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GROWTH AND DIFFERENTIATION OF TRANSPLANTED EPITHELIAL CELL CULTURES*

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In 1952 Billingham and Reynolds demonstrated that sheets of epidermis and of trypsinized suspension of epithelial cells could be transplanted successfully (1). After dissociation of mouse embryonic skin into individual cells, Moscona reported that skin cells aggregated, and that the aggregates differentiated into patches of skin with hair rudiments (2). The skin epithelial cell has also been successfully maintained in cell culture for the past 2 decades (3–6), and retains many of the characteristic properties of the normal skin epithelial cell *in vivo* (7–10).

We have been interested in the induction of somatic changes in the skin epithelial cell *in vitro*, and the use of epithelial cell grafts to study and to evaluate these changes. This paper is a description of a method for the preparation and maintenance of long-term orthotopic grafts of rabbit skin epithelial cells, and a study of the growth and differentiation of the normal rabbit epithelial cell *in vivo* after cell culture.

MATERIALS AND METHODS

Construction of Silicone Rubber Skin Chambers. Silicone rubber sheeting (0.44 mm x 8.0 cm), silicone rubber tubing (¾″ I.D., 1.0″ O.D.), and silicone rubber medical adhesive are obtained from the Dow Corning Corporation. The tubing is cut into 1.0 cm sections, and the sheeting into 4.0 cm diameter circles. A 0.9 cm anulus is cut in the center of each circle of silicone sheeting. The cut tubing and sheeting are cleaned by boiling with detergent, and by rinsing successively with distilled water and with acetone. After drying, one rim of the tubing is sealed over the anulus with the adhesive. The adhesive is allowed to dry for 24 hours at room temperature, and the chamber is sterilized in a boiling water bath for 15 minutes before being inserted on the back of an experimental animal. A completed skin chamber is shown in Fig. 1.

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Growth of Epithelial Cell Monolayers. The back of an anesthetized New Zealand white rabbit is shaved, and a full-thickness section of skin (4 sq cm) is removed and soaked in Hanks' Balanced Salts Solution (containing 500 μg streptomycin and 1000 units of penicillin per ml) for 1 hour at 25° C. The skin is thoroughly rinsed in 0.9 percent saline, and is cut into 1.5 mm × 1.5 mm explants. Four explants are placed on a Corning coverslip secured by a plasma clot, and the explants are maintained in culture as previously described (9). New cell growth is determined daily with a calibrated microscope ocular. When the new cell growth reaches a radius of 1.5 mm from the edge of the explant, the cells are removed from the explant and transplanted to a prepared graft site.

Preparation of a Graft Site. The back of an anesthetized rabbit is shaved, cleaned with 70 percent alcohol, and the epidermis and dermis are excised down to the muscle fascia covering a circular area 2 cm in diameter. The rim of a sterile silicone chamber is placed under the wound edge after undermining the free edges from the muscle fascia. The flexibility of silicone rubber permits the rim of a chamber to be fitted within a 2 cm diameter wound. If a wider wound margin is made or the chamber does not fit tightly within the wound, the skin on opposing sides of the chamber is sutured so that the top of the exposed chamber is held firmly in place by the surrounding skin. Four chambers may be placed on the back of a rabbit (Fig. 2). Two of the chambers are used for grafts, and 2 of the chambers serve as controls for non-graft reactions. The top of each chamber is covered with paper tape, and the rabbit is injected intramuscularly with 250,000 units of benzathine penicillin. A period of 7–14 days is required for sufficient granulation tissue to form within a chamber to support an epithelial cell graft. Before grafting, the dry coagulum over the granulation tissue is removed by soaking with physiologic saline, and the graft bed is washed with sterile saline to remove cellular debris.

Transplantation of Epithelial Cells. Filter papers (Whatman #40) 2 cm in diameter are washed successively with distilled water, ethanol, and ether, and are dried with air. Cylindrical cellulose sponges (1.5 cm diameter × 0.5 cm) are cut from a stock cellulose sponge (Dupont Fine Grain Cellulose Photographic Sponge) and are prepared as a covering for the epithelial cells by boiling with distilled water, by washing with acetone and ether, and by drying in air. The filter papers and sponges are sterilized by autoclaving.

A new growth of epithelial cells, seen as an

opalescent halo surrounding each explant, is cut away from 4 explants with a #15 scalpel blade and the explants are discarded. The large cell sheet is then cut into smaller 0.5 mm × 0.5 mm sections.

