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A porcine model using skin graft chambers for studies on cultured keratinocytes

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SUMMARY. **In wound healing research, animal models permit an extensive tissue analysis which is not normally possible in clinical studies. A morphological comparison of human and porcine skin was made in order to identify those aspects of the wound healing process where a porcine model may help our understanding of clinical problems. We describe a porcine model for evaluating the growth of cultured keratinocytes on a variety of wound beds. Polytetrafluoroethylene skin graft chambers were used to isolate wounds and prevent epidermal healing from the skin edge. The chambers remained *in situ* for 5–7 weeks. We detail the surgical technique, the method of porcine keratinocyte culture and highlight some practical measures that were taken to optimise the "take" of the cultured keratinocyte grafts.**

Clinical studies have reported the use of cultured keratinocyte grafts to resurface burn wounds[1] and other skin defects.[2,3] However, there are difficulties in understanding the biological basis of changes in such wounds following grafting, due principally to the unacceptability of performing repeated skin biopsies in patients. *In vivo* animal studies are able to meet this need by virtue of their ability to provide tissue data in a planned and controlled fashion. Cultured keratinocytes have been grafted on athymic mice[4,5] and Eisinger reported the use of a porcine model.[6] Our department previously established a porcine model and studied the rejection of allogeneic cultured keratinocytes,[7] but having encountered some practical problems we have sought to improve the model by changes to its surgical aspects.

Since cultured keratinocytes replace the epidermis, they should ideally be tested on full-thickness skin defects where there are no endogenous epidermal remnants. Nonetheless, migration of epidermal cells from the wound edge may complicate the results as cultured keratinocytes produce cytokines that promote epidermal growth.[8] Isolated "test beds" within an *in vivo* environment, such as intra-peritoneal diffusion chambers[9] and granuloma air pouches,[10] have been used previously in experimental models. Worst and co-workers reported the use of cutaneous silicone implants in mice to study epidermal cell cultures.[11] We have developed similar chambers using polytetrafluoroethylene (PTFE) to isolate full-thickness wounds in pigs and prevent epidermal migration from the skin edge. These chambers were used successfully in a recent study of cultured keratinocyte growth on a dermal wound bed.[12] This present paper provides the practical details of this wound healing model and discusses its advantages and limitations. Furthermore we judge the clinical relevance of a porcine model for studies on cultured keratinocytes by a morphological comparison of porcine and human skin.

Materials and methods

Under Home Office licence, 30 Large White pigs 20–25 kg in weight (Bury Farm, Edgware, Middx) were used in the study. After 5–7 weeks the animals weighed 65–75 kg.

Skin graft chambers

Skin graft chambers were made from PTFE (Bioengineering Dept, MRC Clinical Research Centre). The chambers were 4 cm in diameter, 2 cm high and had a 1 cm flange at the base. Four pairs of holes, each 1 mm in diameter in the walls of the chambers, allowed for the passage of sutures (Fig. 1).

Anaesthesia

The animals were given a sedative mixture of xylazine 1 mg/kg (Rompun®, Bayer UK Ltd, Bury St. Edmunds, Suffolk) and ketamine 5 mg/kg (Vetalar®, Parke-Davis Veterinary Ltd, Pontypool, Gwent) by intramuscular injection. Anaesthesia was induced and maintained with 2–5% enflurane and 3–5 l/min of nitrous oxide and oxygen (50:50). Postoperative analgesia was provided with subcutaneous buprenorphine (Temgesic®, Laboratories RoC Ltd, Welwyn Garden City, Herts) at a dosage of 100–200 μg by subcutaneous injection. Topical lignocaine gel B.P. 2% (Biorex Laboratories Ltd, London) was applied to skin graft donor sites. Three hundred and

