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Inhibition by Tunicamycin of Mucin Synthesis, Not Morphological Changes, in Epidermis During Retinol-Induced Mucous Metaplasia of Chick Embryonic Cultured Skin

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ABSTRACT ***Background:*** **Our previous studies have shown that epidermal mucous metaplasia of chick embryonic skin can be induced by culture in medium containing 20 μM retinol for only 8 hr and then in a chemically defined medium without retinol for 2 days and that retinol primarily affects the dermal cells, which then transform the epithelial cells into mucus-secreting cells.**

Methods: **Tarsometatarsal skin of 13-day-old chick embryo was cultured with 20 μM retinol for 1 day and then without the vitamin but with 0.1 μg/ml tunicamycin for 5 days. Effect of tunicamycin on epidermal mucous metaplasia was studied biochemically and morphologically.**

Results: **Tunicamycin, which prevents the formation of N-glycans and inhibits maturation or morphological organization of various epithelial cells, irreversibly inhibited the synthesis of sulfated glycoproteins (O-glycans, mucin) in the epidermis only when applied to retinol-pretreated skin. Microvilli on the surface of the cells were well developed, but mucous granules surrounded by a limiting membrane were not observed in the upper cell layer of the epidermis, and many vesicles without electron-dense materials (mucin) and dilated rough endoplasmic reticulum were seen in the intermediate cell layers of the epidermis. When recombinants of 13-day-old normal epidermis and cultured dermis, which had been treated with retinol for 24 hr and with only tunicamycin for 2 days, were cultured without the antibiotic for 5 days, epidermal mucous metaplasia was induced.**

Conclusion: **These results suggest that tunicamycin did not prevent morphological changes induced by retinol but inhibited mucin synthesis by a direct action on the epidermis of retinol-pretreated skin. Because in some cell-line mucin precursors contain high mannose N-linked oligosaccharides side chains, tunicamycin may have inhibited mucin synthesis. Interaction between epidermal basal cells and retinol-pretreated dermal fibroblasts is prerequisite for epidermal mucous metaplasia. Thus, the present study suggests that N-linked protein glycosylation is not required for this interaction.**

Key words: Retinol, Tunicamycin, Epidermal mucous metaplasia, Epithelial–mesenchymal Interaction

Retinoids affect differentiation of epithelial cells. In human keratinocytes grown on the emerged dermal equivalent (collagen lattice), retinoic acid negatively regulates epidermal keratinization (Asselineau et al., 1989). We have found previously that when either recombinants of 13-day-old tarsometatarsal epidermis and retinol-treated dermis (Obinata et al., 1987b) or explants of normal tarsometatarsal skin of 13-day-old chick embryos that had been placed for 1 day on retinol-pretreated dermal fibroblasts (Obinata et al., 1994) were cultured for 7 days in a chemically defined medium (BGJb; Biggers et al., 1961) in the absence of retinol and hormones, they showed altered epidermal differentiation (mucous metaplasia). Therefore, retinol primarily affects the dermal cells, which then transformed the epithelial cells into mucus-secreting cells by interactions with epidermal basal cells (Obinata et

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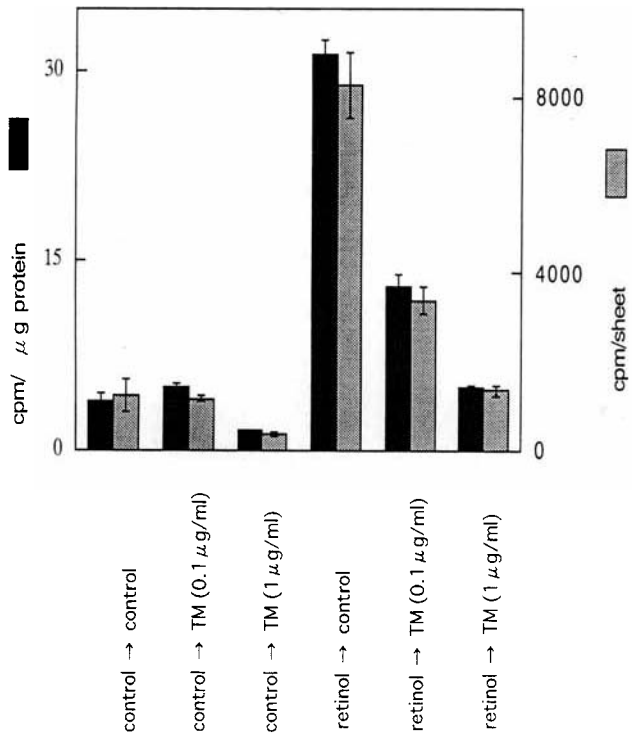

Fig. 1. Inhibition by tunicamycin (TM) of retinol-induced synthesis of sulfated glycoproteins in cultured chick embryonic skin. Thirteen-day-old chick embryonic skin was cultured for 1 day in BGJb containing 5% delipidized FCS and 20 nM hydrocortisone with or without 20 μM retinol and then in BGJb containing carrier-free 20 μCi/ml of [^{35}S]sulfate with 2 mM Bt_2cAMP in the presence or absence of 0.1 or 1 μg/ml of TM for 2 days. Epithelial [^{35}S] acid glycoproteins were determined as described in Materials and Methods. Values are means ± standard errors for triplicate measurements.

