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We hope that making available the relevant information on Pachyonychia Congenita will be a means of furthering research to find effective therapies and a cure for PC.
Re-expression of disease-characteristic features of non-bullous congenital ichthyosiform erythroderma (CIE) after grafting of the pathological keratinocyte cultures to athymic mice


Epidermal keratinocytes separated from skin lesions of non-bullous congenital ichthyosiform erythroderma were investigated in an attempt at experimental reproduction of this keratinization disorder. In vitro studies on growth and differentiation of pathological keratinocytes isolated from the influence of the host’s dermal and humoral components were performed using the immersed epidermal cell culture technique. Ten to 25-day-old confluent and stratified cultures were examined by means of photonic and electron microscopy, and stained with various differentiation markers for indirect immunofluorescence studies. The cultured epidermis showed low-grade differentiation and no clear-cut evenly distributed signs of the original disease. Grafting on congenitally athymic nude mice allowed further differentiation of the epidermal sheets and re-expression of the histologic and ultrastructural features of non-bullous congenital ichthyosiform erythroderma. Thus, the purely epidermal origin of this particular form of autosomal recessive ichthyosis could be confirmed. Large amounts of pathological keratinocytes generated from small skin biopsies may be used for experimental purposes after grafting on several athymic animals.

In vitro epidermal cell (EC) cultures represent an attractive model for studies of keratinocyte growth and differentiation in the absence of the host’s dermal and humoral influences. In their elegant experiments with dermal—epidermal recombinants, Briggaman and Wheeler (1) obtained evidence that lamellar ichthyosis is a skin disease of purely epidermal origin. We used keratinocytes from lesional skin of nonbullous congenital ichthyosiform erythroderma (CIE), another entity which has recently been individualized in the autosomal recessive ichthyosis group (2, 3), for reproduction of the pathological epidermis in vitro. If successful, EC cultures of pathological keratinocytes would represent an excellent source of the biological material for in vitro biochemical and pharmacological investigations.

In the second part of our study, congenitally athymic nude mice, which do not reject skin xenografts (4), were grafted with cultured human epidermis as described by Banks-Schlegel and Green (5). This in vivo experimental model allows for a better differen-
Fig. 1. Histopathology of a CIE skin biopsy used for experiments. Note hyperkeratosis, acanthosis, and slightly accentuated granular layer (paraffin embedding, H&E, × 250).

entiation of the human epidermis than the *in vitro* immersed keratinocyte cultures (6). We thus investigated the possibility of a long-term maintenance of pathological epidermis in a nonhuman living system.

**Material and methods**

**Skin specimens.** Lesional skin of CIE was taken from an axillary region of 2 unrelated girls (4 and 6 years old) who presented characteristic clinical and histological signs of this variety of autosomal recessive ichthyosis. Patients displayed a marked erythroderma and a generalized scaling (fine, small scales). The main histological features of the lesional epidermis were: 1) hyperkeratosis resulting in a compact, thick horny layer with a focal parakeratosis; 2) acanthosis; and 3) accentuated granular layer (Fig. 1). Three normal skin biopsies from healthy donors (plastic surgery) were used for the control experiments.

**Epidermal cell cultures.** The skin specimens of approximately 2 cm² were separated from most of the connective tissue with scissors and treated with 0.3% trypsin (Difco, USA) at 37°C for 60 min. The epidermis was then separated from the dermis and ECs were teased apart by gentle pipetting. Primary EC cultures were realized for every skin specimen according to the slightly modified method of Green, Kehinde and Thomas (7). ECs were seeded on 25 cm² plastic dishes (3 × 10⁴ cells per 1 cm²) containing a lethally irradiated 3T3 mouse fibroblast feeder layer and were cultured in an enriched 3:1 mixture of Dulbecco’s modified Eagle/Ham F-12 medium (Gibco, USA). The medium was supplemented with 10% fetal calf serum, 0.4 μg/ml hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin, 2 × 10⁻⁸ M triiodothyronine, 10⁻⁶ M cholera toxin (Sigma, USA), 8 × 10⁻⁴ M adenine, and 10 ng/ml epidermal growth factor (Sochibo, France). As an alternative, some cultures were grown on human collagen type III + I (Institute Merieux, France). Confluent primary cultures were dissociated with 0.1% trypsin / 0.02% EDTA (1:1) and multiple first passage cultures were established. In some of these secondary cultures, a 10% supplement of the culture medium with delipidized fetal calf serum (6) was substituted for the normal fetal calf serum in order to increase the culture’s terminal differentiation. Confluent and well stratified 10- to 24-day first passage cultures were detached from their support with Dishes II (Boehringer Mannheim, France) 1.2 U/ml in serum-free medium (1 h at 37°C), and used for further studies.

