In Vitro Pemphigus Vulgaris Model Using Organotypic Cultures of Human Epidermal Keratinocytes

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Using the Combi-ring-dish (CRD), a new culture device, organotypic cultures of human epidermal keratinocytes were grown on bovine eye lens capsules. In these highly differentiated cultures, typical suprabasal acantholysis was induced by pemphigus vulgaris antibodies. This in vitro pemphigus vulgaris model may be used to analyse keratinocyte-derived factors causing acantholysis in experimental pemphigus. J Invest Dermatol 93:263–267, 1989

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kin organ cultures and submerged keratinocyte cultures have been used as in vitro models of pemphigus [reviewed in Ref 1]. Skin organ culture is simple and elicits keratinocyte differentiation quite similar to that seen in vivo. Acantholysis is easily documented by light and electron microscopy and immunofluorescence. Skin organ cultures, however, contain other viable cell types, such as fibroblasts and endothelia, as potential sources of error in the interpretation of keratinocyte-derived factors leading to acantholysis. In contrast, submerged keratinocyte subcultures grown without a feeder layer do not contain relevant amounts of other cell types. The differentiation of these cultures, however, is usually low and does not correspond to the situation in vivo [2]. Furthermore, acantholysis can be documented only indirectly by considering the nonspecific phenomenon of dissociated cell detachment from the culture plate as “acantholysis equivalent.” Unequivocal documentation of acantholysis assumes increasing importance in light of the recent report of “spontaneous” blistering in keratinocyte cultures from normal human epidermis grown under standard conditions [3]. The cleavage in such blisters occurs at the cell–substrate interface; no features of acantholysis are demonstrable.

We developed an in vitro pemphigus vulgaris model using organotypic cultures of human epidermal keratinocytes. This model permits morphologic assessment of acantholysis in a single type cell system.

MATERIALS AND METHODS

Materials Adenine was purchased from Boehringer-Mannheim (Rotkreuz, Switzerland), bacto-trypsin from Difco (Detroit, MI), choleratoxin, epidermal growth factor, hydrocortisone, insulin, and mitomycin C from Sigma (St. Louis, MO), Dulbecco’s modified Eagle’s medium, Eagle’s minimal essential medium with Earle’s salts, and Ham’s F 12 medium from Fakola (Basel, Switzerland), Embed 812 and osmium tetroxide from Balzers (Balzers, Fürstenstein Lichtenstein), FCS from Nabi (Basel, Switzerland), glutaraldehyde, sodium cacodylate, and uranyl acetate from Elinis (Fort Washington, PA), penicillin from Hoechst (Frankfurt, F.R.G.), and methylene blue and triiodothyronine from Fluka (Buchs, Switzerland).

Hank’s balanced salt solution and PBS were prepared in our laboratories. Lead citrate was used according to Reynolds. Fluorescein-labeled antihuman immunoglobulins were prepared in rabbits in our laboratories (molar ratio of fluorescein to protein, 3.5). Monospecific antibodies against fibronectin were a kind gift from Dr. J.-J. Morgenthaler [4].

Organotypic Cultivation of Human Keratinocytes Human keratinocytes were isolated from surgical specimens of skin by trypsinization (0.25% trypsin in Hank’s balanced salt solution either overnight at 4°C or for 1.5 h at 37°C) and cultivated as primary cultures following a modification of the methods of Rheinwald and Green [5]. 3T3 cells (CCL 92; American Type Culture Collection, Rockville, MD) treated with mitomycin C (4 μg/ml for 4 h at 37°C) or, in later experiments, irritated human dermal fibroblasts (single dose of 7000 cGy using a Dermopan 2 Siemens, 50 kV, 1.0-mm Al filter, distance 5 cm) (J Invest Dermatol, in press) served as a feeder layer. The feeder fibroblasts were plated at a density of 4–5 × 10⁴/cm². The culture medium consisted of three parts Dulbecco’s modified Eagle’s medium and one part Ham’s F 12. The medium was supplemented with 10% fetal calf serum (FCS), 0.4 μg/ml hydrocortisone, 5 μg/ml insulin, 0.135 mM adenine, 2 mM triiodothyronine, 0.1 mM choleratoxin, 10 ng/ml epidermal growth factor, and 100 U/ml penicillin. The cultures were incubated at 37°C in a humid atmosphere with 5% CO₂.

