

## IN VITRO ORGAN CULTURE OF ADULT HUMAN AND MOUSE SKIN

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**Abstract.** A new method of organ culture of skin has been elaborated which is more convenient and gave better results. Mouse skin cultured for 4 weeks by this method was found to be unaffected as regards its texture and toughness, whereas in other methods these qualities deteriorated even after 2 weeks of culture. Human skin, on the other hand, was found to be comparatively less affected. However, both types of skin were found to grow well by the new method. Various histological differences in proliferation, mitotic activity, parakeratosis, epiboly, necrosis etc. observed in human skin cultured by different methods could be assigned to various environmental conditions prevailing therein. In our new method, solid medium was used and care was taken to provide, as far as possible, in vivo conditions for the skin explants. Despite all efforts, skin did not grow according to the in vivo morphological pattern. Nevertheless, skin could be successfully cultured in solid medium for as long as 6 weeks in case of human and 4 weeks in case of mouse skin, good enough for transplantation purposes.

Ljunggren in 1898 (8) demonstrated for the first time that human skin could survive for many days if stored in ascitic fluid. Subsequently, skin culture remained a forgotten subject until the 1930s, when Bornstein (2), Pinkus (10), Doljanski et al. (4) revived it. However, it was Medawar's (9) observations which gave the subject its great impetus. Since then, Blank et al. (1), Reinertson (12), Reaven & Cox (11), Sarkany et al. (13), Karasek (6), Summerlin et al. (14) have successfully demonstrated skin culture in vitro. In almost all these studies except that of Blank et al. (1) who cultured skin in solid medium for as long as 72 hours, the skin explant is either immersed in or moistened with the liquid medium which makes the skin, especially that of the mouse, too soft to be used for transplantation purposes (5). In addition, other adverse effects of liquid medium on the skin in vitro (12, 15) and the effect of prolonged water exposure in vivo (16) have been discussed. In view of the difficulties encountered in most of the culture techniques and the paucity of the literature regarding the culture of skin in solid medium, we were encouraged to undertake the

present investigation. For this purpose several hundred adult human and mouse skin explants have been cultured.

### MATERIALS AND METHODS

Chick embryo extract, chicken plasma, special agar (Noble) and triple distilled water were obtained from Difco Laboratories (Detroit); Earle's balanced salt solution (BSS), Eagle minimum essential medium (MEM) with Earle's salts and 20 mM Hepes buffer, newborn calf serum (heat-inactivated and filter-sterilised) and L-glutamine (200 mM) from Flow Laboratories (Irvine, Scotland); streptomycin sulphate and sodium benzylpenicillin from Pharmachemie (Haarlem, Holland); nystatin from Labaz (Brussels, Belgium); Millipore filters (0.22  $\mu$ m) from Sartorius (Göttingen, W. Germany) and disposable plastic Petri dishes (4 cm diameter and sterilised) were obtained from Proton (Amsterdam, Holland).

Chick embryo extract and chicken plasma were reconstituted according to the procedure described in Difco Manual (3).

The soaking medium was prepared by adding streptomycin (500  $\mu$ g), penicillin (1000 U) and nystatin (100  $\mu$ g) per millilitre of BSS. The pH of the medium was adjusted to 7.3 with 1 N NaOH and 1 N HCl.

Liquid culture medium was prepared by adding 1 ml L-glutamine and 12 ml calf serum to 100 ml of MEM. Streptomycin (50  $\mu$ g) and penicillin (100 U) were used per ml of culture medium and the pH was adjusted to 7.3 with 1 N NaOH and 1 N HCl.

For solid medium 20 ml agar (5% in distilled water) was sterilised in the autoclave for 15 minutes at 15 pounds pressure at 121°C. The agar, when cooled to 40°C, was thoroughly mixed with 80 ml of liquid medium pre-warmed to 30°C in an incubator for 1 hour. Agar was then poured into the plastic Petri dishes, forming a 3 mm thick layer. When cool, the agar plates were ready for use.

All the solutions were prepared in triple distilled water and sterilised through Millipore filters unless otherwise stated.

A strain of inbred white mice (S. P. F. Cat. No. 203, TNO, Holland) of male sex, weighing 18-22 g was used. Full thickness skin was removed by scalpel after shaving with a safety razor under ether anaesthesia.

The human skin specimens were removed in the operating room from the patients undergoing skin grafting procedures. They were either full thickness grafts thinned in such a manner that no subcutaneous tissue was left attached to the under-

surface, or thick, split thickness skin grafts removed by dermatome. The ages of the patients ranged from 5 to 72 years.