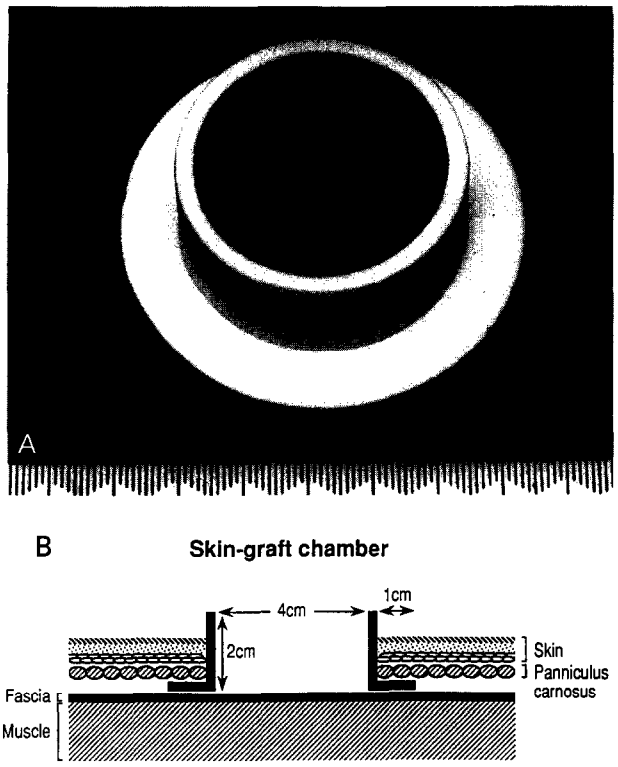

Fig. 1

Figure 1—(A) The PTFE skin graft chamber (scale in cm). (B) Illustration of the skin graft chamber, showing its dimensions and its position on the muscle fascia with the flange buried subcutaneously (suture not indicated).

twelve anaesthetic procedures have been successfully done in this way.

Surgery

Split-thickness skin was harvested from the anterior limb girdle region or the paravertebral area to prepare the cultured keratinocytes. In experiments where split-thickness skin was used to prepare dermal sheets,[12] the paravertebral area was the preferred donor site since the skin at this site is less mobile and it is technically easier to harvest a long, continuous sheet of skin.

Full-thickness wounds were used as the test bed for the skin substitutes. These were positioned overlying the rib cage, avoiding the limb girdles and the paravertebral areas (Fig. 2). The hair on the operative site was clipped and shaved and the skin was prepared with 0.5% chlorhexidine in spirit (DePuy Healthcare Ltd, Leeds) followed by a solution of isotonic saline. A maximum of four circular wounds, each 4 cm in diameter, were marked on each flank of the animal and full-thickness discs of skin, subcutaneous fat and panniculus carnosus were excised, exposing the muscle fascia of the external intercostal muscles. The skin edges were undermined so that the flange of the skin graft chambers could be buried subcutaneously. The chambers were placed on the muscle fascia and they were secured to the surrounding skin with 2/0 silk sutures (Fig. 1B). The wounds were grafted 48 h later. The semi-permeable dressing Surfasoft (Fermentech Ltd, Edinburgh) was used as the wound dressing throughout the study. A rigid lightweight jacket that

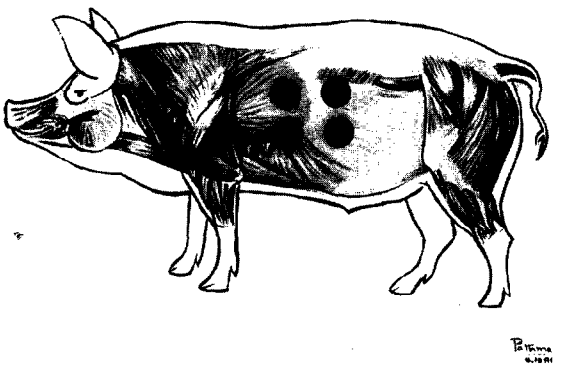

Fig. 2

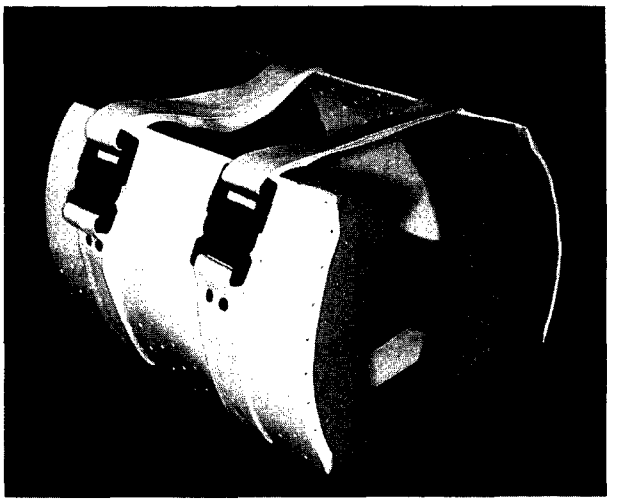

Fig. 3

Figure 2—The full-thickness wounds were positioned overlying the rib cage and avoiding the limb girdles. **Figure 3**—The rigid lightweight jacket put around the trunk of the animals to protect the wounds. (Scale 4 cm).