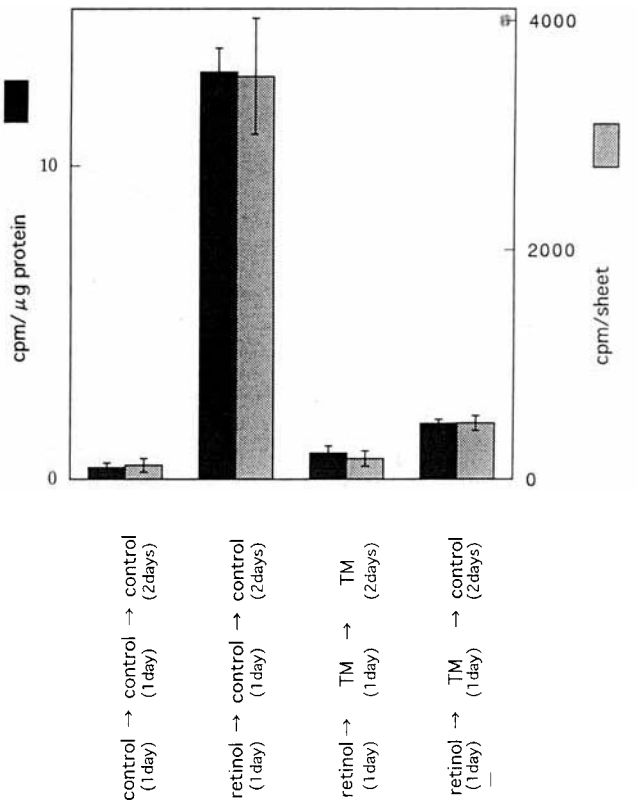

Fig. 2. Irreversible inhibition by tunicamycin (TM) of retinol-induced synthesis of sulfated glycoproteins in cultured chick embryonic skin. Explants of 13-day-old chick embryonic skin that had been cultured for 1 day in BGJb containing 5% delipidized FCS and 20 nM hydrocortisone with or without 20 μM retinol were cultured in BGJb containing 2 mM Bt_2cAMP in the presence or absence of 0.1 μM tunicamycin for 1 day and then cultured in BGJb containing 2 mM Bt_2cAMP and carrier-free 20 μCi/ml of [^{35}S] in the presence or absence of 0.1 μg/ml of tunicamycin for 2 days. Epithelial [^{35}S] acid glycoproteins were determined as described in Materials and Methods. Values are means ± standard errors for triplicate measurements.

al., 1987b, 1994) but not via gap junctional communication (Obinata, 1994). Mucous metaplasia stimulated by application of Bt_2cAMP after retinol treatment (Obinata et al., 1991a) can be induced by culturing 13-day-old chick embryonic tarsometatarsal skin in medium containing 20 μM retinol for only 8 hr and then in BGJb for 2 days (Obinata et al., 1991b).

Tunicamycin, which prevents transfer of oligosaccharide chains to nascent protein (core glycosylation; Heifetzet al., 1979), inhibits maturation or morphological organization of epithelial cells in the testis (Kanai et al., 1991), thyroid (Giraud and Franc, 1989), and lung (Webster et al., 1993). We studied whether tunicamycin inhibits mucin synthesis or morphological changes induced by retinol in epidermis in the course of mucous metaplasia.

MATERIALS AND METHODS

Culture Methods

Skin explants from the tarsometatarsal region of 13-day-old chick embryos were cultured for 1 day in a chemically defined medium, BGJb, containing 5% delipidized fetal calf serum (FCS) and 20 nM hydrocortisone hemisuccinate (Upjohn, Tokyo) with or without 20 μM retinol (Sigma). The explants were washed twice with calcium-free and magnesium-free phosphate buffered saline (PBS(−)) and cultured in BGJb supplemented with 2 mM Bt_2cAMP (Sigma) with or without 0.1 μg/ml of tunicamycin (Wako Chemicals) for 2–5 days by the Millipore filter-roller-tube method (Kojima et al., 1976). In skin recombinant culture, explants of dermis of cultured skin and epidermis of 13-day-old chick embryonic skin were reassociated in various heterotypic recombinants and placed on Millipore filters. The recombinants were left on the filters for 2 hr to allow sufficient mutual adhesion between the dermis and epidermis and were cultured for 5 days in BGJb containing 2 mM Bt_2cAMP.

Serum was delipidized by treatment with an acetone: alcohol mixture (Rothblat et al., 1976). This treatment quantitatively removed glucocorticoid and retinoids, which are soluble in nonpolar organic solvents, with most lipids. The medium was renewed every other day. Because L-ascorbate is very unstable, a stable and active derivative of L-ascorbate, the magnesium salt of L-ascorbic acid 2- phosphate n-hydrate (0.1 mM; water content, 20–29%; Wako Chemicals), was added to BGJb.

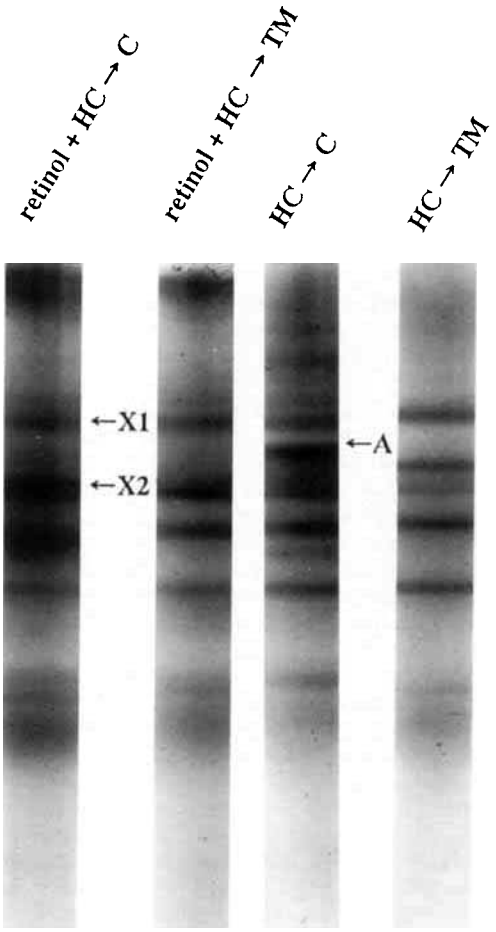

Fig. 3. Inhibition by tunicamycin (TM) of hydrocortisone-enhanced accumulation of A-protein (α-type keratin, A). Explants of 13-day-old chick embryonic skin were cultured for 1 day in BGJb containing 5% delipidized FCS, 20 μM retinol, 20 nM hydrocortisone, and then in BGJb containing 2 mM Bt_2cAMP with or without 0.1 μg/ml of tunicamycin for 5 days. The 40 μg/ml of S-carboxymethylated epidermal protein was separated by polyacrylamide gel electrophoresis, as described in Materials and Methods. C, control; HC, hydrocortisone; X1, X2, characteristic components of undifferentiated epidermis.