**Epidermal sheet grafts.** A total of 70 athymic nude Swiss mice (Ifa-Credo, France) were used in this study. Detached EC cultures were briefly rinsed with 0.9% saline and applied on a sterile vaseline gauze (with the basal layer facing the fascia) to the graft beds prepared by the whole thickness skin excision (2 cm²) on flanks of the mice. The grafts were either fixed with Biender surgical tape (3M, USA) and slightly compressed with a dry gauze tampon and an adhesive cloth band-aid or covered with plaster rounding sequent c formed u Nembuta in aseptic on Days of the gra with a m. A thin α graft and fixed for in for 2 h (Bouin's indic with spc were incl Tissue fluorescence were pured epi sheets to nufluorescence Briefly, min with twice fo

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**Table 1. Antibodies used in the present study.**

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<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Dilution</th>
<th>Conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td>KL 1 murine monoclonal Ab (Immunotech, France)</td>
<td>56.5KDa acidic keratin; suprabasal keratinocytes</td>
<td>1:50</td>
<td>goat anti-mouse (Fab’)2 FITC (Zymed, USA) dilution 1:50</td>
</tr>
<tr>
<td>KM48 murine monoclonal Ab (J. Thivolet, Lyon, France)</td>
<td>desmosome- and differentiation-related antigen; keratinocyte membranes</td>
<td>1:100</td>
<td></td>
</tr>
<tr>
<td>GP37 guinea pig polyclonal antibody (J. Thivolet, Lyon, France)</td>
<td>37KDa cytoplasmic glycoprotein; granular layer keratinocytes</td>
<td>1:100</td>
<td>rabbit anti-guinea pig IgG FITC (Nordic, Holland) dilution 1:50</td>
</tr>
<tr>
<td>anti-involucrin rabbit polyclonal antibody (H. Green, Boston, USA)</td>
<td>human keratinocyte cross-linking envelope; maximum in granular layer</td>
<td>1:200</td>
<td>goat anti-rabbit IgG FITC (Nordic, Holland) dilution 1:50</td>
</tr>
<tr>
<td>anti-H2 rat monoclonal Ab (Hybrilab, USA)</td>
<td>murine H-2 monotypic antigen (M1/42); cell membranes</td>
<td>1:10</td>
<td>rabbit anti-rat FITC IgG (Zymed, USA) dilution 1:50</td>
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</tbody>
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with plastic capsules separating them from the surrounding mouse skin. The grafting and the subsequent change of the dressing on Day 7 were performed under an intraperitoneal anesthesia with Nembutal (Abbott Labs., 50 mg/kg body weight), in aseptic conditions. The grafted mice were killed on Days 10, 15, 20 and 30. The overall appearance of the grafted site was noted, the tissue was excised with a mouse skin margin and divided into 3 parts. A thin central band comprising the center of the graft and the 2 parts of marginal mouse skin was fixed for standard electron microscopy. The remaining 2 side-parts were used for routine histology (Bouin’s fixative) and for immunofluorescence studies (snap-freezing in liquid nitrogen). Three animals with spontaneously healing non-grafted wounds were included in the study for control purposes.