For organotypic cultivation, primary keratinocyte cultures were trypsinized with 0.1% trypsin in phosphate-buffered saline (PBS) free of Ca²⁺ and Mg²⁺ (pH 7.2). Keratinocytes, 1 × 10⁵, were plated as a first subculture on top of bovine eye lens capsules (Fig 1A) mounted in B/C-units of the Combi-ring-dish (CRD) system (Fig

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Abbreviations:
cGy: centi gray
CRD: Combi-ring-dish
FCS: fetal calf serum
PBS: phosphate-buffered saline

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1A). The lens capsules were freshly prepared according to Vermorken and Bloemendal [6]; their thicker anterior parts were then trypsinized with 0.25% trypsin in PBS free of Ca++ and Mg++ (pH 7.2) to remove adhering lens cells, and sterilized by irradiation with a single dose of 10,000 cGy (Dermopan 2 Siemens, 50 kV, 1.0-mm Al filter, distance 5 cm). They could be stored for up to several months at 4°C in Eagle’s minimal essential medium containing 10% FCS and 100 U/ml penicillin. The CRD system is described in detail by Noser and Limat [7]; it consists of two sets of four concentric Teflon rings (A,B,C,D = large; a,b,c,d = small), offering different sizes of culture surface when combined. The lens capsules were mounted expanded on perforated and notched, ethanol-sterilized polypropylene foils (OPP 60, Tecnomara, Zürich, Switzerland) (Fig 1A,B,C) [7]. The central part of the foil was removed with fine tweezers before starting the cultures. Culture media and incubation conditions were the same as for the primary keratinocyte cultures. The keratinocytes were allowed to grow submerged for 4 to 7 d until confluent. Then the culture device was lifted on a Teflon grid (Thomapor, Reichelt Chemietechnik, Heidelberg, F.R.G.) with culture medium added only to the level of the lens capsule (Fig 1D). Lifted cultures were incubated for up to 3 wks.

Morphologic Evaluation of Organotypic Cultures For histologic examination the whole B/C-unit of the CRD system was immersed in 5% glutaraldehyde in sodium cacodylate buffer for at least 3 h. Then the lens capsule with the cultured keratinocytes on top was cut out of the CRD with an 8-mm metal stamp. The specimen was postfixed in osmium tetroxide, dehydrated in graded alcohols, and embedded in Embed 812 (standard techniques). Semithin sections of 1-μm thickness were stained with methylene blue. For electron microscopic examination thin sections were double-stained with uranyl acetate and lead citrate (standard techniques).

Immunofluorescence Direct and indirect immunofluorescence was performed on 7-μm cryostat sections. For cryostat sectioning the whole B/C-unit of the CRD system was frozen. Then the lens capsule with the cultured keratinocytes on top was cut out of the CRD as mentioned above. Further processing of the specimen was according to standard techniques.

Acantholysis Experiments In most experiments, pemphigus antibodies consisted of 200 μl of plasma or a 5-mg/ml ammonium
sulfate (45%) IgG fraction thereof from a patient with severe pemphigus vulgaris. Twelve liters of plasma had been withdrawn from this patient with plasmapheresis. The antibody titer in indirect immunofluorescence varied from 1:256 to 1:64 on human skin. Anticoagulation during plasmapheresis had been performed with 1000 units heparin intravenously. The patient also received prednisone (100 mg/day) and azathioprine (200 mg/day). The plasma and its IgG fraction had induced typical suprabasal acantholysis in more than 95% of skin explants from different donors in various series of experiments in the skin-organ-culture model of pemphigus [4,8,9].

In the present experiments, the 200-μl samples were added for 24 or 48 h to the inner compartment of the CRD system (Fig 1C,D) in lifted cultures. Before use, the ammonium sulfate IgG fraction had been dissolved in and exhaustively dialyzed against PBS. The antibodies were not purified further.