The sections are gently lifted from the coverslip with a scalpel blade, and are uniformly distributed over a washed filter paper kept moist by means of a drop of Eagle's Minimal Essential Medium (MEM) containing 10 percent calf serum. The filter paper and cells are placed over the granulation tissue on a prepared graft site with the cells in direct contact with the granulation tissue. Two cellulose sponges are placed over each filter paper and are moistened with approximately 0.75 ml of Minimal Essential Medium. Sufficient medium is added to the sponges so that they are kept moist; care is taken that the cells are not washed from the filter paper by excess medium.

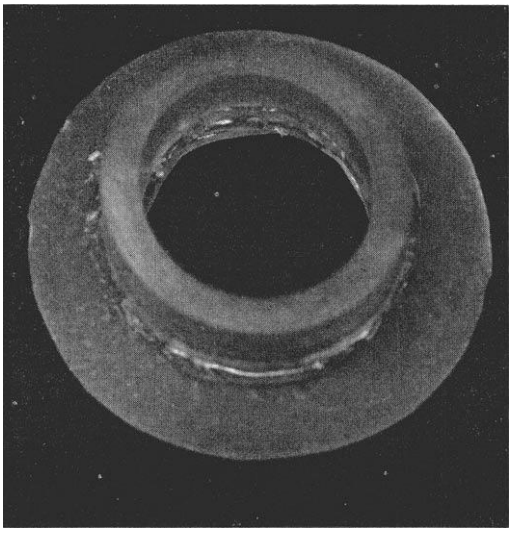

FIG. 1. Silicone rubber skin chamber. A chamber is constructed from silicone rubber medical grade tubing ¾″ I.D., 1″ O.D., and a 0.4 mm non-reinforced silicone rubber sheeting. A cylinder 1 cm in height is cut from the tubing and sealed (with silicone rubber adhesive) over an anulus 0.9 cm in diameter cut in the center of a circle of non-reinforced sheeting 4 cm in diameter.

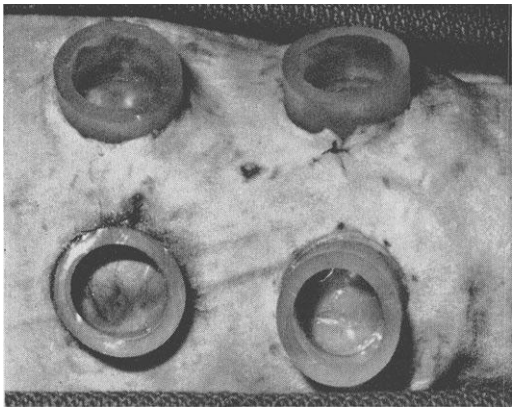

FIG. 2. The position of 4 silicone rubber skin chambers inserted between the muscle fascia and the dermis on the back of an anesthetized rabbit.

The top of each chamber is sealed with paper tape, and the rabbit is injected intramuscularly with 250,000 units of benzathine penicillin. After 7 days, the filter paper and nutrient sponges are removed following soaking in physiologic saline, and the growth of epithelial cells is studied in successive weeks by direct observation and by histiologic preparations from 3 mm punch biopsies.

Biopsies. Skin from 3 mm punch biopsies is obtained from the graft and control sites, fixed in 20 per cent buffered formalin, pH 7.2, sectioned and stained with Hematoxylin and Eosin, and with Periodic acid-Schiff (PAS).

RESULTS

Growth of Transplanted Cells

Week 1. A layer of epithelial cells 3–5 cells in thickness covers the surface of the graft site one week after transplantation (Fig. 3A). A columnar basal layer is seen but a stratum corneum is not yet formed. A thin and irregular basement membrane separates the epidermal cells from granulation tissue. The granulation tissue is highly cellular and infiltrated with a large number of lymphocytes and a smaller number of eosinophils.

Week 2. A further growth and differentiation of the transplanted cells and their progeny has taken place. A well formed stratum corneum over numerous granular cells is observed (Fig. 3B). The basement membrane remains thin and irregular, and the granulation tissue continues to be highly cellular.

Week 3. The epidermis is hyperplastic (10–14 cells in thickness) with numerous keratohyalin granules and with a thick stratum corneum (Fig. 3C).

Week 5. The epidermis is almost completely deteriorated, and only occasional areas of a greatly altered epidermis are observed (Fig. 3D).