Separation of Epidermis and Dermis

The epidermis and dermis of cultured explants were separated by incubating the skin at 37°C for 40 min in BGJb supplemented with 1,000 units/ml of Dispase (Godo Shusei). The epidermis of 13-day-old chick embryonic skin was obtained by treating the skin with 0.4% EDTA in BGJb at 37°C for 25 min. The epidermis and dermis were then washed several times with PBS(−) at room temperature.

Assay of Incorporation of ^{35}S Into Acid Glycoproteins in the Epithelium

Skin was exposed to carrier-free (^{35}S)sulfate (10–20 μCi/ml; Japanese Atomic Institute) during the last 42 hr of culture. The ^{35}S incorporated into acid glycoproteins (mucin) in the epidermis was determined as described elsewhere (Obinata et al., 1991a).

Microscopy

Skin explants were processed for light and electron microscopic observations as described before (Obinata et al., 1994).

Reduction and S-Carboxymethylation of Epidermal Protein

The epidermis of cultured skin explants was homogenized in a glass homogenizer with 5 mM Tris-glycine (pH 8.6) containing EDTA and 8 M urea. Protein in the homogenate (2 mg protein/ml) was solubilized by reduction followed by S-carboxymethylation as described elsewhere (Obinata et al., 1987a; Kojima et al., 1976).

Polyacrylamide Gel Electrophoresis

Gel electrophoresis was performed as described by Davis (1964). The separating gel contained 6% acrylamide (w/v), 0.16% N,N′-methylenebisacrylamide (w/v), 8 M urea, and 1.5 M Tris- glycine (pH 8.9). The stacking gel contained 1.25% acrylamide, 0.3% N,N′-methylenebisacrylamide, and 0.0625 M Tris-HCl (pH 6.7). Disc gel electrophoresis was carried out at 3 mA/0.5 cm diameter for 4 hr.

RESULTS

Irreversible Inhibition by Tunicamycin of Retinol-Induced Synthesis of Sulfated Glycoproteins in Retinol-Pretreated Skin

Explants of 13-day-old chick embryonic skin that had been cultured in BGJb containing 5% delipidized FCS, 20 μM retinol, and 20 nM hydrocortisone for 1 day were washed in PBS(−) and cultured in BGJb containing 2 mM Bt_2cAMP with or without tunicamycin for 2 days. Results showed that tunicamycin at 0.1 and 1 μg/ml dose dependently inhibited retinol-induced synthesis of epidermal sulfated glycoproteins (mucin; Fig. 1). Because tunicamycin seemed to be somewhat toxic at 1 μg/ml, it was subsequently used at a concentration of 0.1 μg/ml. Figure 2 shows that when explants of retinol-pretreated skin were cultured in the presence of tunicamycin for 1 day and then without tunicamycin for 2 days, the synthesis of sulfated glycoproteins during the last 2 days was still inhibited, indicating that this inhibitory effect of tunicamycin was irreversible.

Effect of Tunicamycin on the Electrophoretic Pattern of Epidermal Protein in Retinol-Pretreated Cultured Skin

Because 20 nM hydrocortisone can induce chick embryonic epidermal differentiation toward α-type keratinization during culture of 13-day-old chick embryonic skin (Kojima et al., 1976; Takata et al., 1981), we studied whether accumulation of α-keratin (A-protein) could be seen as a result of inhibition by tunicamycin of retinol-induced synthesis of mucin. Figure 3 shows that when explants of retinol- and hydrocortisone-pretreated skin were cultured with or without tunicamycin for 5 days, the urea–gel electrophoretic pattern of S-carboxymethylated epidermal protein of tunicamycin-treated skin resembled that of control skin. No accumulation of epidermal structural protein (protein A, α- keratin), which is not a glycoprotein (Obinata et al., 1982), was observed, whereas the syntheses of proteins X1 and X2, which are components characteristic of nonkeratinized epidermis (Kojima et al., 1976), were seen. Thus, because accumulation of α-type keratin in

epidermis was not seen by tunicamycin treatment, the antibiotic may not have completely abolished the effect of retinol on skin but only inhibited mucin synthesis. Sulfated glycoproteins (mucin) with high molecular weights (Linsley et al., 1988) probably could not enter the gel.

Abolishment by Tunicamycin of Mucus-Secreting Granules in Epidermis of Retinol-Pretreated Skin

When explants of retinol- and hydrocortisone-pretreated skin were cultured with or without tunicamycin for 5 days, tunicamycin inhibited the appearance of periodic acid-Schiff (PAS)-positive materials in the epidermis (Fig. 4d), which were seen in the intermediate and upper cell layers of the epidermis of control skin (Fig. 4b). Figure 5 shows that, in retinol- and hydrocortisone-pretreated skin in the presence of tunicamycin, microvilli on the surface of the cells were well developed, but mucous granules surrounded by a limiting membrane were not observed in the upper cell layer of the epidermis (Fig. 5a). Many vesicles without electron-dense materials (mucin) and much dilated rough endoplasmic reticulum and Golgi apparatus in the cytoplasm were seen in the intermediate cell layers of the epidermis (Fig. 5b). Furthermore, desmosomes, hemidesmosomes, and tonofilament bundles were poorly developed, with discontinuity of the basement membrane in the basal cell layer of the epidermis (Fig. 5c). In the absence of tunicamycin, many typical goblet cells with abundant secreting granules in their cytoplasm were seen in the superficial cell layer (Fig. 5d). When explant of retinol- and hydrocortisone-pretreated skin were cultured with tunicamycin for 1 day and then in the same medium without the antibiotic for 4 days, the same features as those in cultures with retinol and hydrocortisone for 1 day and then treated with tunicamycin throughout (Fig. 5e) were observed, indicating that the effect of tunicamycin was irreversible, as also shown in Figure 2.

