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STUDIES ON EPIDERMAL-DERMAL SEPARATION AND ENZYME ACTIVITIES IN NEONATAL RAT EPIDERMIS

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ABSTRACT: *Rapid and complete epidermal-dermal separation procedures were determined in neonatal rat skin by light microscopic observation and by compairing enzyme activities in the separated epidermis. Microscopic appearance demonstrated that four different separation procedures used in the study resulted in good separation of epidermis from dermis; heating method(i.e., immersion in 55°C water for 30 sec, followed by immersion in 0-4°C water) and microwave irradiation for 10 sec were saving time. Epidermal activities of lactate dehydrogenase and isocitrate dehydrogenase were preserved by these two separation procedures. However, ornithine decarboxylase activity was significantly higher in microwave irradiated epidermis than in heat separated epidermis. The results suggested that microwave irradiation appears to be a rapid method to separate epidermis with preserving in vivo levels of macromolecules and heat separation procedure seems ideal for the assay of enzymes highly sensitive to environmental factors.*

Key words: *Epidermal separation, Enzyme activity, Neonatal rat epidermis*

INTRODUCTION

Skin is the largest organ of the body and an active site of the biotransformation of a variety of endogenous substances such as steroid hormones, cholesterol, and foreign xenobiotics including drugs and carcinogens (Bickers and Kappas, 1980). The two most important components of skin are the epidermis and the dermis. The epidermis is the surface layer and is essentially a stratified squamous epithelum with a high metabolic rate in direct and continuous contact with the environment (Ackerman, 1978). Very few studies have been carried out to assess the capacity of skin to metabolize drugs and chemicals. Microsomal fraction of skin was reported to contain cytochrome P-450 and drug-metabolizing enzymes such as aryl hydrocarbon hydroxylase (AHH) and epoxide hydrolase (Pohl *et al.*, 1976; Mukhtar *et al.*, 1076). Bickers *et al.*, (1982) emphasized that the epidermis, often considered to be metabolically inert, is an impor-

tant site of xenobiotic metabolism and epidermal microsomes contain inducible mixed function oxidase activities. The epidermis of mouse skin offers a relatively homogeneous population of cells in which to study the metabolic events associated with initiation and promotion stage of carcinogenesis (Boutwell, 1974). The studies of early metabolic events occuring in the epidermis in response to the application of tumor promoters became interesting research areas in skin tumorigenesis (Perchellet *et al.*, 1987; Fisher *et al.*, 1988). These studies necessitate rapid and complete separation techniques of epidermis from dermis with a minimum of chemical or thermal exposure, preservation of the *in vivo* levels of macromolecules and small molecules, and reducing concomitant damage to enzymes.

This study was designed to determine a rapid epidermal separation method in neonatal rat skin by light microscopic appearance and by comparing enzyme activities in epidermis which was separated by heating procedure or microwave irradiation.

MATERIALS AND METHODS

Materials

DL-(1-^{14}C) ornithine hydrochloride(58 mCi/mmole) were purchased from Amersham International plc(Buckinghamshire, England). Lactate dehydrogenase (LDH) and isocitrate dehydrogenase (ICD) test kits, pyridoxal 5-phosphate, dithiothreitol, L-ornithine, citric acid, methyl benzethonium hydroxide, citric acid, 2,5-diphenyl oxazole (PPO) and 1,4-bis(5-phenyl-2-oxazolyl) benzene(POPOP) were obtained from Sigma Chemical Co. (St. Louis, MO. U.S.A.). All other reagents were of reagent grade commercially available.

Animals

Sprague-Dawley rats were obtained from the animal breeding room of the Korea Ginseng and Tobacco Research Institute (KGTRI). Female neonatal rats born *in situ* were allowed to suckle for one day after birth. The neonatal rats were sacrificed and whole skin was excised.

Epidermal Separation by Dithiothreitol (DTT)

Whole skin was immediately placed in ice-cold 0.15 M KCl solution and epidermal side down on a covered glass Petri dish cotnaining crushed ice. The skin was scraped with a sharp scalpel blade (No. 21, Keisei Medical Industrial Co., Tokyo, Japan) to remove subcutaneous fat and muscle. The skins were floated in 0.1 M potassium phosphate buffer, pH 7.4, containing 10 mM dithiothreitol in a glass beaker. The beaker was placed on a rotary shaker with gentle shaking in a cold room at 4°C. After 2 h of shaking the epidermis was peeled away from the dermis using forceps. (Dixit *et al.*, 1983). The whole skin, epidermis, and dermis were washed twice using fresh 0.15 M KCl, blotted with paper towels and used for microscopic observations.