Growth Conditions

Nutrient Sponges. The rate of epithelial cell growth and the extent of differentiation was studied in 8 cell grafts in which daily replacement of nutrient sponges was made. More fre-

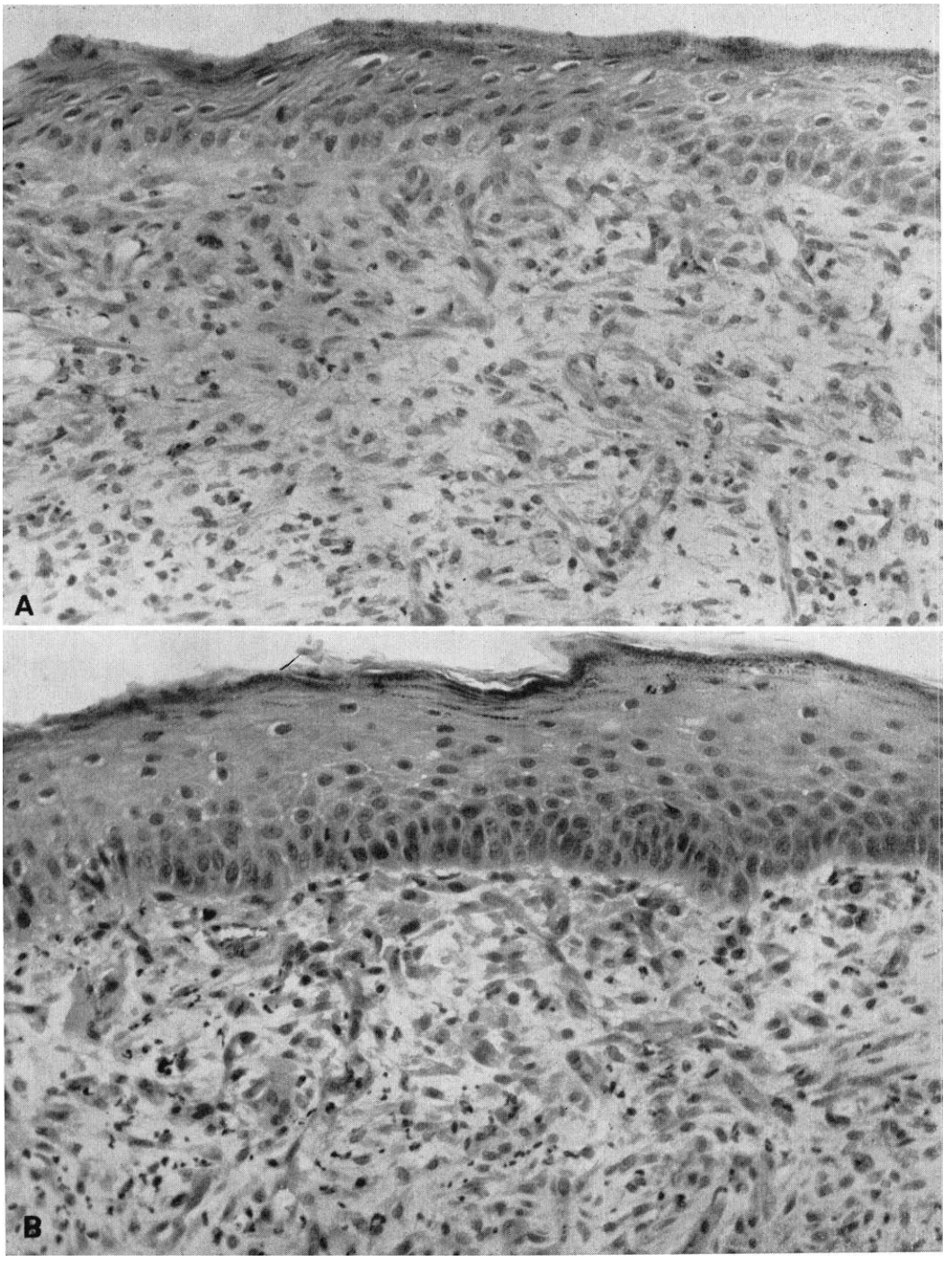

FIG. 3A. Light micrograph of a 3 mm punch biopsy of skin 1 week after transplantation of rabbit skin epithelial cells. Hematoxylin and Eosin × 194. The cells have organized into a columnar basal layer. A stratum corneum is not formed. Granulation tissue shows the presence of large numbers of round cells, mainly lymphocytes and eosinophils.