was made from Orthoplast (Johnson & Johnson, Ascot, Berks) and lined with foam (Fig. 3), was strapped around the trunk of the animals to protect the wounds.

Keratinocyte culture

Split-thickness skin was transported to the cell culture laboratory in Dulbecco's modified Eagles' medium (DMEM) containing 5% foetal calf serum and antibiotics (benzylpenicillin 600 units/ml, streptomycin 600 μg/ml, gentamicin 250 μg/ml and amphotericin B 1.25 μg/ml). The skin was washed in ethylenediaminetetraacetic acid (EDTA) (Sigma Ltd, Poole, Dorset), cut into 2–3 cm wide strips and incubated in 0.25% trypsin (Sigma Ltd, Poole, Dorset) for 2 h at 37°C to cleave the skin at the dermo-epidermal junction. The epidermis was then mechanically separated and a single epidermal cell suspension was obtained by gentle pipetting and filtration through a fine wire mesh.

A feeder layer of Swiss 3T3 mouse fibroblasts was irradiated with 6000 rads and seeded at a density of 2×10^6 cells per 75 cm^2 tissue culture flask (Falcon,

Table 1 Medium for porcine keratinocyte culture

*DMEM:Hams F12	3:1 ratio
*Foetal calf serum	10%
**Cholera toxin	1×10^{-10} M
**Epidermal growth factor	10 ng/ml
†Hydrocortisone	0.4 μg/ml
†Adenine	1.8×10^{-4} M
†Transferrin	5 μg/ml
†Insulin	5 μg/ml
‡Tri-iodothyronine	2×10^{-11} M

* Gibco Ltd, Uxbridge, Middx.
** ICN-Flow Ltd, High Wycombe, Bucks.
† Sigma Ltd, Poole, Dorset.
‡ Koch-Light Ltd, Haverhill, Suffolk.

Marathon Ltd, London). Each flask was then seeded with 2×10^7 keratinocytes. The culture medium was prepared as outlined in Table 1; the epidermal growth factor was added after 48 h. Colonies of keratinocytes were present after 3–4 days and grew to form stratified, confluent sheets of 6–8 layers of cells by 12–14 days.

Prior to grafting, the keratinocyte sheets were treated with Dispase® (Boehringer Mannheim, Lewes, Sussex) at 37°C for 1 h to separate them from the floor of the tissue culture flasks. Finally, intact sheets of cultured keratinocytes were lifted off with the aid of a Tricotex backing dressing (Smith & Nephew Ltd, Chessington, Surrey) and then transported to the operating theatre in DMEM.

Testing PTFE toxicity to keratinocytes

Since the skin graft chambers were made from PTFE, a cell proliferation assay was done to test if PTFE was toxic to cultured keratinocytes. Porcine keratinocytes were plated in "6 well" tissue culture plates at a density of 2×10^5 cells/ml. After 48 h when cell colonies were established, the cultures were divided into two groups. In one group, blocks of PTFE each $3 \times 3 \times 10$ mm were suspended on metal grids into the culture medium. The grids prevented the blocks of PTFE abrading the keratinocyte layer. The second group had no additives. After 2, 5 and 7 days cells were disaggregated by trypsin (0.125%) and then counted using a haemocytometer in triplicate. The experiment was repeated twice more using keratinocytes from different pigs.

Sampling of tissue

A comparison was made using light microscopy of 8 samples of pig skin with 8 samples of adult human skin taken from the thoracic region. Three skin samples from each species were processed for study by both transmission electron microscopy and immunohistochemistry.