Tissue examinations. Routine histology, immunofluorescence, and standard electron microscopy were performed on all the initial skin biopsies, cultured epidermis, and after grafting of the epidermal sheets to mice. Immunoreagents used in the immunofluorescence studies are detailed in Table 1. Briefly, 4.5 µm frozen sections were incubated 45 min with a primary antibody, washed with PBS (twice for 10 min), and reacted with an appropriate conjugate for 30 min. After a final 30 min wash in PBS, the sections were mounted in buffered glycerin and examined on a Zeiss fluorescence microscope. Tissue fragments intended for standard electron microscopy were fixed with 2% glutaraldehyde in sodium cacodylate buffer, washed, postfixed in 1% osmium tetroxide, dehydrated in ethanol, and embedded in epon. Ultrathin sections were contrasted with uranyl acetate and lead citrate.

Results

The confluent secondary EC cultures of CIE keratinocytes did not differ from the normal keratinocyte cultures of the same age under light microscope (phase contrast) examination. In the normal medium, they were 7 to 10 layers thick and composed of keratinocytes that were polygonal at the bottom layers to become flat at the culture surface. The cultures grown in lipid-free medium were approximately twice as thin because of the precocious and increased flattening of keratinocytes beginning already at the basal layer. Histological examinations did not disclose any specific differences between the pathological and normal EC sheets used for grafting, regardless of the level of the culture differ-
Fig. 3. Indirect immunofluorescence with anti-involucrin rabbit anti-human antibody; a) suprabasal labelling of a 15-day CIE keratinocyte culture: peripheral cytoplasmic pattern of staining with the maximum of intensity at the culture surface (× 500); b) a 10-day graft of the CIE keratinocyte culture to a nude mouse. Several involucrine layers in the grafted epidermis (right) show positive peripheral staining. The animal epithelium (left) remains completely negative (× 250). Broken lines outline the upper and lower limits of epidermis.

entiation (normal or delipidized medium). A closer, ultrastructural study revealed a focal occurrence of peculiar aspects resembling the original in vivo lesions: the characteristic superposition of well adhering cornocytes (Fig. 2). This was an uncommon event, unlikely to occur in the immersed EC cultures which rapidly shed cornified cells into the culture medium. There was no difference between the cultures grown on collagen and on 3T3 feeder layer.

All the immersed epidermal cell cultures of normal and pathological origin gave the same results when tested with differentiation-related antibodies. They were KM48- and GP37-negative, whereas in the initial CIE biopsies KM48 labelled the spinous layer and GP37 bound to the thicker than normal granular layer. The KL1 monoclonal antibody uniformly stained all but the basal cells. The anti-involucrin polyclonal antibody labelled the suprabasal keratinocyte layers with the maximum of immunofluorescence at the culture surface (Fig. 3a), as was also the case in the lesional skin.

The anti-involucrin antibody, which was human-specific and positive on cultured epidermal sheets, was chosen as a marker of the grafted tissue and used for confirmation of the graft survival (Fig. 3b). The graft receiver’s cells were traced using an antibody to H-2 mouse histocompatibility antigen. No histologic or clinical signs of the immune graft rejection were observed in any of the 67 grafts performed. The failures were due to the premature loss of the wound dressing and to the insufficient adherence of the dressing whereby the epidermal graft slipped off the graft bed. The rest of the grafted normal and pathological epidermal sheets survived at least until day 10 after grafting, as confirmed by the anti-involucrin and anti-H-2 labelling. At that time the grafted normal epithelium became thicker and developed clearly distinguishable granular and squamous cell layers. The grafts re-acquired the KM48- and GP37-specific antigen expression characteristic of a normal epidermis. Later on, considerable retraction of the graft sites not protected with plastic caps was observed: on Day 20, the surface of the grafts was reduced on average by 75%, and most human keratinocytes in the remaining part were replaced by mouse epidermal cells migrating along the reconstituted basement membrane (immunofluorescence findings). The capsule-protected epidermal graft the expres and histol prevented grafted e Macroscoq observed 4, were si excessive, could not easily det CIE grafti strauctu order. Tyf cornified l of the nori CIE epid prominence ues of ker
CIE re-expression by grafted keratinocytes

