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Secretion of Type IV Collagen by Keratinocytes of Human Adult

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Secretion of type IV collagen by keratinocytes was studied by anti-type IV collagen immunoglobulin in pure keratinocyte culture of the human adult. The cultivated keratinocytes secreted type IV collagen into the space between the cell growth and the underlying fixed film of human skin collagen type I. The secreted collagen was accumulated on the cell surface to form junction structures.

Type IV collagen is the collagenous component of basal lamina in various tissues [1]. A previous paper disclosed type IV collagen in subepidermal basal lamina [2]. Keratinocytes form junction structures anchoring the epidermis to the dermis [3–5]. Melanocytes may also be capable of producing basal lamina, since pigment cells of nevi are surrounded by basal lamina [6]. This study intends to demonstrate production of type IV collagen by keratinocytes of the human adult.

MATERIALS AND METHODS

Pure Cultures of Keratinocytes of the Human Adult

Normal skin of adults was obtained under operation in the Department of Plastic Surgery of this hospital. Keratinocyte cultures on a film of human skin collagen type I were prepared by the routine technique of these laboratories.

1. Sheets of epidermis were separated from skin specimens using clostridial collagenase 2 mg/ml complete culture medium shaken for 1½ h at 36°C. The epidermis was then peeled by a forceps [7].

2. Epidermal cells were dispersed by a dithioerythritol solution 3 mg/ml Hanks' balanced salt solution, pH 7.6, for 7 min at 37°C, followed by a quick treatment with 0.25% trypsin (Difco), pH 7.3, during vigorous shaking for 3 min [8].

3. Viable keratinocytes in the suspension were separated from dendritic cells and keratinized cells by differential centrifugation in Percoll, first in a continuous and, thereafter, a discontinuous gradient for 25 min at 800 *g* each. Viable keratinocytes have a density of 1.087 g/ml [9].

4. A fixed film of human skin collagen type I was prepared in Falcon flasks. The type I collagen solution, 1 mg/ml 0.5 M acetic acid, was placed in a thin layer on the flask bottom and hardened in ammonia vapor for 3 days. The collagen film was then fixed with a 1% glutaraldehyde solution of distilled water for 20 min, washed, and the flasks filled with distilled water were kept in a refrigerator until use. The flasks with collagen film were washed with Hanks' balanced salt solution for 10 min before use.

Culture medium contains 10% fetal calf serum, 5 mg streptomycin, 10,000 units penicillin, and 2 ml of 200 mM L-glutamin in 100 ml of Eagle's minimum essential medium with Earle's salt (GIBCO). pH was adjusted at 7.3 by 0.5 M HEPES buffer. Cultivation was carried out at 35°C. Ninety-nine percent of the implanted cells settled down on the collagen film and grew in membrane within 12 h.

Type IV Collagen and Its Anti-immunoglobulins

Type IV collagen was extracted from porcine kidney by acid pepsin digestion and precipitated by 1 M NaCl at pH 7.4. The precipitate was dissolved in 0.5 M acetic acid, dialyzed against 0.05 M Tris-HCl buffer, pH 7.4, and precipitated again by 1 M NaCl. The precipitate was then purified by DEAE-cellulose and CM-cellulose filtration [10]. The collagen was identified by polyacrylamide gel electrophoresis and amino acid analysis [10]. Rabbits were immunized with type IV collagen mixed with Freund's complete adjuvant 1:1. Anti-type IV collagen immunoglobulins were precipitated from antisera by ammonium sulfate at 30–50% saturation and dissolved in phosphate buffer 0.5 M, pH 7.4, with 0.001 M sodium azide. The immunoglobulins were characterized by the enzyme-linked immunosorbent assay (ELISA) technique to ensure the antibody's specific activity and the titer for the antigen. They did not react with human serum fibronectin (Sigma) and laminin from rat yolk sac tumor (kindly provided by Drs. U. Wewer and R. Albrechtsen, Institute of Pathological Anatomy of the Rigshospital) [11]. The immunoglobulins were demonstrated by positive staining of human dermal-epidermal junction at 4000–5000 times dilution. Control sera were prepared with the solvent of the collagen solution and Freund's complete adjuvant mixture 1:1. Immunoglobulins were isolated and kept in a similar way as the anti-type IV collagen immunoglobulins.