FIG. 3B. Light micrograph of a 3 mm biopsy of skin 2 weeks after transplantation of rabbit skin epithelial cells. Hematoxylin and Eosin × 194. Stratum corneum is well formed, and numerous granular cells with keratohyalin granules are observed. Epidermis is hyperplastic and 10–12 cells in thickness.

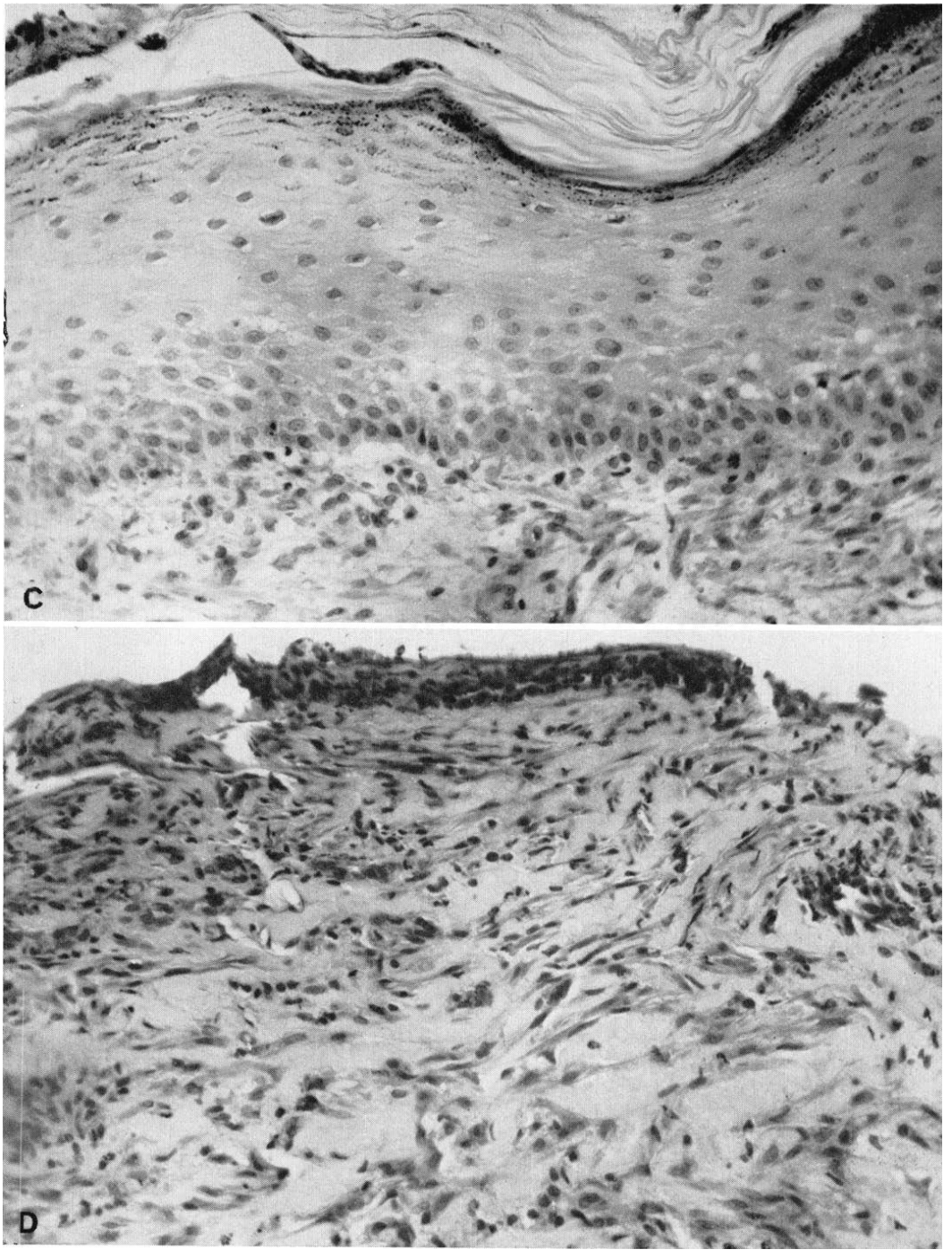

FIG. 3C. Light micrograph of a 3 mm biopsy of skin 3 weeks after transplantation of rabbit epithelial cells. Hematoxylin and Eosin × 194. Epidermis is 10–14 cells in thickness. There is a thick stratum corneum with numerous keratohyalin granules.

