the cell cycle

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Sample Preparation and DNA Staining Sample preparation was in principle as described earlier [22]. In brief, the procedure was as follows: The biopsies were incubated in a few drops of 0.15 M phosphate buffer containing 1% crude trypsin (Difco 1:250) and 0.3% dithioerythritol (Sigma) at 37°C for 20 min. After incubation, the piece of skin was transferred to a small test tube containing 0.5 ml phosphate buffer with propidium iodide (20 mg/liter, Calbiochem) and sonicated for 2 s (Sonifier B-12, Branson Sonic Power Co) to dissociate the epidermis. Then 0.1 ml fetal calf serum (to inhibit traces of trypsin), a drop of dimethyl sulfoxide (DMSO), and 0.1 ml RNAse (Type 1-A, Sigma) was added. The stratum corneum and the dermis remain intact with this procedure so that no admixture of dermal cells occurs. After filtration through gauze (mesh approximately 50 μm, Phywé, BRD) and a staining period of 15 min, cellular DNA was determined with the flow cytometer. The cell concentration was about 4×10^5 cells/ml.

Flow Cytometry Cell cycle analysis was performed using a flow cytometer 50H (Ortho Instruments, Westwood, MA) equipped with a 5 W argon ion laser (164-05, Spectra Physics, Mountain View, CA). The propidium iodide fluorochrome was excited at the 488 nm laser line and the fluorescence was measured with a high-pass filter RG 630 (Melles Griot, Zevenaar, The Netherlands). At least 5×10^3 cells were counted at a flow rate of about 100 cells per second. The data were stored and analysed with a PDP11/34 computer (Digital Equipment, Galway, Republic of Ireland).

Both area and peak value of each fluorescence signal were measured. The ratio area to peak is an excellent discriminator between artifacts that result from doublets of diploid cells and real single tetraploid (or late S) cells [23]. Typically the value of clumped cells was 0.5–1%. In the DNA frequency distribution histogram, the contribution of the S-phase cells is approximated by a rectangle between the diploid maximum and its double. The height of the rectangle is indicated by the level of the mid-S phase. The remaining area on the right of mid-S is the G_2M contribution. The percentages of S and G_2M cells are obtained by integrating the appropriate areas of the histogram.

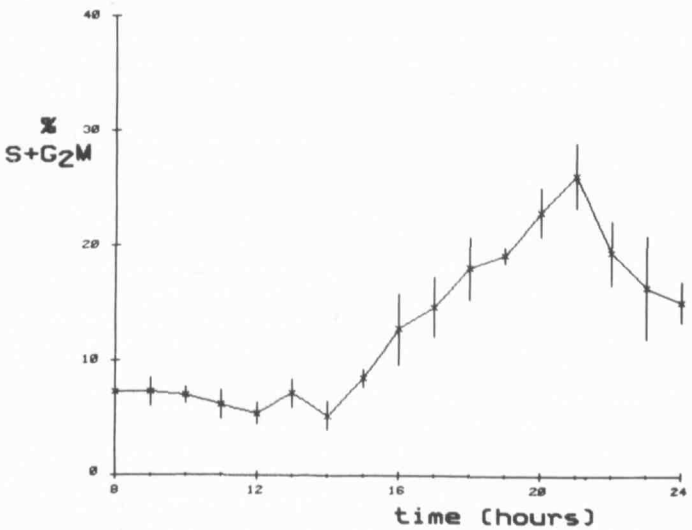

Figure 1. SG_2M cells as a percentage of the total epidermal population at different times after the application of PMA to the skin of nude mice (hr/hr). The backs and flanks of the animals were treated with a solution of PMA in acetone and specimens of skin were removed at intervals. Keratinocytes were isolated by trypsinization, stained with propidium iodide, and the cellular DNA content determined as described in Materials and Methods. Points represent means ± SEM.

RESULTS

PMA Initiates Recruitment of G_0 Cells Figure 1 shows the number of cells in the S, G_2, and M phases of the cell cycle (SG_2M cells) as a percentage of the total epidermal population at different times after the application of PMA to nude mice. It is seen that at 8 h after application the level of SG_2M cells is comparable with the unstimulated value of about 8%; this figure rises between 18 and 24 h after application of PMA with a maximum of 26% at 21 h. These data are consistent with a cohort of recruited G_0 cells entering the S-phase in an ordered fashion.

Amiloride Inhibits the Regenerative Response The fraction of SG_2M cells in normal human epidermis is rather constant, averaging 2.7 ± 0.8% [22]. The value measured 40 h after tape stripping, in the absence of amiloride, was 19.0 ± 1.6%; this is close to figures reported previously [3], indicating that the base cream does not essentially influence the repair process. Although an increase in SG_2M cells is not per se direct evidence for an enhanced rate of cell production, the assumption is justified by data presented in our earlier report [3].

It is seen from Table I that amiloride, applied to the skin surface immediately after stripping, inhibits the regenerative response in a dose-dependent manner. At a concentration of 0.6% the response is almost wholly abolished, the percentage of SG_2M cells remaining being close to the figure found in control (uninjured) epidermis. Although it is difficult to estimate cutaneous drug levels after topical administration, our data seem compatible with the K_i of about 1 mM reported for the inhibition of the Na^+-H^- exchanger [10,24,25].

