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Increases in Dihydronicotinamide Adenine Dinucleotide (NADH) Content and α-Glycerophosphate Dehydrogenase Activity in Epidermal Wound Healing[1] (41602)

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Abstract. Alterations in NADH content and NAD-dependent dehydrogenase activity were determined in regenerating epithelium during wound healing in young guinea pigs. Regenerating epithelium exhibited increased levels of NADH. The migratory and proliferative phases of epidermal wound healing were characterized by increases in glycolytic enzyme activities, including an increase in lactate dehydrogenase (LDH). The maturation phase of epidermal wound healing was characterized by a maximal increase in α-glycerophosphate dehydrogenase (GOPDH) activity. The contrasting changes in LDH and GOPDH activity suggest that increased levels of NADH are utilized first by LDH in glycolysis during epidermal migration and proliferation and then by GOPDH in triacylglycerol synthesis during epidermal differentiation.

Epidermal keratinization is accompanied by a decrease in phospholipids and an increase in certain neutral lipid esters such as triacylglycerols and sterol esters (1–3). Fatty acids liberated by the hydrolysis of phospholipids may be utilized by reesterification as neutral lipid esters during epidermal keratinization (4). Epidermal wound healing is composed of three phases: cell migration, cell proliferation, and cell maturation. These phases serve as a model for studying epidermal differentiation and cornification. The migratory and proliferative phases occur during the initial 3–4 days following wounding and epidermal maturation occurs during the period following epithelial wound closure (5).

In the present study, the different phases of epithelization have been characterized by assaying enzyme activities and tissue concentrations of coenzymes. The contribution of glucose metabolism to triacylglycerol production during epidermal differentiation is suggested by increased activity of α-glycerophosphate dehydrogenase and increased levels of NADH.

Materials and Methods. Twelve Hartley male guinea pigs, 7 weeks old, from Buckberg (Tomkins Cove, N.Y.) were utilized for the experiment. Five linear incisions, 10 mm in length, were made to the right side of the midline on the back of each animal under ether anesthesia at different time intervals. The contralateral sites on the left side of the midline on the back were used as the unwounded controls. The wounds and the controls in a given animal were biopsied at one time under light ether anesthesia so as to obtain wound samples at 2, 3, 4, 7, and 10 days postwounding. Full-thickness skin blocks, measuring 10 × 10 mm, were obtained from the wound and control sites after freezing the sites with a cotton-tipped applicator soaked in liquid nitrogen, and the frozen skin blocks were dropped immediately in liquid nitrogen. The frozen specimens were cross-sectioned 20 μm in thickness perpendicular to the long axis of the incision and lyophilized at −30°C. The freeze-dried tissue sections were stored in vacuum tubes at −20°C until the enzymes and coenzymes were assayed.

Unstained, freeze-dried sections demonstrated the histological integrity of the skin in terms of the original wound margin and the regenerating epithelium. The entire length of the migrating and maturing epithelium in a freeze-dried tissue section was utilized for the enzyme and coenzyme assays. Microdissection isolated relatively pure, intact migrating and maturing epithelium from the dermis, granulation tissues, and wound exudate (5). Isolated epithelial segments were weighed (0.5–2.0 μg) on a quartz fiber microbalance (6) and transferred into homemade test tubes (2.5 × 30 mm) for subsequent enzyme and coenzyme assays.

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Measurement of NAD(H). NAD(H) content in normal and regenerating epidermis was assayed by an enzymatic cycling method (6) coupled with alcohol and malate dehydrogenase activities (7). One microliter of cold 0.02 *N* NaOH containing 0.5 m*M* cysteine was added to each tissue sample (0.5 μg). The total NAD was measured in the alkaline suspension without further treatment. To measure NADH, the suspension was heated for 10 min at 60°C. Twenty microliters of the cycling reagent mixture was added to each test tube and incubated for 60 min at room temperature. The cycling reagent consisted of 0.1 *M* Tris–HCl buffer (pH 8.0), 0.3 *M* ethanol, 2 m*M* oxalacetate, 2 m*M* mercaptoethanol, 0.02% bovine serum albumin (BSA), and 50 μg/ml alcohol dehydrogenase (pig heart, Boeringer-Mannheim, Indianapolis). After incubation, the cycling reaction was stopped by boiling the tubes for 3 min. Seventeen-microliter aliquots were transferred to 1 ml of the indicator reaction mixture which consisted of 0.02 *M* imidazole buffer (pH 7.0), 20 μ*M* NADP, 0.1 m*M* $MnCl_2$, 1 m*M* DL-dithiothreitol, and 5 μg/ml malic enzyme (Sigma Chemical, St. Louis). The fluorescence of NADPH was read directly in a fluorometer until the reaction was stabilized. Standard and blank tubes were used throughout the entire analysis.

Enzyme assays. α-Glycerophosphate dehydrogenase (GOPDH) and lactate dehydrogenase (LDH) activities in isolated regenerating epidermal segments (1–2 μg) were measured fluorometrically in a volume of 10 μl of an appropriate substrate reagent. The optimal assay conditions for each enzyme were predetermined for the skin (8, 9). The reaction mixture for GOPDH consisted of 50 m*M* Tris–HCl buffer (pH 7.6), 0.5 m*M* dihydroxyacetone phosphate, 0.5 m*M* NADH, and 0.02% BSA. The reaction mixture for LDH consisted of 50 m*M* Tris–HCl buffer (pH 7.5), 1 m*M* sodium pyruvate, 2 m*M* NADH, and 0.02% BSA. The incubation (37°C) times were 120 and 30 min for GOPDH and LDH, respectively. Enzyme reactions were linear for up to 5 μg of freeze-dried epidermal tissue. The amount of NAD formed during the enzyme reactions was determined fluorometrically as described previously (8–10). Tests using standard (containing 1–5 nmole of NAD) and blank tubes were carried out simultaneously with the samples.