In order to detect the possibility of epidermis migrating from the outer skin edge and passing beneath the chambers to the test area, large blocks of tissue that contained the wound site and the surrounding skin were taken for light microscopic analysis from studies where the chambers had been in place for up to 7 weeks.[12]

The appearances of both porcine cultured keratinocytes and human cultured keratinocytes that were grown under similar conditions[2] were compared using transmission electron microscopy.

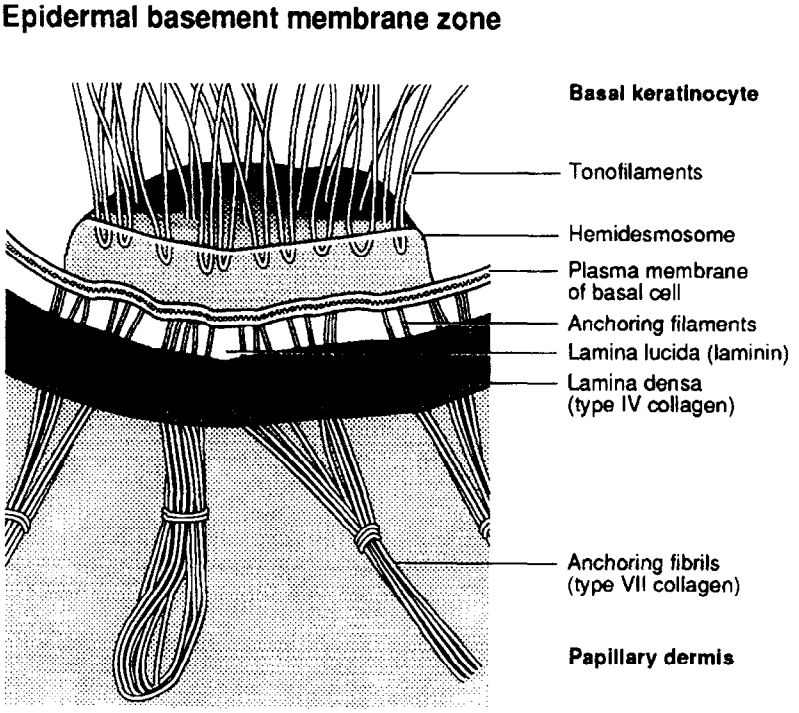

Fig. 4

Figure 4—Illustration of some of the ultrastructural features of the epidermal basement membrane (lamina lucida, lamina densa and anchoring fibrils) in the region of the hemidesmosome of the basal keratinocyte. The constituent basement membrane proteins that were detected by immunohistochemistry in this study are shown in parentheses. (Based on a painting by Geras AJ. Dermatology, a medical artist's interpretation. Switzerland: Sandoz Medical Publications, 1990: 38.)

Table 2 Antibodies used for epidermal basement membrane immunofluorescence

Primary anti-serum to	*Dilution of primary antiserum*	*Fluorescein conjugated secondary antibody*	*Dilution of secondary antibody*
*Type IV collagen (polyclonal rabbit)	1:50	†Swine anti-rabbit	1:20
*Laminin (polyclonal rabbit)	1:50	†Swine anti-rabbit	1:20
**Type VII collagen (LH7.2, monoclonal mouse)	Undiluted culture supernatant	†Rabbit anti-mouse	1:50

* Euro-path, Bude, Cornwall.
** Royal London Hospital, London.
† Dako, High Wycombe, Bucks.

Histological preparations

Tissue was fixed in 10% formal saline and embedded in paraffin. Sections were stained with both haematoxylin and eosin and also elastic van Gieson to demonstrate the collagen and elastin in the tissue.

Transmission electron microscopy

The appearance of the epidermal basement membrane zone (Fig. 4), the basal keratinocytes and the superficial dermis approximately 2–3 μm below the dermo-epidermal junction was studied using transmission electron microscopy. Tissue was fixed in 3% gluteraldehyde (in 0.1 M phosphate buffer at pH 7.4) and post-fixed in 1% osmium tetroxide followed by dehydration in a graded acetone series. Samples were embedded in Spurr resin and 50–80 nm ultrathin sections were stained with uranyl acetate and lead citrate. (All reagents were obtained from Agar Scientific Ltd, Stanstead, Essex).