No Abolishment by Tunicamycin Given at the Same Time as Retinol of Mucus-Secreting Granules in the Epidermis of Skin

In explants in culture with retinol, hydrocortisone, and tunicamycin for 1 day and then without the substances for 5 days, tunicamycin did not inhibit the retinol-induced appearance of PAS- positive materials in the epidermis (Fig. 6). Electron microscopic study showed no difference between explants of skin pretreated with retinol in the absence and presence of tunicamycin for 1 day and then without tunicamycin for 5 days (Fig. 5f).

Inhibition of Epidermal Mucous Metaplasia by a Direct Effect of Tunicamycin on the Epidermis, not the Dermis

Figure 7a,b show that when recombinants of normal epidermis and dermis of skin, which had been cultured with retinol and hydrocortisone for 1 day and then with tunicamycin for 2 days, were cultured without tunicamycin for 5 days, epidermal mucous metaplasia was induced. Because inhibition of retinol-induced epidermal mucous metaplasia by tunicamycin was irreversible, as indicated in Figures 2 and 5e, its inhibition was due to a direct action on the epidermis, not the dermis.

Inhibition by Tunicamycin of Hydrocortisone-Induced Keratinization in Cultured Skin

In explants of skin that had been cultured with hydrocortisone for 1 day, culture with tunicamycin for 5 days inhibited the accumulation of A-protein (α-keratin) in the epidermis (Fig. 3) and prevented epidermal keratinization (Fig. 4g), whereas accumulation of A-protein (Fig. 3) and keratinization (Fig. 4e) were observed in control skin.

Electron microscopic examination showed that tunicamycin prevented induction of α-type keratinization by hydrocortisone. Although thickening of the cell membrane and degeneration of intracellular organelles, which are induced by hydrocortisone during α-type keratinization of epidermis, were observed (Fig. 5g), the superficial region of the epidermis did not form either the filament bundles or the electron-dense amorphous masses (Fig. 5g) seen in the cytoplasm of the glucocorticoid-induced keratinized layer, and the tonofilament bundles in the cytoplasm of the basal and intermediate epidermal cells were not as conspicuous as those in the glucocorticoid-induced keratinized epidermis (Fig. 5h,i). Detachment of the basal lamina from the basal surface of the epidermis was observed in tunicamycin-treated skin (Fig. 5i) but not in control skin. When recombinants of normal epidermis and dermis of skin that had been cultured with hydrocortisone for 1 day and then with or without tunicamycin for 2 days were cultured without tunicamycin for 5 days, tunicamycin-treated dermis inhibited epidermal keratinization (Fig. 7e), whereas keratinization was observed in control recombinant skin (Fig. 7c), indicating that N-glycosylation of dermal proteins is required for the epidermal keratinocytes to differentiate properly toward α-type keratinization.

DISCUSSION

In this study, we showed that (1) tunicamycin irreversibly inhibited the synthesis of sulfated glycoproteins (mucin) in the epidermis of retinol-induced mucous metaplasia of chick embryonic skin; (2) the inhibition was observed only when tunicamysin was added after retinol treatment and not with retinol; (3) the inhibition was direct on the epidermis, not the dermis; and (4) the antibiotic did not prevent morphological changes in epidermis induced by reitnol. In the presence of tunicamycin in retinol-pretreated skin, microvilli on the surface of the cells were well developed, but mucous granules surrounded by limiting membrane were not observed in the upper cell layer of the epidermis, and many vesicles without electron-dense materials and dilated rough endoplasmic reticulum and the Golgi apparatus were seen in the intermediate cell layers of the epidermis. Dilation of the rough endoplasmic reticulum was also observed in tunicamycin-treated tissue (Kanai et al., 1991), presumably because nascent proteins, whose glycosylation is blocked by tunicamycin, accumulate in the rough endoplasmic reticulum. Mucin is a highly glycosylated protein, and carbohydrates are attached to the protein core primarily by O-linkages (Linsley et al., 1988). In a breast carcinoma cell line, the mucin precursors contain high mannose N-linked oligosaccharide side chains, and initiation of N-linked oligosaccharide synthesis thus pre-