In further experiments, plasma samples from two other patients with active pemphigus vulgaris (antibody titers in indirect immunofluorescence on human skin of 1:256 and 1:64, respectively) were tested in the same way. Previously, both samples had been found to induce typical suprabasal acantholysis in the skin-organ-culture model of pemphigus.

Control experiments were conducted with 200 μl of a 10-mg/ml IgG preparation (Sandoglobulin, see Ref 9) and 200 μl of plasma from a patient with active bullous pemphigoid (antibody titer of 1:512 on human skin).

RESULTS

Characterization of Organotypic Culture of Human Epidermal Keratinocytes A culture surface of 78 mm² is obtained with the B/C-units of the CRD system. Confluency of keratinocytes was reached in such culture devices after 4 to 7 d of submerged incubation, resulting in monolayers of polygonal cells 5 to 10 μm in average diameter. When lifted to the air–medium interface during the following 2 to 3 wks of incubation, the cultures developed into multilayered sheets showing some retraction from the borders of the CRD. In phase-contrast microscopy, these cultures assumed a slightly brownish color, transparency was diminished, and several cell layers could be focused on. Semithin sections showed a stratified, epidermislke organization with a basal, up to 5 intermediate, and several cornified cell layers (Fig 2). The average thickness was 0.05 mm and varied from area to area, as apparent in phase-contrast microscopy. The basal cells were of columnar shape and measured 5 to 10 μm. The overlying intermediate cells were slightly larger and polygonal. They formed a mosaic with a progressively flattened cell shape, the long axis of which was usually parallel to the culture surface. Especially in the intermediate layers, the intercellular spaces were traversed by well recognizable intercellular bridges (Fig 2). The cornified cell layers were built up of mostly anuclear, extremely flattened, and densely packed cells.

Pemphigoid antigen and fibronectin could not be identified in the basal membrane zone by immunofluorescence techniques using pemphigoid plasma and fibronectin antibodies (see Materials and Methods). Because bovine eye lens capsules consist mostly of type IV collagen, this component of the basal membrane zone was not looked at.

Electron microscopy documented the following signs of differentiation: cuboidal basal cells with abundant ribosomes and polyribosomes and some lipid vacuoles were organized in a single layer. Clear-cut formation of hemidesmosomes joining the bovine eye lens capsule was not demonstrated. In the intermediate layers, the flattened cells showed increased amounts of keratin filaments associated with the numerous desmosomes. The cells were densely packed with moderate interdigitation. They contained the organelles typically associated with cellular metabolism, i.e., mitochondria and the Golgi apparatus, as well as glycogen granula. Desmosomes often exhibited the intercellular dense line (Fig 3). Keratohyaline granules and lamellar bodies were only rarely detected in suprabasal cells, no true granular layer was formed. Upper layer cells were tightly packed. They showed cornified cell envelopes, usually complete nuclear destruction, and sparse organelles with degenerative changes.

Acantholysis in the Organotypic Culture System During some experiments, bullae were already detected by phase-contrast microscopy. After 24 and 48 h incubation with the three different pemphigus vulgaris antibodies, light microscopy of organotypic cultures showed areas of widened intercellular spaces leading to suprabasal separation (Fig 4). Single cells in widened intercellular spaces appeared rounded. The basal cells remained attached to the bovine eye lens capsule, forming the "tombstone row" familiar in pemphigus vulgaris lesions. In some specimens, only this tombstone row was left, especially after 48 h incubation (Fig 5).

Electron microscopy (Fig 6) also demonstrated widened intercellular spaces with dissolution of the intercellular cement substance and microvilli formation by the keratinocytes. Desmosomes were rarified and tonofilaments were retracted to the perinuclear area, losing contact to the attachment plaques of the desmosomes.

Figure 2. Morphologic evaluation of organotypic cultures. Stratified organotypic keratinocyte culture forming a basal and several intermediate and cornified cell layers (methylene blue, X150). * = bovine eye lens capsule.

Figure 3. Morphologic evaluation of organotypic cultures. Complete desmosomes in intermediate cell layers of an organotypic keratinocyte culture showing the intercellular dense line (reduced from X85,000).
Direct immunofluorescence performed on organotypic cultures after 24 h incubation with the pemphigus vulgaris antibodies revealed the typical honey-comb pattern of intercellular antibody binding in the intermediate layers.