Immunohistochemistry

Three of the structural proteins of the epidermal basement membrane zone,[13] type IV collagen, laminin and type VII collagen (Fig. 4) were detected using immunohistochemical methods. Tissue was oriented in cryogel and snap frozen in isopentane cooled in liquid nitrogen. Five μm cryostat sections were fixed in 100% acetone at 4°C for five minutes, air dried and then incubated overnight with the primary antibodies at room temperature in a humid chamber (Table 2). The sections were then stained with fluorescein conjugated secondary antibodies at room temperature for 1 h (Table 2). Finally the sections were counterstained to show cell nuclei using propidium iodide (Sigma Ltd, Poole, Dorset) for 4 min and mounted in glycerol.

Results

Biocompatibility of chambers

After 2–4 weeks, the skin edge retracted from the walls of the skin graft chambers and a sero-purulent exudate lined the intervening space. (A bacteriological analysis of wound swabs is given elsewhere).[12] After 5–7 weeks, approximately one third of all the chambers had marked erythema of the surrounding skin and partial or complete extrusion of the chambers. This adverse reaction was not dependent on the site of the wound, although in pilot studies when wounds were made along the paravertebral regions where the skin tension is higher than in the loose skin overlying the rib cage, extrusion of the chambers occurred much sooner.

PTFE toxicity to keratinocytes

The three sets of proliferation assays showed that the growth rate of the cultured keratinocytes in the presence of PTFE was identical to the controls (Fig. 5), indicating that PTFE was not toxic to keratinocytes in this *in vitro* system.

Histology

Using light microscopy, porcine skin (Fig. 6A) appeared similar to human skin (Fig. 6B) with a stratified epidermis overlying a moderately thick collagenous dermis and adnexal structures. However eccrine sweat glands were not present in the porcine skin and, whereas human dermis has an abundance of elastin fibres, in pig skin there were only a few short elastin fibres scattered in the dermis. In porcine skin, but not in human skin, there was an elastic membrane below the subdermal fat and an underlying panniculus carnosus.

Analysis of the wound sites where the chambers had been in place for up to 7 weeks[12] showed that there was no epidermal migration from the skin edge to the test area, except on those occasions where the chambers had become dislodged.

Transmission electron microscopy

The ultrastructure of porcine epidermis and superficial dermis appeared virtually identical to that of human

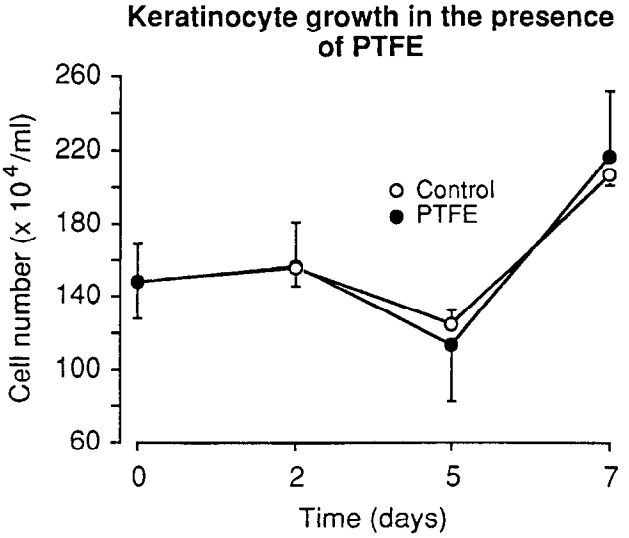

Fig. 5

Figure 5—Representative results from one of the cell proliferation assays of porcine keratinocytes, indicating there was no toxic effect from PTFE.