Fig. 6. Ultrastructural comparison between CIE and normal EC cultures 10 days after grafting to nude mice (× 3,000; bar = 2 μm); a) upper part of an acanthotic CIE epidermis showing characteristic corneocyte superposition and increased keratohyalin expression (arrows) also observed in the initial biopsy; b) neither similar hyperkeratosis nor acanthosis can be observed in normal EC graft of the same age. Keratohyalin granules are small and infrequent. SG = stratum granulosum; SC = stratum corneum; BM = basement membrane.

dermal grafts of pathological origin were studied for the expression of the disease-characteristic clinical and histologic features up to Day 30. The plastic cap prevented any intermingling and colonization of the grafted epidermis with the host keratinocytes. Macroscopic aspects of the grafted epidermis, as observed at the time of removal of the dressing (Fig. 4), were similar in the normal and CIE grafts. The excessive, ichthyosis-like scaling of the graft surface could not be observed but a thick horny layer was easily detached with the occlusive dressing. The CIE grafts expressed histological (Fig. 5) and ultrastructural (Fig. 6) features of the original disorder. Typical for CIE lamellar superposition of the cornified keratinocytes could not be observed in any of the normal grafts. As early as Day 10, the grafted CIE epidermis was acanthotic and displayed a prominent granular layer. Characteristic large granules of keratohyalin were observed ultrastructurally in CIE grafts but not in the grafts of normal keratinocyte cultures nor in the murine epidermis.

Discussion

Previous studies on the expression of epidermal pathology in vitro using keratinocyte cultures have been performed in psoriasis and ichthyosis vulgaris. Fleckman et al. (9) have demonstrated the persistence of biochemical and structural characteristics of this latter disease in vitro. Baden, Kubilius, and MacDonald (10) as well as Liu and Parson (11) failed to reproduce the stigmates of psoriasis in EC culture. This may be not very surprising since psoriasis is at present considered as a systemic disease with a sizeable immunological background (12, 13). Moreover, the immersed EC cultures are known to be relatively low-differentiated (6, 14) and may not
attain the degree of differentiation necessary for the expression of recognizable pathological traits. Indeed, there was no clear-cut reproduction of the ichthyosis in our immersed EC cultures. The ultrastructural evidence of an increased cornocyte cohesion was infrequent and occurred in better stratified cultures from lipid-free medium only. Nevertheless, it seems that the ichthyosis had to be coded in the cultured CIE keratinocytes since it could be re-expressed after grafting of the cultured epidermis to athymic mice. To our knowledge, this is the first report describing such a re-production of a skin disease in the favorable conditions of differentiation after a latent period of non-expression in vitro.

Indirect immunofluorescence study confirmed the low-grade differentiation of EC cultures which failed to express a desmosome- and differentiation-related keratinocyte membrane antigen KM48 (15), and GP37 glycoprotein normally found in the stratum granulosum cells (16). Both differentiation antigens were found re-expressed together with histological and ultrastructural evidence of CIE in the grafted epidermis.

It is known that vitamin A is a factor that inhibits keratinocyte differentiation both in vitro and in vivo. Application of delipidized medium eliminates any influence of the lipid-soluble vitamin A on cultured cells. We used this procedure in an attempt to increase the degree of differentiation of some of the cultures studied. Retinoids, the vitamin A derivatives which proved to be efficient in the management of several keratinization disorders, have been demonstrated to reduce epidermal differentiation (17). In the context of non-expression of lamellar ichthyosis in insufficiently differentiated cultures, it is tempting to think that the beneficial effect of retinoid treatment in vivo is due to its inhibitory action on the process of epidermal differentiation.

The model of epidermal cell culture and grafting of epidermal sheets to athymic mice may be used for studies on the origins and pathological mechanisms of several other skin diseases. Since this experimental method allows in vitro multiplication of the biological material obtained from small skin biopsies, it affords also the possibility of studies on pharmacological treatments of purely epidermal dermatoses after grafting of the pathological cultures on several animals.

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References