Separation Using Acetic Acid

The skins were stretched dermis side down against a piece of filing card, and placed

in a beaker containing 200 m*l* acetic acid at 5°C for 24 h. The skins were then placed on a Petri dish containing crushed ice, and the epidermis was peeled off with tweezers (Slaga *et al.*, 1973).

Heating Procedure

The skin was excised and plunged into ice-cold water. The skin was then placed in 55°C water for 30 sec, cooled in ice-water, blotted dry with filter paper, and placed flat on a cooled glass plate. The epidermis (0.08 to 0.15 g) was scraped off the dermis with a scalpel blade (Connor and Lowe, 1983).

Microwave Irradiation

A microwave oven (Goldstar Model No. ER-710 MB) was used to irradiate the neonatal rats. Rats were killed and immediately placed in the center of the microwave oven where it was irradiated for 10 sec. After irradiation, it was quickly removed and packed in ice or water slurry. Whole skin was excised and epidermis was seperated from dermis by gentle scraping with a scalpel (Mufson *et al.*, 1977).

Histological Examination

To monitor the separation techniques, whole skin, epidermal scrapings and dermis were subject to histological examination after exposure to separation procedures. Tissue was fixed in 10% neutral formalin and dehydrated with a graded series of alcohols before embedding in paraffin. Sections 5 μ in thickness were stained with hematoxylin and eosin.

Enzymatic Determinations

For enzyme assay, each epidermis, which was separated by heating method or microwave method, was homogenized in ice-cold 50 mM potassium phosphate buffer, pH 7.2, containing 0.1 mM pyridoxal phosphate and 0.1 mM EDTA at 0-4°C. Homogenates were centrifuged at 700 × g for 10 min at 0°C and supernatant was used for the assays of lactic dehydrogenase (LDH) and isocitrate dehydrogenase (ICD). The epidermis from 4 animals were pooled and enzyme activities in soluble epidermal extracts were measured by commercial test kits(Sigma Chemical Co., St. Louis, MO).

The supernatant fraction obtained after further centrifugation at 30,000 × g for 30 min at 0°C was used for estimation of ornithine decarboxylase (ODC) activity. ODC activity was determined by measuring the release of $^{14}CO_2$ from DL-(1-^{14}C) ornithine, essentially as described by O'Brien *et al.* (1975). Incubations were carried out in 25 m*l* Erlenmyer flasks equipped with a polyethylene cup-fitted center well assembly containing 0.2 m*l* methylbenzethonium hydroxide. Incubation mixtures consisted of 0.4 μmole pyridoxal phosphate, 1.0 μmole dithiothreitol, 0.2 μmole L-ornithine, 100 μmoles potassium phosphate, pH 7.2, 0.2 m*l* epidermal extract, and 0.5 μCi DL-(1-^{14}C) ornithine (58 mCi/mmol) in a final volume of 2.0 m*l*. All components except DL-(1-^{14}C) ornithine were incubated at 37°C for 10 min prior to the addition of radio isotope. Incubations were routinely carried out for 30 min at 37°C. The reaction was stopped by addition of 1.0 m*l* 2 M citric acid and incubations were continued for an additional 30 min to insure complete absorption of $^{14}CO_2$. Polyethylene cups con-

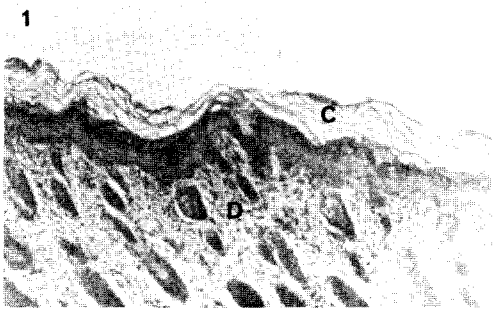

Fig. 1. Light microscopy of newborn rat skin and cornified cells. Tissues were fixed in 10% neutral formalin, paraffin embedded, cut and stained with hematoxylin and eosin. C, cornified cells; E. epidermis; D, dermis (× 100).