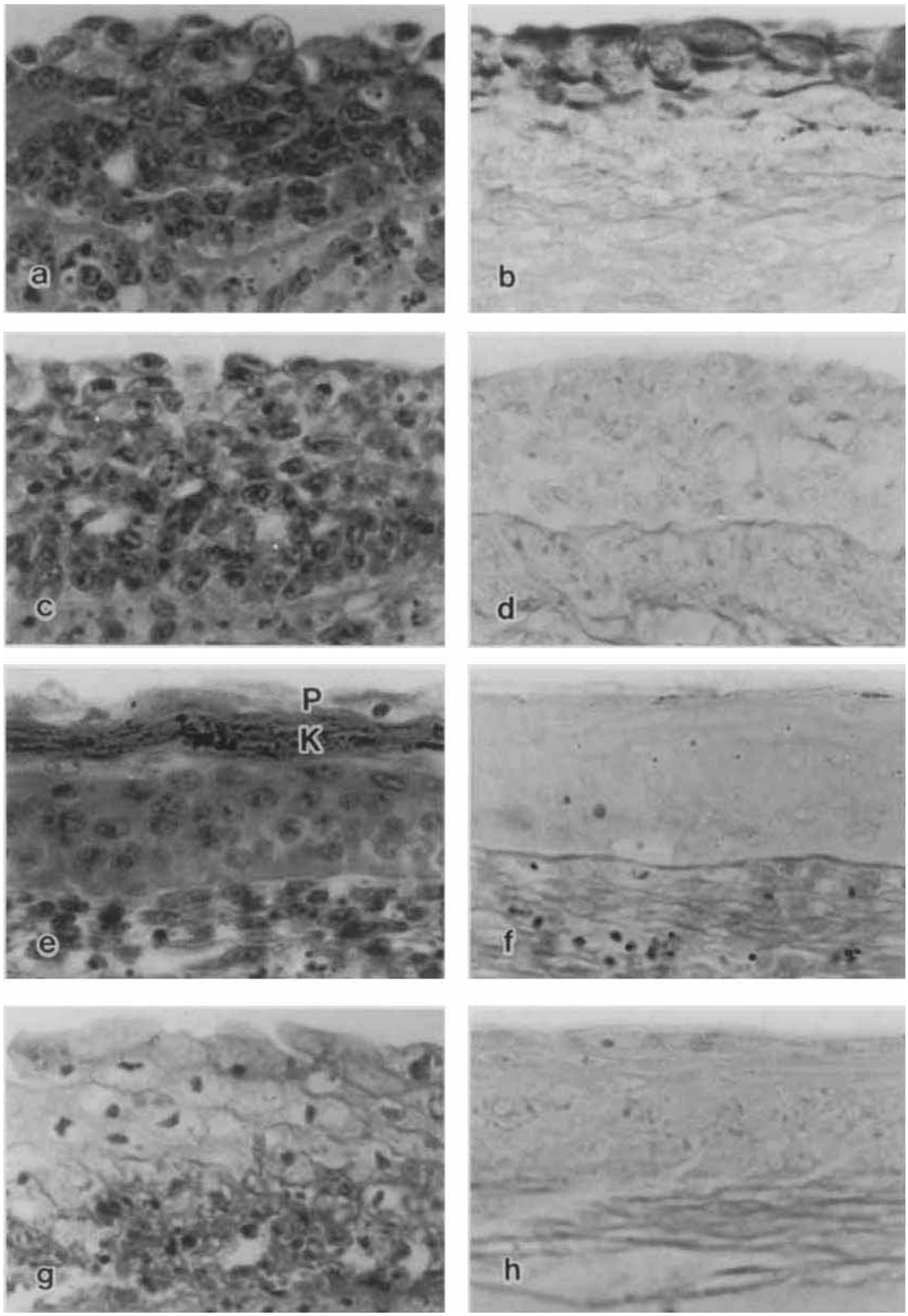

Fig. 4. Light micrographs of cultured 13-day-old chick embryonic skin. Skin explants were incubated for 1 day in BGJb containing 5% delipidized FCS and 20 nM hydrocortisone with (**a–d**) or without (**e–h**) 20 μM retinol and then for 5 days in BGJb containing 2 mM Bt_2cAMP in the absence (a,b,e,f) or presence (c,d,g,h) of 0.1 μg/ml of tunicamycin. Sections were stained with hematoxylin and eosin (a,c,e,g) or PAS (b,d,f,h). b: PAS-positive metaplastic cells (arrows) are seen in the superficial and intermediate layers of the epidermis. d,f,h: The PAS reaction is negative in all layers of the epidermis. The keratinized layer (K) is clearly recognized under the embryonic peridermal cell layer (P) in e but not in a, c, or g. Magnification, ×640.