In the control experiments with purified IgG from healthy donors or pemphigoid plasma, no signs of acantholysis could be detected by light or electron microscopy.

DISCUSSION

Pemphigus acantholysis is believed to be caused by intraepithelial activation of protease systems induced by binding of the pemphigus autoantibodies to the antigen on keratinocytes [reviewed in Ref 1]. A series of experiments aiming to inhibit acantholysis in pemphigus models by adding various protease inhibitors has produced in part controversial results [reviewed in Refs 9 and 10]. Protease activation in correlation with the time-course of pemphigus acantholysis is less well documented [11,11]. Such experiments are hampered by the disadvantages of the presently available in vitro pemphigus models. The problems relate to documentation of acantholysis in submerged keratinocyte cultures, and to the possibility that in skin organ cultures cell types other than keratinocytes might contribute to protease activation. Further problems include difficulties in repeated selective sampling of supernatants and dilution of secreted culture products by culture media.

We present organotypic cultures of human epidermal keratinocytes in a new culture device, the Combi-ring-dish [7]. Used as a pemphigus vulgaris model, they combine the advantages of earlier systems, i.e., well differentiated keratinocytes as the sole cell type and clear documentation of antibody-induced acantholysis by direct immunofluorescence and light and electron microscopy.

The supporting membrane mounted in the CRD is an important part of this model. Bovine eye lens capsules [6] can be sterilized and rendered free of viable cells by irradiation, and enhance keratinocyte growth in the absence of a feeder layer. The lens capsules allow feeding of the cultures by diffusion from below during lifted incubation, and remain stable supports for several weeks. They are transparent, making phase-contrast microscopic control and documentation during incubation and experiments possible. In histologic processing we had problems with unfixed specimens rolling up, even after several weeks of incubation, and with the keratinocyte layers splitting off during sectioning of paraffin-embedded specimens. These problems stemmed from the rigidity of the lens capsules. The problems were avoided by fixation or freezing of the whole CRD before cutting out the lens capsule, and by embedding in Embed 812.

The CRD containing the keratinocyte culture is easily and repeatedly transferable from one culture dish to another. Thus, initial incubation can be carried out on different substrates such as feeder layers or dermis equivalents. These might enhance growth and differentiation [7], but can be removed before starting the experiments. Culture medium ingredients, such as hydrocortisone, which might inhibit acantholysis [reviewed in Ref 8], or FCS, which might interfere with protease analysis, are easily omitted before starting an experiment. Furthermore, substances, such as protease inhibitors or complement components, or even cellular elements, such as leuko-
cytes, may be repeatedly added during an experiment. By using the small inner compartment of the CRD (Fig 1C,D), supernatant solutions chosen for protease analysis can be sampled repeatedly, and substances such as those mentioned above can be added during an experiment, keeping dilution by the culture medium to a minimum.

All strains of organotypically cultured keratinocytes from different donors showed signs of acantholysis in the course of 24 to 48 h incubation with the pemphigus antibodies used, but they exhibited a varying sensitivity and time-course. We have reported this phenomenon for different skin samples in the skin-organ-culture model of pemphigus [8]. Therefore, pemphigus antibody preparations should be controlled in the skin-organ-culture model, preferably using skin explants from the donor of the cultured keratinocytes, before starting experiments with the new model system.

In the model presented here, protease activation in an experiment using protease-free preparations of pemphigus antibodies can result only from the target of these antibodies, the keratinocytes. It should be possible to study the correlation of protease activation with the time-course of acantholysis. The use of a defined pemphigus antibody with a single keratinocyte cell strain should eliminate the problems of donor-dependent varying sensitivity to acantholysis [8], and thus allow to develop exact dose-response and time-course studies.

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REFERENCES


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The meeting will be held in New York, New York on September 1 – 2, 1989. For further information, please contact: Nicholas A. Soter, M.D., Department of Dermatology, New York University Medical Center, 562 First Avenue, New York, NY 10016