Results. Alterations in total NAD and NADH content during epidermal wound healing are presented in Table I. The concentration of total NAD in normal epidermis was 1.1 mmole/kg dry weight, of which 7% was present in its reduced form. Although no significant change in the total NAD content was observed, the concentration of NADH showed a twofold increase on Day 2 and a 2.4 times normal increase on Days 3 and 4 following wounding. The NADH content was maintained at the increased level through Days 7 and 10 postwounding.

GOPDH and LDH activities in the normal epidermis of young guinea pigs were 0.079 ± 0.002 SD (n = 4) and 15.1 ± 2.5 (n = 10) mole/hr/kg dry weight, respectively. Alterations in GOPDH and LDH activity in regenerating epithelium are illustrated in Fig. 1. GOPDH activity gradually increased to a maximum of 500% of normal on Day 7 postwounding. LDH activity increased to 250% of normal on Days 2–4 postwounding.

Discussion. In this investigation, the cell

TABLE I. NAD(H) CONTENTS IN REGENERATING EPITHELIUM DURING WOUND HEALING

Days after wounding	NADH[a] (mean ± SD)	Total NAD[a] (mean ± SD)	Percentage in reduced state
0	73 ± 9.3 (8)[b]	1118 ± 220 (8)	7
2	151 ± 7.1 (4)	1102 ± 121 (4)	14
3	177 ± 13.6 (6)	1239 ± 380 (6)	14
4	175 ± 32.6 (4)	1126 ± 258 (4)	16
7	199 ± 23.9 (6)	1303 ± 474 (6)	15
10	180 ± 23.4 (4)	1276 ± 284 (4)	14

[a] Tissue content is expressed as micromoles per kilogram dry weight.
[b] Number of wounds (animals) used, each assayed in quintuplicate.

multiplication and migration phases occur during the initial 4 days of wound healing, and the cell maturation phase occurs during Days 7–10 postwounding. The regenerating epithelium sampled during the first 4 days of wound healing includes actively migrating cells in the distal end of the regenerating epidermal "tongue" and the cells undergoing mitosis at the proximal regions. The keratin layer increases rapidly after epithelial wound closure on Day 4, reflecting active epidermal differentiation (5). A difference in the times when peak levels of LDH and GOPDH activity occur in regenerating epithelium was observed. LDH activity reaches its maximum during the cell multiplication and migration phases, whereas GOPDH exhibits its greatest activity during the cell maturation phase. An increased NADH content (approximately 2.5 times normal) is maintained during cell migration and maturation. This illustrates bienzymatic competition for NADH in epidermis during wound healing. Energy metabolism in regenerating epithelium is most active on 3 and 4 days postwounding (11). The increased amount of NADH appears to be utilized by LDH for energy metabolism during cell migration and proliferation and by GOPDH for neutral lipid synthesis during cell maturation. In the present study, no attempt has been made to isolate the actively migrating cells from the proliferating (proximal) cells in regenerating epithelium to reflect the metabolic patterns in cell migration versus cell proliferation.

Epidermal differentiation is associated with a shift in lipogenic patterns: an enhanced production of neutral lipid ester is accompanied by a decline in phospholipids (3). Intermediates of carbohydrate metabolism and glycerol 3-phosphate may play a regulatory role in lipid synthesis in skin (12, 13) by stimulating fatty acid esterification (14). In the present study, a striking (5 times normal) increase in GOPDH activity together with increased NADH content during epidermal maturation suggests that GOPDH may play a role in epidermal keratinization by supplying glycerol 3-phosphate from the trioses of carbohydrate metabolism for the synthesis of neutral lipid esters. We have demonstrated a maximal increase in total lipid contents during epidermal maturation of wound healing in guinea pigs

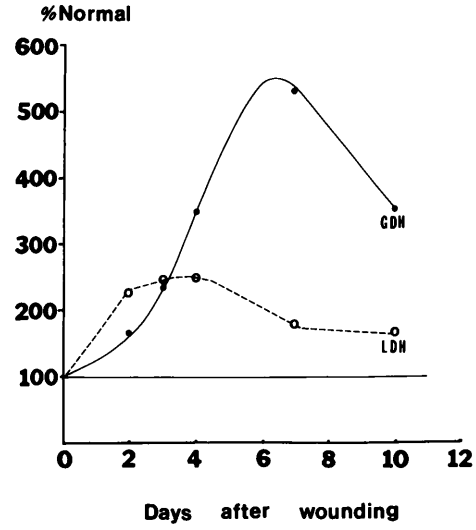

FIGURE 1

(5). Assays of triacylglycerol synthesis in regenerating epithelium should provide more meaningful interpretation of the data obtained in this study.

The ratios of GOPDH to LDH activity correlate with the degree of epidermal differentiation. The GOPDH to LDH ratio is 5.2 ($\times 10^{-3}$) in normal guinea pig epidermis, 2.1 in the cell migration phase on Day 2, and 9.8 during the cell maturation phase on Day 7. The ratios of GOPDH to LDH activity in normal epidermis of man (10) and macaques (8, 9) are 5.3 and 5.9, respectively. The data provide an enzymatic basis for a possible shift in glucose metabolism from glycolysis to fatty acid esterification during epidermal differentiation. The important role of GOPDH in lipid metabolism is also apparent as evidenced by a 57 times greater activity in sebaceous glands than in the epidermis (10). The ratios of GOPDH to LDH activity in sebaceous glands are 291 ($\times 10^{-3}$) in human sebaceous glands (10), 182 in the glands of rhesus, and 122 in those of stump-tailed monkey (8, 9).

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