Immunohistochemical Stain of Type IV Collagen for the Keratinocyte Cultures

The membranous growth of keratinocytes was fixed at start, hours 2, 8, and 24, and days 2, 7, 10, and 14 of culture in 2% paraformaldehyde solution in 0.05 M phosphate buffer, pH 7.3, with 7.5% sucrose and lysine–periodic acid–paraformaldehyde solution [12] for 30 min at 4°C and washed in phosphate buffer 0.1 M, pH 7.3, for several hours. The fixed keratinocyte growth then reacted with anti-type IV collagen immunoglobulins. Peroxidase-antiperoxidase technique was applied for visualization of the immunoreactant. After osmication, the growth was dehydrated and embedded in epoxy resin without a transit solvent. Ultrathin sections with or without counterstain of lead citrate for 30 s were observed using a JEOL electron microscope 100 CX at 80 kV. For control, growths stained with control unspecific immunoglobulins were used.

RESULTS

The Junction Structures in the Control (Fig 1)

Blurry threadlike material is located in the space between the basal cell membranes of keratinocytes and the collagen film. The material is distributed diffusely on the collagen film and accumulated on the thickened cell membrane and semidesmosomes. Cytoplasmic filaments gather at the cytoplasmic surface of the thickened parts of the cell membrane.

The Junction After Immunohistochemical Staining for Type IV Collagen (Fig 2)

After peroxidase-antiperoxidase staining and osmication, type IV collagen antibody conjugates are sufficiently contrasted to be visualized by electron microscopy. A short-term staining with lead citrate increases the contrast. The conjugates are first detected at 2 h after cultivation and increase their intensity up to 24 h. The conjugates are found in the space between the basal cell membrane of keratinocytes and the collagen film, especially close to the dense parts of the cell membrane. The conjugates form masses and bands without showing detailed fine structures. The dense parts of the cell membrane show a sharp border to the extracellular space, while the border to the cytoplasm is blurry. A limiting membrane of intracytoplasmic vesicles is contrasty.

The Growing Edges of the Cell Growth (Fig 3)

The immunostaining is found covering the growing edges of the cell growth.

Control sections show no staining.