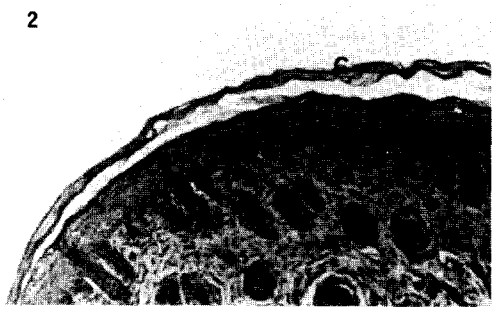

Fig. 2. Whole skin after all separation procedures, but prior to scraping with a scalpel. Epidermal-dermal junction(arrows) looks intact but epidermal sheet was easily peeled away with tweezers or scalpel blades (× 100).

taining $^{14}CO_2$-absorbed methyl benzethonium hydroxide were removed and placed directly into scintillation vials. Cocktail solution contains 2 m*l* ethanol and 10 m*l* of a toluene-based scintillation fluid (4 g PPO and 50 mg POPOP per liter of toluene). Radioactivities were measured in a Beckman LS-1800 liquid scintillation spectrometer. All enzyme activities were corrected against a blank. ODC activity is expressed as nmole CO_2 released for 30 min per mg protein. Protein concentration in epidermal extract was measured by the method of Lowry *et al.* (1951).

RESULTS AND DISCUSSION

Comparison of Epidermal-dermal Separation by Light Microscopic Appearance

Mammalian skin contains two major stuructural components: the outer epidermis and the inner underlying dermis. The epidermis is an actively replicating, compact and stratified squamous epithelium, whereas the dermis has a relatively loose structure consisting of collagen fibers embedded in a ground substrance rich in glycosyl aminoglycans (Bickers *et al.*, 1982).

Epidermis is tightly adherent to the dermis and separation without concomitant damage to enzymes, seemed difficult to achieve. In this study, neonatal rat skin was used because it is frequently used in xenobiotic metabolism studies. Neonatal rats take an advantage; it is hairless, which precludes the need of shavng, much less resistant to homogenation procedures and minimal environmental pollutant-induced alterations in drug metabolizing enzymes.

Fig. 1 shows a 1 day-old newborn rat skin. The whole skin consisted of epidermis (E) and dermis(D). The outermost cornified cells(C) of the epidermis appear anucleated and filled with a fibrous material. The fibers of cornified cells are formed in precursor epidermal cells, and no major alteration takes place in these proteins during epidermal cell differentiation (Inoue *et al.*, 1976). The cornified cells were loosely attached to epidermis after all separation procedures and epidermis could be peeled

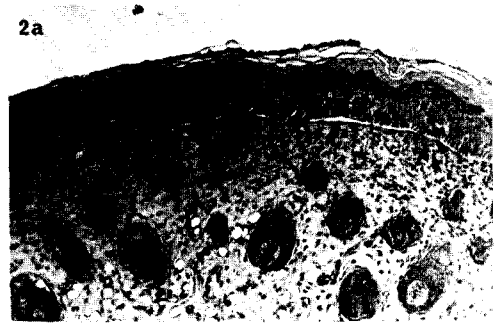

Fig. 2a. Epidermal-dermal junction(arrows) was separated after microwave irradiation (×100).

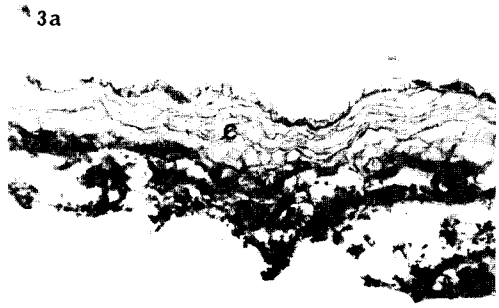

Fig. 3a. In some cases, epidermis attached to cornified cells was shown (×100).

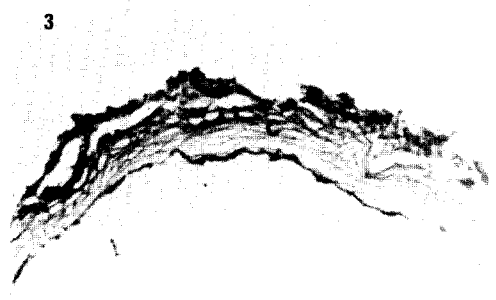

Fig. 3. The separated cornified cells after 24 h of immersion in 1% acetic acid at 5℃ (×100).