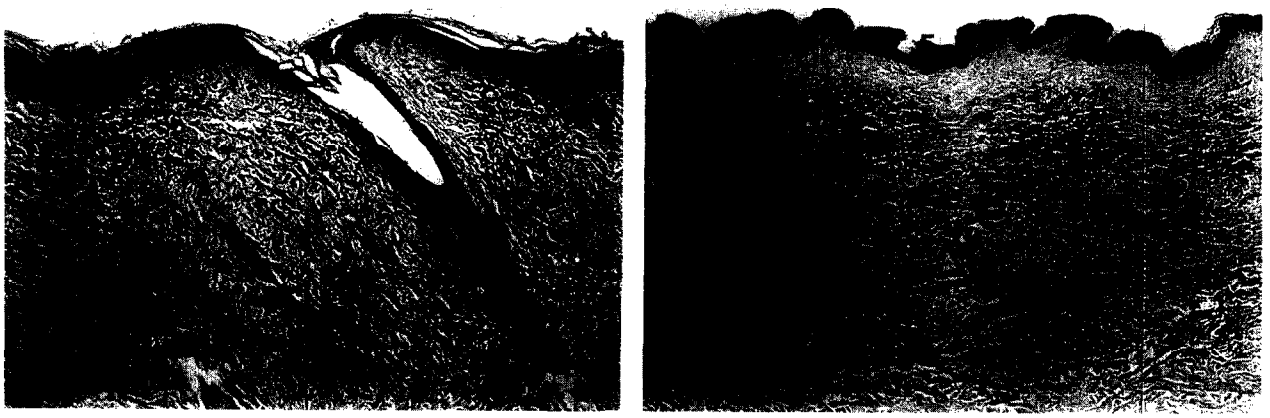

Fig. 6

Figure 6—(A) Porcine and (B) human thoracic skin sections, illustrating histological similarities in the appearance of the epidermis (E) and dermis (D). (H&E × 36).