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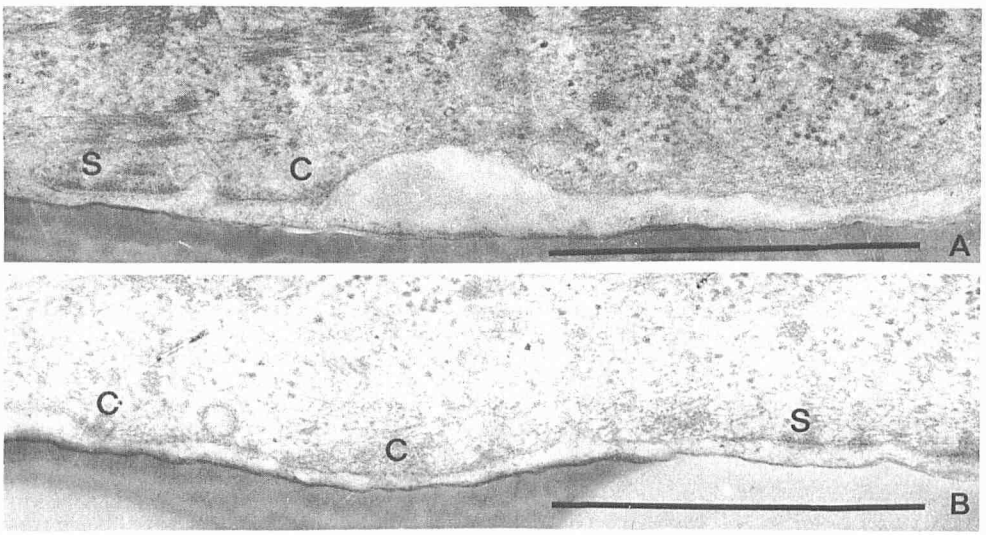
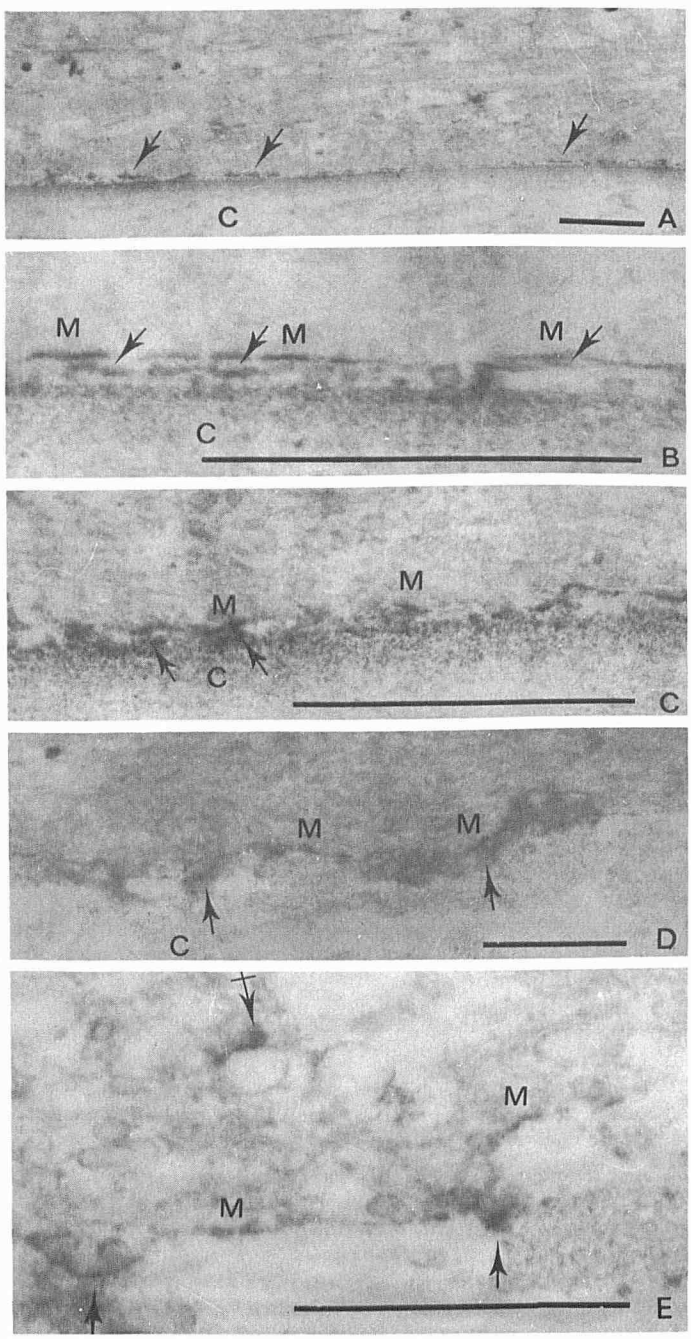


FIG 1. *A*, *B*, Seven-day culture. Uranyl acetate and lead citrate staining. Semidesmosomes (*S*) and thickened cell membrane (*C*) showed threadlike material in the extracellular spaces. *Bar* = 1.0 μm.



DISCUSSION

Previous papers have revealed positive immunostain of type IV collagen in basal lamina and cisternae of endoplasmic reticulum in parietal yolk sac cells of mouse embryo [13], epithelial cells of duodenum, and the enamel-organ, as well as vascular endothelial cells in Sherman rat embryo [14]. These authors maintained the theory of type IV collagen secretion either via the Golgi apparatus [14] or directly through the cell membrane [13]. Since the present authors used whole cultures for staining and since anti-type IV collagen immunoglobulins can only with difficulty penetrate through the cell membrane, the visualization of type IV collagen in the intracellular organelles is uncertain.