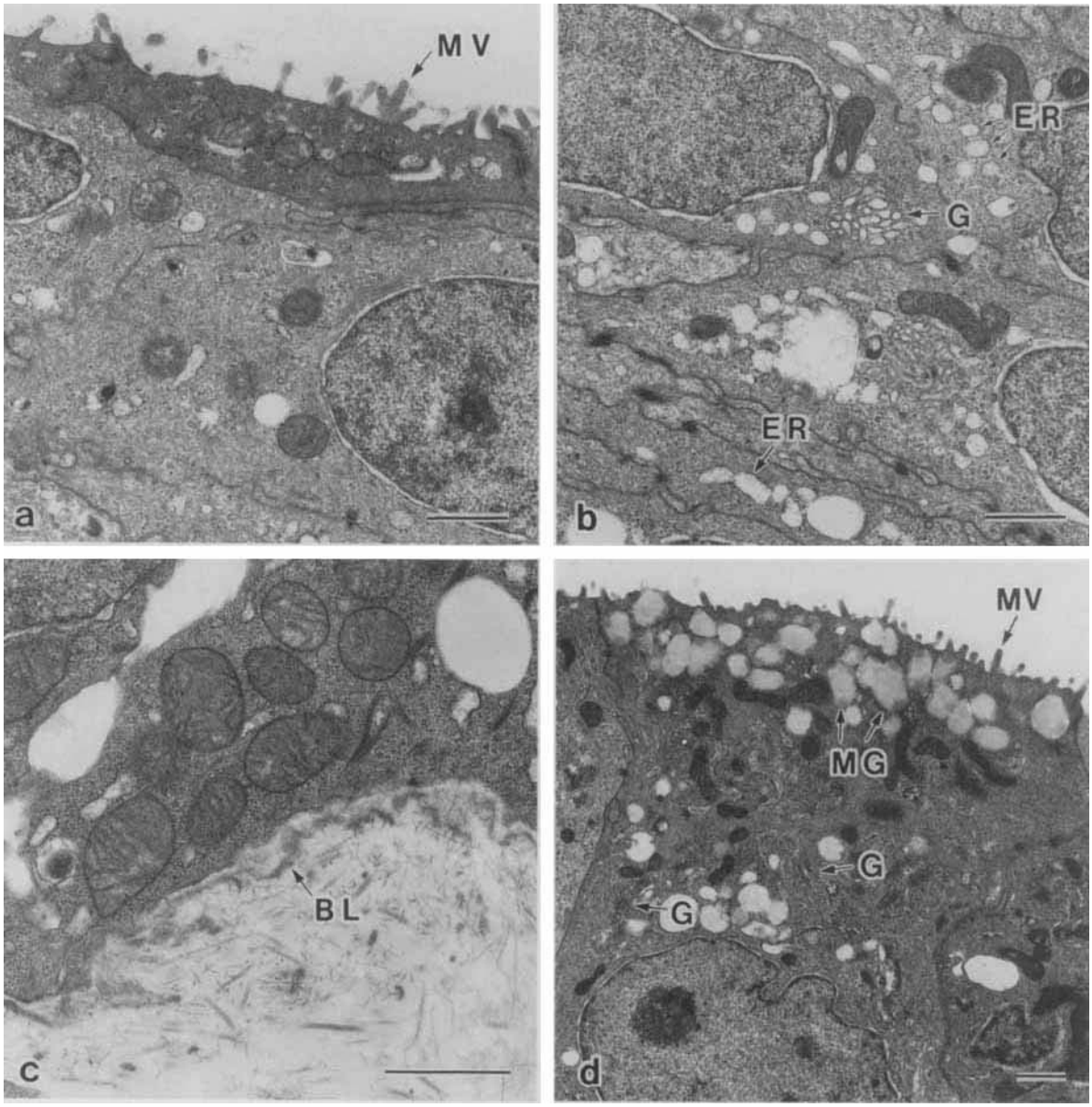

Fig. 5. Electron micrographs of cultured 13-day-old chick embryonic skin. Skin explants were cultured for 1 day in BGJb containing 5% delipidized FCS, 20 μM retinol, and 20 nM hydrocortisone and then for 5 days in BGJb containing 2 mM Bt_2cAMP with (**a–c**) or without (**d**) 0.1 μg/ml of tunicamycin. **e:** Skin explants cultured for 1 day in BGJb containing 5% delipidized FCS, 20 μM retinol, and 20 nM hydrocortisone were cultured for 1 day in BGJb containing 2 mM Bt_2cAMP and 0.1 μg/ml of tunicamycin and for another 4 days in the same medium without tunicamycin. **f:** Skin explants were cultured for 1 day in BGJb containing 5% delipidized FCS, 20 μM retinol, 20 nM hydrocortisone, and 0.1 μg/ml of tunicamycin and in BGJb containing 2 mM Bt_2cAMP for another 5 days. **g–i:** Skin explants were cultured for 1 day in BGJb containing 5% delipidized FCS and 20 nM hydrocortisone and then for 5 days in BGJb containing 2 mM Bt_2cAMP and 0.1 μg/ml of tunicamycin. **a,e:** Enlargement of a portion of the superficial cells in the epidermis. Scarecely any mucus-secreting granules are observed. Microvilli (MV) on the surface of the epidermis are well developed. **b:** Enlargement of a portion of intermediate cells in the epidermis. Dilated rough endoplasmic reticulum (ER) and the Golgi (G) apparatus are seen. **c:** Enlargement of basal cells of the epidermis. Desmosomes, hemidesmosomes, and tonofilament bundles are poorly developed. The basal lamina (BL) is discontinuous. **d,f:** Enlargement of a portion of the superficial cells in the epidermis. Many mucus-secreting granules (MG) are observed. Microvilli on the surface of the epidermis are well developed. **g:** Enlargement of superficial cells in the epidermis. Thickening of the plasma membrane is conspicuous (arrows). The electron-dense amorphous masses seen in the cytoplasm of the glucocorticoid-induced keratinized layer are not observed. **h:** Enlagement of intermediate cells in the epidermis. Few tonofilament bundles are observed. Dilated ER is observed (arrowheads). **i:** Enlargement of basal cells in the epidermis. Desmosomes, hemidesmosomes, and tonofilament bundles are poorly developed. The basal lamina is detached from the surface of the basal cells and folded, protruding into the dermis (arrowheads). **j:** Enlargement of basal cells of the epidermis. Desmosomes (D), hemidesmosomes (HD), and tonofilament bundles (T) are well developed. Basal lamina (BL) is continuous along the basal surface of the basal cells. Scale bars = 1 μm.