In both procedures skin pieces were cut approx.  $0.4 \times 0.4$  cm and were soaked in soaking medium for 2 hours. Then in one set of experiments each explant was placed over the agar surface with the dermis side down, in a number of agar plates. Each agar plate was then placed separately in a larger glass Petri dish (sterilised) filled with triple distilled water containing streptomycin (50 µg), penicillin (100 U) and nystatin (25 µg) per ml of distilled water to maintain a high humidity and sterility in the environment. The plates were placed in an incubator at 37°C and every third day 2 ml of liquid medium was added to avoid dehydration of the agar medium. After every 8 to 10 days explants were transferred to new agar plates aseptically.

In another set of experiments, the liquid medium method (6) was followed for the culture of skin.

Explants were harvested from both sets of experiments on 2nd, 3rd, 4th, 10th, 13th, 21st, 28th, 35th and 42nd days. They were fixed immediately in 10% buffered formalin (pH 7.3). Sections were cut and stained with hematoxylin and eosin. All experimental procedures were performed in an UV chamber.

Histological changes in human skin explants were studied at regular intervals as mentioned, whereas specimens of mouse skin were checked only occasionally to determine whether the tissue was alive or not.

## RESULTS

The detailed account of sequential histological features of the skin specimens cultured *in vitro* are summarised in Table I. Histological studies readily revealed whether the tissue was alive or dead (1).

Table I. *In vitro* histology of human skin

Histological changes	Culture medium	
	Liquid	Solid
Proliferation of epidermal cells	24-48 hours	24-48 hours
Maximum proliferation	5th-6th day	4th-5th day
Appearance of epiboly	4th-6th day	4th-5th day
Completion of epiboly	3rd-5th week	2nd-3rd week
Mitotic activity	3rd day	2nd day
Maximum mitotic activity	4th-5th day	3rd-4th day
Vacuolated cytoplasm	4th day	4th day
Parakeratosis	4th-5th day	2nd-3rd day
Complete parakeratosis	24th-28th day	19th-21st day
Necrosis by end of 2nd week	In almost all skin explants	In few of the skin explants
General appearance of dermis after 1 week	Sporadic nuclear pyknosis occurred and dermis began to disintegrate	skin explants
Skin texture after 5 weeks	Became a little soft	Remained normal

In our studies the skin cells were found viable and alive.

Adequate growth occurred in almost all human skin explants cultured in liquid as well as in solid medium, with minor differences (Table I). The earliest notable histological change was proliferation of the epidermal cells by 24 to 48 hours which was judged by a uniform increase in epidermal thickness as compared with the control skin biopsies. Maximum proliferation occurred on the 4th to 6th day. Usually by the 4th to 6th day the most striking feature was the migration of the proliferating epidermis over the incised margins of the explant to cover the cut surface of the dermis, partially or completely encasing it; this was the beginning of epiboly which was completed by the end of the 2nd to 5th week. At the base of the epidermis the single cell layer remained adherent and thickened, but no marked distortion resulted up to the 2nd to 3rd day. At this time mitotic figures appeared, indicating regeneration. Maximum mitotic figures were found on the 3rd to 5th day. However, mitotic figures were by no means confined to the basal layer, as cells of Malpighian layer also contained mitotic figures, though not as frequently as in the basal layer. Some of the cells of the superficial Malpighian layer often contained pyknotic nuclei. Cytoplasm was found to be vacuolated in the entire epidermis by day 4.

Parakeratosis was evident throughout the stratum corneum after 2 to 5 days. Initially, a parakeratotic layer consisting of two layers of cells was formed above the basospinous cell layer, characterised by cells showing retention of intensely stained nuclei in the horny layer, absence of granular layer and an oedematous eosinophilic cytoplasm. The parakeratotic layer continued to grow until it attained a thickness of 8 to 10 cells whereas basospinous cells diminished finally to a thickness of 1 to 3 cells by the 19th to 28th day.

At the end of the second week scattered necrosis was observed in some of the explants in solid medium while in liquid medium it occurred in almost all of the explants.

Dermis did not undergo any appreciable change during the first week but thereafter in some of the cells certain changes were observed which included nuclear pyknosis and fragmentation.

By the end of the sixth week the epidermis remained only one cell thick and only small foci of basal cells remained and a large number of pyknotic nuclei containing keratin overlay the epidermis. The

epidermis seemed to be growing well, with occasional mitotic figures and stratum corneum formation. Fibroblasts seemed to be normal but the general architecture of the dermis was found to be progressively disintegrated.

In the case of mouse skin, although all the skin explants in liquid as well as in solid medium were found to be alive during the first 2 weeks, conspicuous change, such as has been observed in the case of human skin, could not be observed properly due to lack of sufficient samples and thinness of the skin to make histological preparations. However, some of the skin explants in solid medium exhibited proliferation, mitotic activity, epiboly, etc., whereas in liquid medium these changes were observed only occasionally. In liquid medium the skin as a whole lost its texture even during the third week of *in vitro* culture whereas in solid medium it maintained its texture as long as 4 weeks and sometimes even longer.