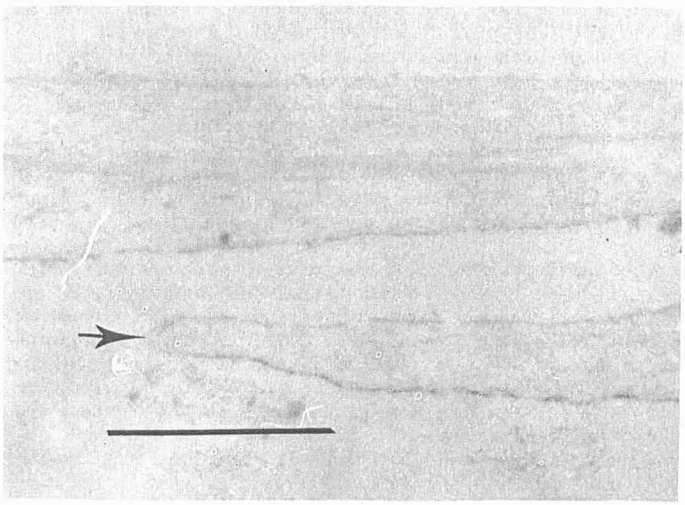


FIG 3. Two-week culture. Anti-type IV collagen immunoglobulin and peroxidase-antiperoxidase reactions. No counterstain. Dense staining of the cell surface facing the collagen film covers the growing edge (*arrow*). *Bar* = 1.0 μm.

FIG 2. Anti-type IV collagen immunoglobulin and peroxidase-antiperoxidase reactions. No counterstain. *Bars* = 1.0 μm. *A*, Seven-day culture. Dense cell membrane is seen on the cell surface facing the collagen film (*arrows*). *C* = collagen film. *B*, Seven-day culture. Thickened cell membrane (*M*) and bandlike mass of type IV collagen (*arrows*) in the space between the cells and the collagen film (*C*). *C*, Seven-day culture. Thick cell membrane (*M*) and dense mass of type IV collagen in the space (*arrows*). *C* = collagen film. *D*, Two-week culture. Dense mass of type IV collagen (*arrows*) on the cell surface facing the collagen film (*C*). *E*, Two-week culture. Mass of type IV collagen in the space (*arrows*). *M* indicates cell-membrane thickening of type IV collagen. An *arrow with a cross* shows type IV collagen on the limiting membrane of a vacuole.

In the extracellular space, basal lamina with anchoring filaments and fibrils is made up of type IV collagen and some proteoglycans [1]. The present study disclosed that keratinocytes are capable of secreting type IV collagen and form basal lamina in the space between the keratinocytes and the underlying collagen film.

After routine electron microscopic contrasting, human embryonal skin of 15 weeks showed formation of adult type dermal-epidermal junction on the basal lamina of stratum germinativum. Dense thickenings were found on the cell membrane (primitive attachment plaques), the opposing parts of the basal lamina being connected by anchoring filaments [15]. The junction structures are completed in about the 25th fetal week [15].

In organ culture studies, when the epidermis and dermis were recombined after previous separation, the lamina with anchoring filaments was formed under the preserved semidesmosomes [5]. However, it is a common phenomenon in keratinocyte culture that dispersed desmosomes and semidesmosomes are engulfed in the cytoplasm [16]. Basal lamina under the epiboly (newly grown keratinocytes) appears before and at the same period as attachment plaque formation. Anchoring filaments are seen between the basal lamina and the primitive attachment plaques [4]. Pieces of basal lamina and semidesmosomes were found on the plastic surfaces of the flasks under keratinocyte outgrowths [4]. It is, therefore, understood that type IV collagen produced to the extracellular space from the keratinocytes accumulated and was organized in basal lamina and anchoring filaments under the newly formed attachment plaques. However, it is obscure why type IV collagen appears only in the extracellular space facing the collagen film.

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