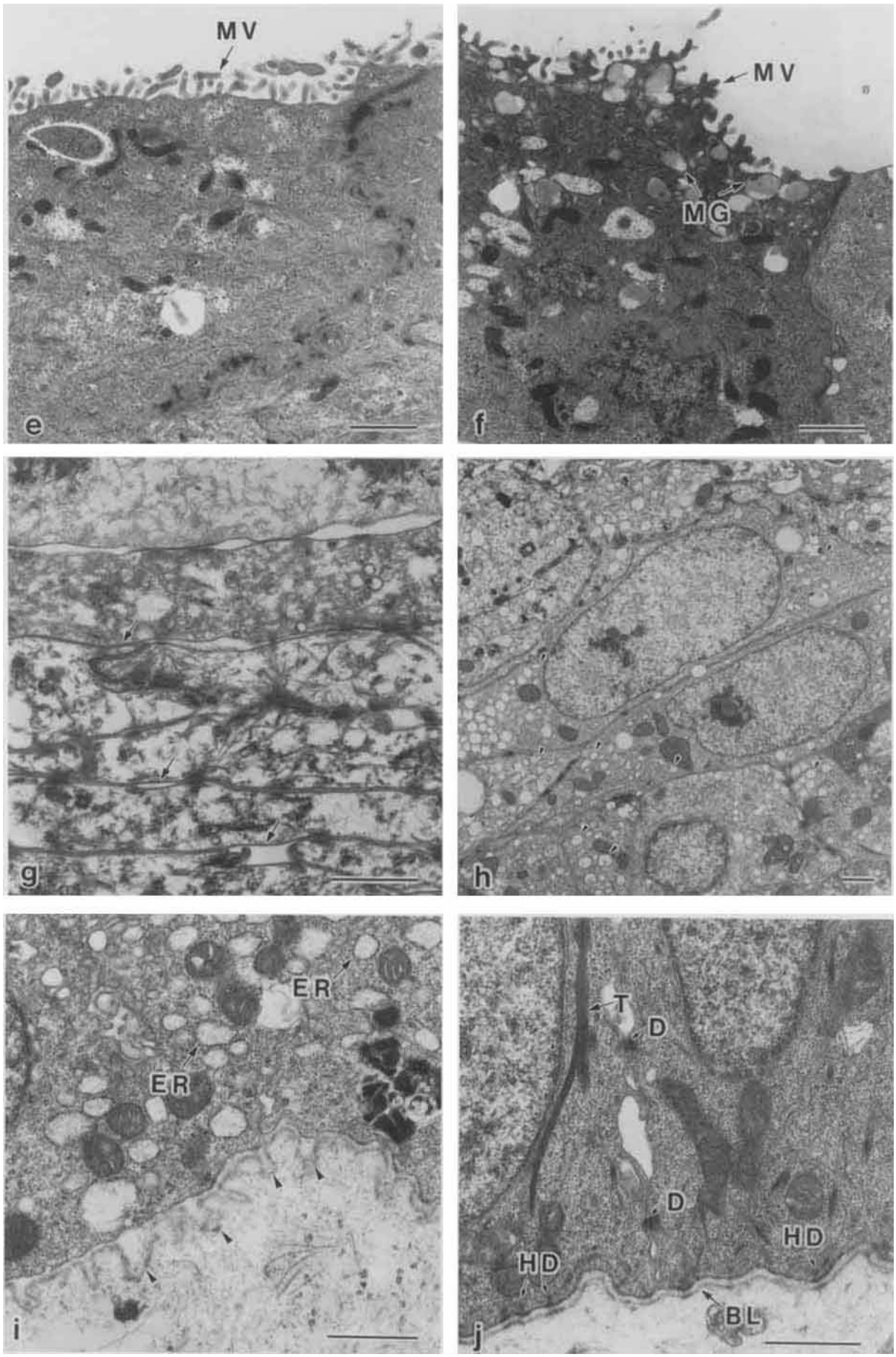

Fig. 5e–j.

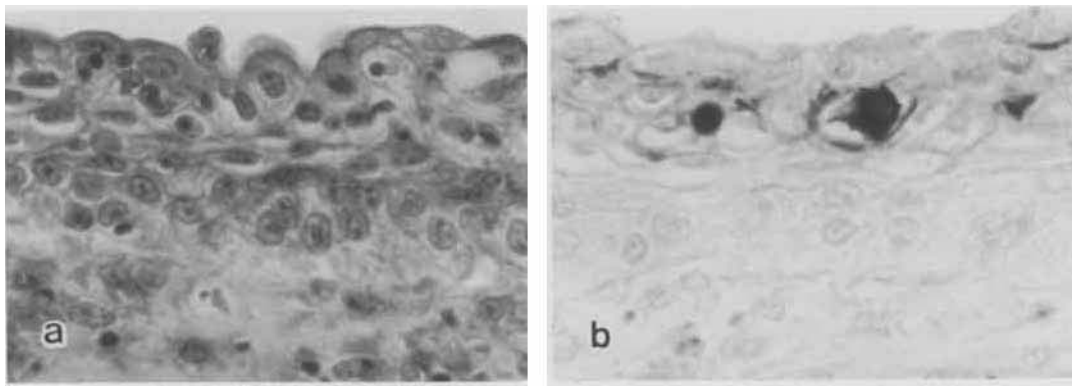

Fig. 6. Appearance of mucus-secreting granules in the epidermis of skin treated with tunicamycin and retinol. An explant of 13-day-old chick embryonic skin was cultured for 1 day in BGJb containing 5% delipidized FCS, 20 μM retinol, 20 nM hydrocortisone, and 0.1 μg/ml of tunicamycin and then in BGJb containing 2 mM Bt_2cAMP for 5 days. The section was stained with hematoxylin and eosin (**a**) or PAS (**b**). Magnification, ×800.