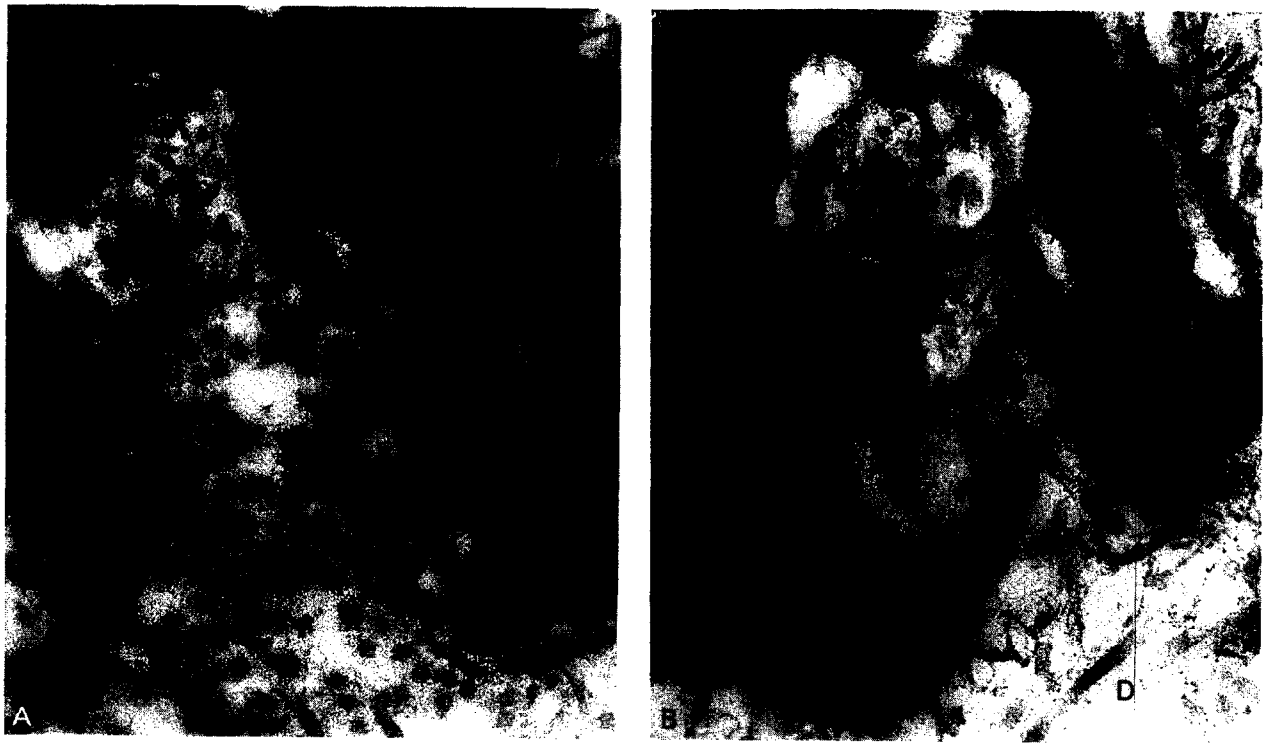

Fig. 7

Figure 7—(A) Porcine and (B) human skin dermo-epidermal junction seen by transmission electron microscopy, illustrating the virtually identical appearance of the epidermal basement membrane zone. Basal epidermal cell (E), papillary dermis (D), hemidesmosomes (h), lamina lucida (hollow arrow), lamina densa (dense arrow) and anchoring fibrils (a). (× 50000).

skin. In both species, the hemidesmosomes, lamina lucida, anchoring filaments, lamina densa and anchoring fibrils of the basement membrane zone were clearly seen. The anchoring fibrils were seen intertwined with dermal collagen fibres (Fig. 7). The only notable ultrastructural difference in the skin was that in the granular layer of the epidermis, keratohyalin was less conspicuous in pig skin as compared with human skin.

Comparison of porcine and human cultured keratinocytes showed that there is no detectable difference between the two species, with stratification of the keratinocytes, formation of desmosomes between adjacent cells and of hemidesmosomes at the bases of the cultures. Rudimentary basal lamina material was present subjacent to the hemidesmosomes.

Immunohistochemistry

The pattern and intensity of immunoreactivity to type IV collagen, laminin and type VII collagen was identical in both the porcine (Fig. 8A) and human (Fig. 8B) skin. There was a linear immunoreactivity

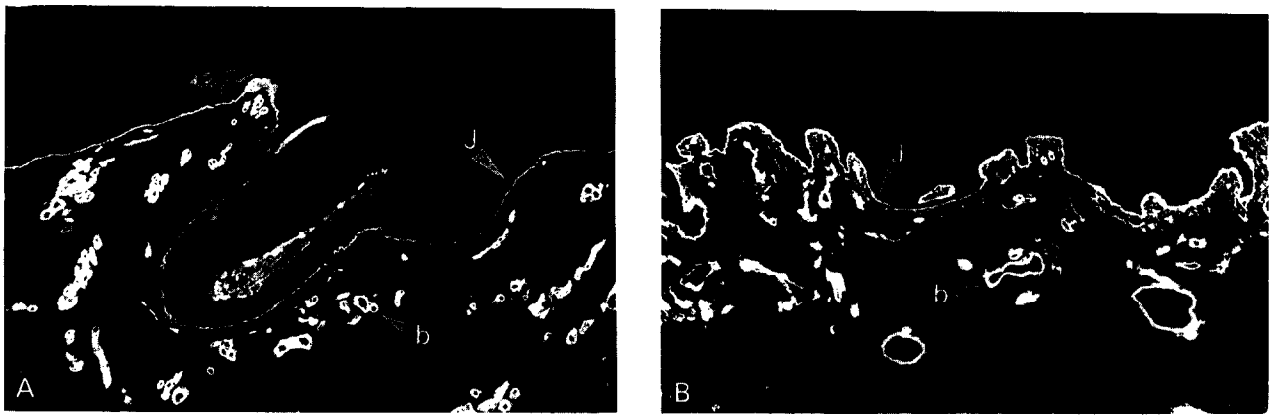

Fig. 8

Figure 8—(A) Porcine and (B) human skin showing the similar immunoreactivity to type IV collagen along the dermal-epidermal junction (J) and the blood vessels (b) in the dermis. (× 93).

along the dermal-epidermal junction to all three proteins, as well as perivascular immunoreactivity to both type IV collagen and laminin in the dermis.