## DISCUSSION

The most important observation in the present investigation from the point of view of transplantation was that mouse skin explant became too soft for grafting purposes after 3 weeks of *in vitro* culture in liquid medium, although seemingly viable and histologically alive. It became practically useless for grafting (5), while human skin explants were found to be less affected. On the other hand both types of skin explants in solid medium were found to be tough in texture and viable even after 4 weeks of *in vitro* culture.

As regards morphological changes in human skin explants they tended to follow those observed by Reinertson (12), Reaven & Cox (11), Sarkany et al. (13) and Summerlin et al. (14). Minor histological differences could be detected between those explants cultured in liquid and those cultured in solid medium (Table I). Mitotic figures, maximum mitosis and parakeratosis appeared later in liquid medium. It took longer for epiboly to be completed in liquid medium.

Proliferation and mitotic activity were also found to be slow and less extensive in liquid medium. The only possible reason for all these observations could be less cell damage in the liquid medium due to less friction and the lesser generative effort required when the skin is protected from dehydration. On the other hand, in solid medium, the skin surface was

exposed to air and large number of cells were liable to damage with the result that new cells were more quickly formed to replace the damaged ones. This replacement of cells resulted in an accelerated upward migration of the epidermal cells (7) which influenced the mitotic activity and thus explains the early formation of the parakeratotic layer and epiboly in solid medium.

Not only basal cells but also the cells of the Malpighian layer were found to be self-differentiating as has been observed by Reaven & Cox (11). Lever (7), however, found mitotic activity throughout the squamous layer during the period of regeneration of the epidermis at the margin of the wounds. It seems likely the *in vitro* cultured skin graft possesses the same tendency of regeneration as the edges of the wound caused by the removal of the graft. In solid medium skin was protected from prolonged exposure to water which would otherwise have caused some adverse effects (16).

Despite very many efforts to provide *in vivo* conditions for skin explants *in vitro*, necrolysis could not be prevented. Neither could true keratinisation be produced according to the *in vivo* morphological pattern. No definite conclusions could be drawn as far as morphological changes are concerned in mouse skin explants because no regular data have been collected. Nevertheless, mouse skin could be cultured successfully as long as 4 weeks and human skin as long as 6 weeks in solid medium.

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## REFERENCES

1. Blank, H., Sagami, S., Boyd, C. & Roth, F. J., Jr: The pathogenesis of superficial fungus infections in cultured human skin. *Arch Derm (Chicago)* 79: 524, 1959.
2. Bornstein, K.: Über Gewebezucht menschlicher Haut. *Klin Woch* 19: 1119, 1930.
3. Difco Manual of Dehydrated Culture Media and Reagents for Microbiological and Clinical Laboratory Procedures, 9th ed. Difco Labs., Detroit, Mich., 1971.
4. Doljanski, L., Hoffman, R. S. & Tenebaum, E.: The effect of extracts of heterologous adult tissue on cell growth *in vitro* and their use in wound healing. *Nature* 150: 23, 1942.

5. Janssen de Limpens, A. M. P., Siddiqui, A. H. & Cormane, R. H.: Studies on the allogeneic transplantation of organ culture of adult mouse skin. *Acta Dermatovener (Stockholm)* Unpubl. data.
6. Karasek, M.: In vitro culture of human skin epithelial cells. *J Invest Derm* 47: 533, 1966.
7. Lever, W. F.: Histology of the skin. *In Histopathology of the Skin*, 4th ed. Pitman Medical Publishing Co., London, J. B. Lippincott Co., Philadelphia, 1967.
8. Ljunggren, O.: *In Cell and Tissue Culture* (ed. J. Paul). Williams and Wilkins Co., Baltimore, 1961.
9. Medawar, P. B.: The cultivation of adult mammalian skin epithelium in vitro. *Quart J Micros Sc* 89: 187, 1948.
10. Pinkus, H.: Gewebekulturen menschlicher Epidermis. *Arch Derm Syph* 165: 53, 1932.
11. Reaven, E. P. & Cox, A. J.: Organ culture of human skin. *J Invest Derm* 44: 151, 1965.
12. Reinertson, R. P.: Stratum corneum formation in auto implants and in explants of human skin. *J Invest Derm* 36: 345, 1961.
13. Sarkany, I., Grice, K. & Caron, G. A.: Organ culture of adult human skin. *Brit J Derm* 77: 65, 1965.
14. Summerlin, W., Charlton, M. & Karasek, M.: Reconstruction of epidermis by transplanted organ cultures of adult human skin. *J Invest Derm* 55: 310, 1970.
15. Szabo, G.: Cultivation of skin, pure epidermal sheets and tooth germs in vitro. *In Fundamentals of Keratinisation*. Publ. 70, Am Assoc Adv Sci, Washington, D.C., 1962.
16. Willis, I.: The effects of prolonged water exposure on human skin. *J Invest Derm* 60: 166, 1973.

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