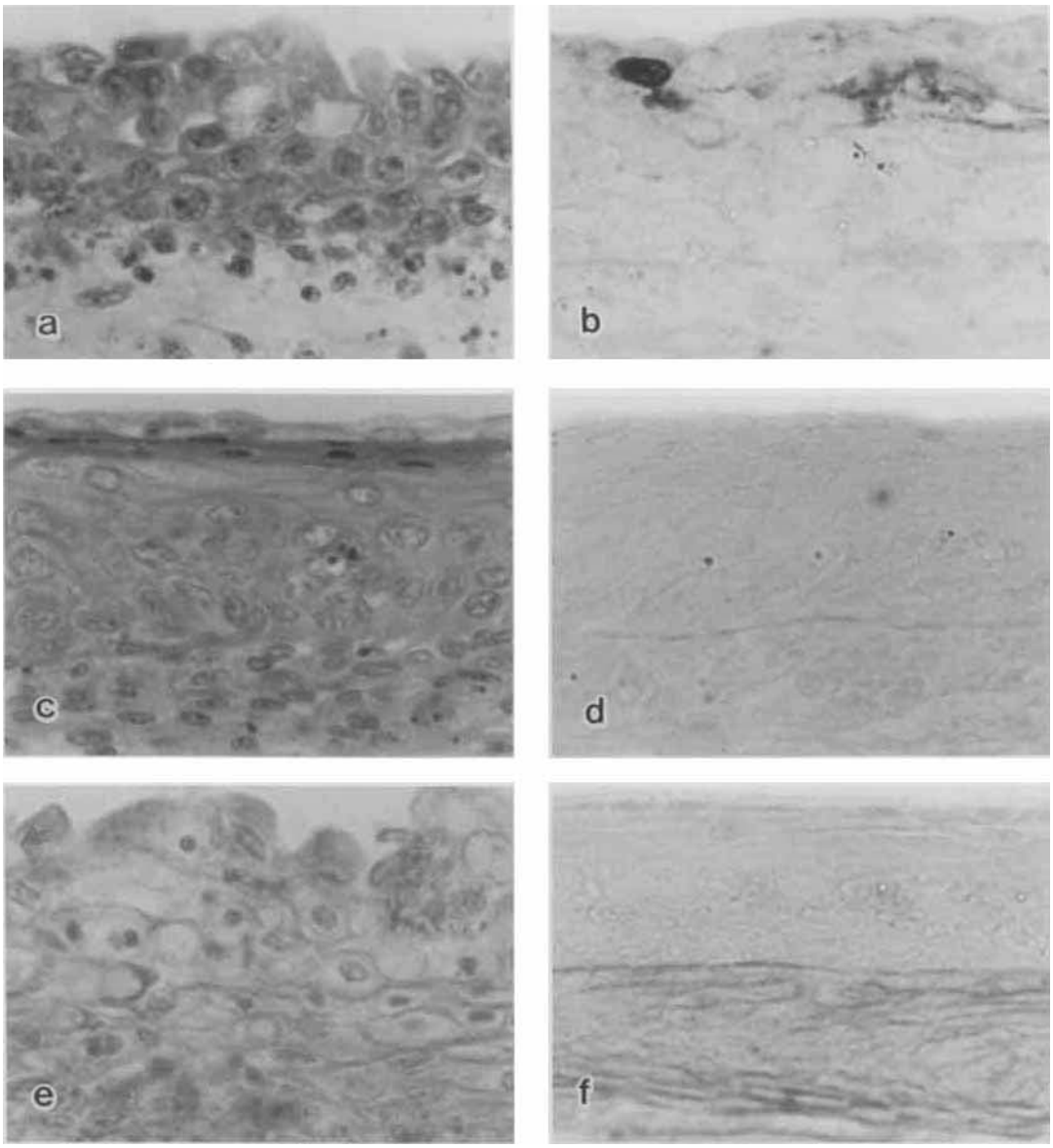

Fig. 7. Light micrographs of recombinants of cultured 13-day-old chick embryonic skin. Hematoxylin and eosin (a,c,e), and PAS (b,d,f) stains. **a,b:** Mucous metaplasia of a recombinantion of epidermis and retinol-pretreated and tunicamycin-treated cultured dermis maintained for 5 days in BGJb are seen. Dermis was obtained from skin that had been cultured for 1 day in BGJb containing 5% delipidized FCS, 20 μM retinol, and 20 nM hydrocortisone and then in BGJb containing 2 mM Bt_2cAMP and 0.1 μg/ml of tunicamycin for 2 days. **c,d:** Epidermal keratinization of a recombinant of epidermis and cultured dermis maintained for 5 days in BGJb. Dermis was obtained from skin that had been cultured for 1 day in BGJb containing 5% delipidized FCS and 20 nM hydrocortisone and in BGJb containing 2 mM Bt_2cAMP for 2 days. **e,f:** Absence of keratinization of a recombinant of epidermis and tunicamycin-treated cultured dermis maintained for 5 days in BGJb. Dermis was obtained from skin that had been cultured for 1 day in BGJb containing 5% delipidized FCS and 20 nM hydrocortisone and in BGJb containing 2 mM Bt_2cAMP and 0.1 μg/ml of tunicamycin for 2 days. Magnification, ×640.

cedes initiation of most O-linked oligosaccharide (Linsley et al., 1988). β1-6GlcNAc transferase, which forms critical branches in O-glycans, has potential N-glycosylation sites (Bierhuizen and Fukuda, 1992), but glycosylation of the enzyme may not be essential for its activity. Hence, tunicamycin inhibited the biosynthesis of mucin glycoproteins presumably at the steps of biosynthesis of mucin precursors and/or O-glycan synthesis. These findings suggest that N-linked protein glycosylation is not required for interaction between epidermal basal cells and retinol-pretreated dermal cells during epidermal mucous metaplasia.

Because the coexistence of hydrocortisone in the physiological concentration of 20 nM with retinol increased the mucin synthesis (data not shown), hydrocortisone was always present during the treatment of the skin with retinol. On culture of explants of 13-day-old chick embryonic tarsometatarsal skin in BGJb, 20 nM hydrocortisone induces epidermal α-type keratinization (Kojima et al., 1976; Takata et al., 1981). Application of tunicamycin to cultured skin with hydrocortisone or after steroid treatment prevented induction of α-type keratinization by hydrocortisone, but thickening of the cell membrane and degeneration of intracellular organelles were observed. Dermis of a skin explant that had been cultured with hydrocortisone for 1 day and then with tunicamycin for 2 days also inhibited epidermal α-type keratinization on recombination with 13-day-old chick embryonic tarsometatarsal epidermis on culture for 5 days. Thus, N-glycosylation of dermal proteins is important in epithelial–mesenchymal interaction for epidermal α-type keratinization. The basement membrane, which contains N-glycosylated protein constituents such as fibronectin, laminin, nidogen and type IV collagen (Eady et al., 1994), became discontinuous in tunicamycin-treated skin, as was seen in tunicamycin-treated gonadal explants (Kanai et al., 1991). Because epidermal growth factor (EGF) in the presence of delipidized FCS inhibits glucocorticoid-induced epidermal α-type keratinization, with discontinuity of the basement membrane in which rapid degradation of α-keratin is observed (Obinata et al., 1987a), continuity of the basement membrane might be required for the epidermal basal cells to accumulate α-keratin (tonofilament) in their cytoplasm. Further studies must be done to dissolve epithelial–mesenchymal interaction at the molecular level.

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