Fig. 4. Epidermis-free surface of the dermis, demonstrating a complete separation of the epidermis from the dermis after scraping skin exposed to various separation procedures (×100).

away with tweezer or scalpel blades(Fig. 2). After 24 h of immersion in 1% acetic acid at 5℃, cornified cells were cleft from epidermis (Fig. 3). All separation procedures used in the study showed a complete separation of epidermis from dermis(Fig. 4). In general, biochemical determinations on epidermis require harvesting tissue from a large number of animals, and there is thus a corresponding large expenditure of time. With this point of view, heating procedure (55℃, 30 sec) and microwave irradiation (10 sec) appear favorable in the studies using epidermis and/or dermis.

Epstein *et al.* (1979) firstly used the disulfide-reducing agent dithiothreitol(DTT) in an attempt to stabilize glucocorticoid receptors of newborn mouse skin during enzymatic dermal-epidermal cleavage and found unexpected separation of epidermis from dermis without the addition of proteolytic enzymes. Even though the mechanism by which DTT effects the split is unknown, it was conceivable that DTT may inactivate some enzyme inhibitor or may activate directly an enzyme that cleaves a molecule(s) necessary for adhesion. The distinctive feature of DTT procedure was retainment of an intact basal layer in epidermis in contrast to mechanical separation. To identify the attachment of basal cells either to epidermis or to dermis, which was separated by dif-

Table 1. Comparison of enzyme activities in neonatal rat epidermis separated by microwave irradiation or heat procedure

Separation procedure	LDH	ICD	ODC
Microwave	5.300 ± 0.420	1.200 ± 0.310	0.4644 ± 0.095
Heat	5.818 ± 0.503	1.636 ± 0.352	0.2339 ± 0.072*

Data represent mean ± SD of four animals.
Statistical significance was assessed by student's t-test, *; $p < 0.005$. Epidermal enzymes are expressed as Sigma unit/μg protein for LDH(lactate dehydrogense) and ICD(isocitrate dehydrogenase) and nmoles CO_2/30 min/mg protein for ODC(ornithine decarboxylase).

ferent procedures, further studies using electron microscopy should be done.

Enzyme Activities in Neonatal Rat Epidermis Separated by Heat or Microwave Irradiation

Lactate dehydrogenase (LDH) is one of the most active enzymes in the skin of primates (Im and Adachi, 1966) and 40 to 70% of glucose is converted to lactic acid in human and rat skin (Freinkel, 1960; Pomerantz and Asbornsen, 1961). In contrast to most tissues, epidermis converts most of the glucose to lactic acid even in the presence of oxygen (Halprin and Ohkawara, 1966). Isocitrate dehydrogenase (ICD), which belongs to aerobic Krebs cycle and is one of the sources of NADPH in the cell, also highly active in human epidermis (Cruiokshank *et al.*, 1958). As shown in Table 1, epidermal separation method, either microwave irradiation or heating, had no effect on LDH and ICD activities. However, ornithine decarboxylase (ODC) activity in epidermis was significantly different by the separation procedures. ODC is present in all nucleated cells and is the rate-limiting enzyme for synthesis of polyamines (Janne *et al.*, 1978). ODC activity is normally low in the epidermis but may be elevated during proliferation and can be induced by trophic hormones, carcinogens, tumor promoters, mitogens and by wounding (Conner *et al.*, 1985). Mufson *et al.* (1977) reported that recoveries of DNA, RNA, and protein on a per area basis of epidermis were the same for the microwave and heat separation procedures, and the TPA-induced ODC levels were not significantly different between the two methods. In this study, ODC activity was higher in epidermis separated by microwave than by heating. It is a possible reason for that ornithine decarboxylase activity can change markedly and rapidly in response to extracellular signals. Similar observation was reported by Byus *et al.* (1988) in which ODC activity was increased in cultured cells following a transient exposure to microwave field.

As a tentalive conclusion, exposure of skin to microwave irradiation for 10 sec can be a rapid methodoloy to separate epidermis and preserve macromolecules and some enzyme activities closed to *in vivo* states. Heat separation procedure (*i.e.*, immersion in 55°C water for 30 sec followed by immersion in 0-4°C water) seems to be good for assy of enzymes which are highly sensitive to environmental factors and having specific membrane receptors.

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