Discussion

An animal model should parallel the clinical situation as closely as possible. In a comparative study of large and small laboratory animals, both the anatomy of porcine skin and the wound healing process in pigs appeared to be most similar to that in humans.[14] Nonetheless wound healing seems to be faster in pigs than in clinical situations[12] and on close histological scrutiny there are differences between the skin of both species. In both pig and human skin, the relative thickness of the epidermis and the dermis is similar, both containing a papillary dermal layer, epidermal rête ridges, sebaceous glands, apocrine sweat glands, subdermal fat, and a similar density of hairs. In contrast, porcine skin has an elastic membrane in the hypodermis and an underlying panniculus carnosus. Furthermore, in porcine skin the dermis is less vascular and, most significantly, there are no eccrine sweat glands.[15–17] Our results show that in pig dermis, there is a lower content of elastin than in human dermis. This observation differs from that of others,[16] but may be explained by a variation in the age or breed of animal under study. Ultrastructurally, the only distinguishing feature between either the epidermis or the cultured keratinocytes of both species, is the lack of keratohyalin in the granular layer of pig epidermis. As shown in this study and also by others,[18] there is cross-reactivity to pig skin of some antibodies raised against human basement membrane zone proteins; a convenient feature for studies on the basement membrane.

PTFE is a perfluorocarbon polymer compound that is a common alloplastic implant material due to its inert, non-adhesive and anti-friction properties.[19] It proved in this present study to be non-toxic to keratinocytes *in vitro* (Fig. 5). As it is also easy to machine it was selected for the manufacture of the skin graft chambers. Having created full-thickness wounds, cultured keratinocytes can be grafted in a number of different ways, such as in combination with a variety of dermal substrates. Although the wound healing environment within the skin graft chambers is artificial, the chambers were effective in preventing epidermal migration from the skin edge onto the test area. Cultured keratinocytes do not inhibit the contracture of full-thickness wounds and this fact alone can limit the duration of a study.[20] In this regard, the chambers had the added advantage of extending the period of study by inhibiting skin contracture. Another benefit of the chambers was that they protected the grafts from loss due to abrasion.

However bio-incompatibility of the chambers did limit the period of reliable study to 5–7 weeks since by this time a third of the chambers showed signs of extrusion. Studies elsewhere have identified the causes for the failure of percutaneous implants as marsupialisation, which is the formation of a sinus tract along the smooth surface of an implant; avulsion, due to a mechanical disruption of the interface between soft tissue and the implant; and infection.[21] The survival of the implants may be increased by using a material that encourages soft tissue integration; one option is porous PTFE[19,22] and another is a biocompatible metal. At present we are evaluating chambers made from niobium, a metal that is used for orthopaedic and dental implants.[23] An alternative strategy to the problem of chamber longevity may be to bury the entire implant beneath a cutaneous[24] or musculocutaneous plane. This may require a preliminary period of tissue expansion. What effect an abnormal environment such as this or any chamber system has on epidermal growth can only be determined by specific studies on epidermal differentiation.

Several precautions were taken to optimise the "take" of the cultured keratinocytes. The grafts are susceptible to loss from infection[25] and most topical antiseptic solutions are toxic to the cells.[26,27] Accordingly, we maintained a sterile theatre discipline for all the operative procedures and routinely rinsed off the chlorhexidine that was used for the skin preparation with a solution of isotonic saline. All wounds were grafted after a 48 h delay to decrease the risk of graft loss from haematoma or seroma. The epithelium that develops from cultured keratinocytes is initially fragile and dressing changes can be traumatic. Hence

we selected a semi-permeable dressing (Surfasoft), which when wet was non-adherent. Desiccation of dermis is thought to be a risk when dermis does not have a protective covering of epidermis and generally an occlusive dressing is the preferred option. However we did not detect any problems using the Surfasoft. Finally, the rigid jackets were essential in protecting the wounds[28] from the less than delicate, habitual desire of the animals to rub their wounds against the walls of their enclosures.

Disadvantages with pigs are their rapid growth rate and the subsequent difficulties in their restraint. In this respect, female pigs are less boisterous than male pigs and are easier to manage. In addition, animal husbandry is more involved and expensive when using pigs rather than small laboratory animals, a farm area is required, animal technicians and skilled animal anaesthetists are mandatory and there should be access to a veterinarian.

A significant proportion of a research tenure may be spent in establishing a model and the methods detailed in this paper are the product of much trial and error. Once the relative similarity of human and pig skin is appreciated, the model can be used to help answer specific clinical problems associated with cultured keratinocyte grafts. We believe that wound healing models such as this one are valuable to further our understanding of cultured keratinocyte grafts, so that we may define their precise therapeutic role in clinical practice.

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