Lag Period Between Injury and Time of Amiloride Inhibition To get more detailed information about the time at which amiloride exerts its inhibitory action on the recruitment, we designed an experiment in which amiloride was applied at different times after stripping and percentages of SG_2M cells were again measured 40 h after stripping (Table II). A delay up to 15 h had no significant effect on the degree of inhibition. After this time the inhibitory effect diminished, half maximum inhibition occurring between 25 and 30 h.

Specificity and Toxicity of Amiloride To find out if amiloride selectivity inhibits G_0 cells recruitment and to exclude the possibility that amiloride inhibition was a toxic effect, we did the following experiments (all of which were based on the fact that hyperproliferation continues at least 72 h after stripping) [26]: We stripped the epidermis and applied the 0.6% amiloride or the base cream 40 h later on 3 areas in each of 2 subjects. Biopsies were taken at 64, 68, and 72 h after stripping. The 6 amiloride-treated samples were measured and compared with the 6 base cream–treated samples. If 0.6% amiloride cream was used, SG_2M fractions were 13.8 ± 1.5 (SEM), using base cream, SG_2M fractions were 12.4 ± 1.2 (SEM).

Clearly sustained proliferative activity that resulted from stripping is not inhibited by amiloride exposure from 40 to 64, 68, or 72 h. Once recruited (after 40 h), cells appeared to be insensitive to this concentration of amiloride, whereas amiloride application from

Table I. Effect of Amiloride on Postinjury Cell Renewal

Application	Percentage $S + G_2M$ Cells	Percentage Inhibition of Proliferative Response
Cream base only	19.0 ± 1.6	0
Base + 0.06% amiloride	14.5 ± 0.8	22
Base + 0.2% amiloride	10.6 ± 1.4	49
Base + 0.6% amiloride	3.8 ± 0.3	94

Sites on the back of healthy volunteers were subjected to repeated "stripping" with adhesive tape. A base cream containing 0, 0.06, 0.2, or 0.6% amiloride was applied immediately after stripping and 20 h later. Biopsies were taken from each site 40 h after injury and the cellular DNA content of keratinocytes measured as described in Materials and Methods. Values are the means and SEMs of 6 separate experiments. Inhibition of the proliferative response by amiloride was calculated using a value of 2.7% for SG_2M cells in normal epidermis [22].

Table II. Relation Between Time of Amiloride Application and Suppression of Proliferative Response

Interval Between Stripping and Amiloride Application (h)	Percentage SG_2M Cells	Percentage Inhibition of Proliferative Response
15	5.0 ± 1.2	86
20	5.2 ± 1.0	85
25	8.5 ± 1.8	65
30	13.5 ± 3.5	35
Cream base only	19.2 ± 0.8	0

Base cream was applied to test sites immediately after stripping, and, after 15, 20, 25, and 30 h, replaced by cream containing 0.6% amiloride. Biopsies were taken 40 h after stripping and cellular DNA content of keratinocytes measured as described in Materials and Methods. Values are means and SEM's of 5 separate experiments. Inhibition was calculated as in *Table I*.

15 to 40 h after stripping had a very strong inhibitory effect on cell proliferation (Table II). This shows that the inhibition by amiloride under these conditions is selective for the transition of cells from a G_0 to a cycling state and thus not a toxic effect.

DISCUSSION

The original observation that epidermal injury caused by ultraviolet radiation stimulates phospholipid turnover, coupled with our present data regarding the effects of PMA and amiloride, strongly suggest that activation of the phosphoinositol cycle may be responsible for postinjury cell renewal in epidermis; the alternative possibility, that the primary target is the differentiation process, seems to be ruled out by the rapidity of the response. The sequence of events thus is

injury → chemical signal → activation of phosphoinositol cycle → elevation of pH → recruitment.

Assuming that the events between cleaving of phosphatidylinositol bisphosphate and the elevation of cytosolic pH are rapid, as suggested by in vitro experiments [27–32], our figures indicate that the interval of 40 h between injury and the appearance of the recruited G_0 cells in S and G_2M in human epidermis comprises a relatively long lag period of 25 to 30 h for transmission of the chemical signal and 10 to 15 h for entry of the G_0 cell into the cycle and subsequent progression to S and G_2M.

Many questions remain, however, the most obvious being the nature of the chemical signal that triggers the phosphoinositol cycle. In different cell types evidence has been gathered that growth factors, such as platelet-derived growth factor and platelet-activating factor can act as these signals by binding to a membrane-bound receptor [28,32]. The role of growth factors for epidermal cells is still uncertain. The early investigations of Bullough et al [33] led to the idea that cell replication in normal epidermis is inhibited by a protein synthesized by the differentiated keratinocyte ("chalone"). The regenerative response after injury was believed to be mediated by a local fall in the concentration of the chalone. Recently, this concept has gained renewed interest by the isolation of a pentapeptide [34] that inhibits cell proliferation in skin. It is a speculative but interesting possibility that the chalone molecule may be required to maintain the phosphoinositol cycle in the inactive state, its dissociation from a membrane-bound receptor leading to the hydrolysis of phosphatidylinositol bisphosphate.

It would clearly be of value to bring the various aspects of this study together to allow direct comparison in a single animal. Unfortunately, sellotape stripping presents technical difficulties using the nude mouse, and PMA may not be applied safely to human skin because of its cancer-promoting potential. Work is therefore in progress to repeat these experiments using the pig as model; in particular, we aim to demonstrate that amiloride can block the response to PMA in addition to the response to physical injury.

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