FIG. 3D. Light micrograph of a skin specimen 5 weeks after transplantation. Hematoxylin and Eosin × 194. Almost all of the epidermis has deteriorated leaving patches of greatly altered epidermis.

quent replacement of sponges did not influence epithelial cell growth. In 4 cell grafts made without subsequent covering with sponges, the wound rapidly dried and no growth of cells was observed.

Closed Grafts. The attachment and growth of epithelial cells in chambers sealed with plastic film to decrease the rate of drying was studied in 4 grafts. When the chamber was tightly sealed, large amounts of fluid accumulated over the granulation tissue and only an occasional area of transplanted cell growth was seen.

Type of Chamber. Chambers made from glass and silicone rubber were compared. Inflammatory reactions occurred less frequently with silicone rubber and produced less damage to surrounding skin than chambers made from glass. Glass chambers could not be successfully maintained in rabbits for periods longer than 4 weeks and were not useful for the long-term study of transplanted cells.

Frequency of Successful Grafts. In 30 epithelial cell grafts made on 17 rabbits by the method described in this report, growth and differentiation were observed in 24 of the graft sites; in 30 control sites not grafted with epithelial cells, only granulation tissue was observed.

DISCUSSION

The studies described in this report have shown that cultures of postembryonic rabbit epithelial cells retain the ability to differentiate and to form an epidermis when transplanted to a suitable graft site. Several factors influence the initial growth of the transplanted cells. When grafted to either poorly developed granulation tissue, or to a graft site without a nutrient sponge covering, epithelial cells do not survive. Extended feeding with nutrient sponges does not appear to accelerate epidermal growth, and an external source of nutrients is needed only during the initial growth period.

Wound contraction and reepithelialization from a wound edge has been prevented by the use of silicone rubber skin chambers. Although similar chambers may also be made from glass, we have found that inflammatory reactions are more frequent with glass chambers, and that the long-term maintenance of glass chambers on a rabbit is more difficult to achieve.

The ease with which skin epithelial cells can be grown in explant culture and transplanted orthotopically can provide an experimental approach to a study of the factors that affect somatic stability of epithelial cells in cell culture. For example, in the present work, it may be concluded that the observed absence of keratohyalin granules in cell culture (10) is the result of a cellular modulation rather than a somatic mutation since growth under physiologic conditions causes the formation of the granules (Fig. 3B and 3C).

The reactions that lead to a complete deterioration of the transplanted cells within 6 weeks are unexplained. Although we have taken the precaution to use only autologous cells and to make all grafts orthotopically, an intense hyperplasia was common in every transplantation site. To account for the accelerated growth that is observed in experimental wounds, Bishop (11) presented evidence that extensive destruction of the dermis may eliminate a tissue element necessary to regulate the normal growth of connective tissue as well as to support a normal epidermal growth. Studies by McLoughlin (12) and Wessells (13) have also shown that connective tissue strongly affects growth, differentiation, and survival of embryonic epidermis.

The absence of a normal connective tissue environment within the skin chambers may also account for the observation that transplanted epithelial cells fail to form adnexal structures. Since a dermal papilla is important in the maintenance of the hair follicle throughout its cycles of activity (14), any change in the ability of connective tissue cells to form a normal papilla would be expected to seriously affect hair development. When normal rabbit wounds were permitted to epithelialize slowly, Breedis observed that scar epithelium organized into hair follicles and sebaceous glands, and that the size and organization of the papillae paralleled the development of the hair follicles (15). That the persistent hyperplasia of the transplanted cells and their progeny, the absence of adnexal structures, and the eventual deterioration of the epidermis may occur as a direct consequence of a change in the normal connective tissue environment is a possible explanation for all of these observations. A study of the chemical composition of granulation

tissue with emphasis on the mucopolysaccharide content preceding and following epithelial cell changes would perhaps be a useful experimental approach to help establish a regulatory function for postembryonic connective tissue in epithelial cell growth and differentiation.

SUMMARY

A method for the transplantation of primary cultures of adult rabbit epithelial cells has been developed, and the behavior of transplanted epithelial cells after a limited period of growth in cell culture has been determined. One week after transplantation of cells, the wound site is covered by a new growth of transplanted epithelial cells and their progeny. In subsequent weeks, the transplanted cells and their progeny continue to differentiate and to form an epidermis that contains a thick stratum corneum over numerous granular cells. Within six weeks after transplantation, the epidermis begins to deteriorate until only traces of a greatly altered epidermis are present. No evidence for the formation of adnexal structures is observed during the growth period of the transplanted cells.

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