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The effect of all-*trans*-retinoic acid on the synthesis of epidermal cell-surface-associated carbohydrates

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1. all-*trans*-Retinoic acid at concentrations greater than 10^{-7}M stimulated the incorporation of D-[^{3}H]glucosamine into 8M-urea/5% (w/v) sodium dodecyl sulphate extracts of 1M-$CaCl_2$-separated epidermis from pig ear skin slices cultured for 18h. The incorporation of $^{35}SO_4^{2-}$, L-[^{14}C]fucose and U-^{14}C-labelled L-amino acids was not significantly affected. 2. Electrophoresis of the solubilized epidermis showed increased incorporation of D-[^{3}H]glucosamine into a high-molecular-weight glycosaminoglycan-containing peak when skin slices were cultured in the presence of 10^{-5}M-all-*trans*-retinoic acid. The labelling of other epidermal components with D-[^{3}H]glucosamine, $^{35}SO_4^{2-}$, L-[^{14}C]fucose and U-^{14}C-labelled L-amino acids was not significantly affected by 10^{-5}M-all-*trans*-retinoic acid. 3. Trypsinization dispersed the epidermal cells and released 75–85% of the total D-[^{3}H]glucosamine-labelled material in the glycosaminoglycan peak. Thus most of this material was extracellular in both control and 10^{-5}M-all-*trans*-retinoic acid-treated epidermis. 4. Increased labelling of extracellular epidermal glycosaminoglycans was also observed when human skin slices were treated with all-*trans*-retinoic acid, indicating a similar mechanism in both tissues. Increased labelling was also found when the epidermis was cultured in the absence of the dermis, suggesting a direct effect of all-*trans*-retinoic acid on the epidermis. 5. Increased incorporation of D-[^{3}H]-glucosamine into extracellular epidermal glycosaminoglycans in 10^{-5}M-all-*trans*-retinoic acid-treated skin slices was apparent after 4–8h in culture and continued up to 48h. all-*trans*-Retinoic acid (10^{-5}M) did not affect the rate of degradation of this material in cultures 'chased' with 5mM-unlabelled glucosamine after 4 or 18h. 6. Cellulose acetate electrophoresis at pH 7.2 revealed that hyaluronic acid was the major labelled glycosaminoglycan (80–90%) in both control and 10^{-5}M-all-*trans*-retinoic acid-treated epidermis. 7. The labelling of epidermal plasma membranes isolated from D-[^{3}H]glucosamine-labelled skin slices by sucrose density gradient centrifugation was similar in control and 10^{-5}M-all-*trans*-retinoic acid-treated tissue. 8. The results indicate that increased synthesis of mainly extracellular glycosaminoglycans (largely hyaluronic acid) may be the first response of the epidermis to excess all-*trans*-retinoic acid.

Vitamin A plays an important role in the differentiation of epithelial tissues. It appears to modulate the expression of either a mucous-secreting or a keratinizing phenotype. In the absence of vitamin A, mucous-secreting epithelia develop a squamous metaplasia (Hicks, 1968; Beitch, 1970; Marchok *et al.*, 1975), whereas in the presence of excess vitamin A, keratinizing epithelia undergo a mucous metaplasia (Fell & Mellanby, 1953).

The effects of excess vitamin A on both embryonic and adult epidermis *in vitro* have been well documented at the ultrastructural level (Jackson & Fell, 1963; Barnett & Szabo, 1973; Hardy *et al.*, 1974; Peck *et al.*, 1977). The process of differentiation (keratinization) during which living epidermal cells synthesize filamentous 'keratin' proteins and are transformed into the highly resistant dead cells of the stratum corneum is inhibited. Desmosomes are decreased, resulting in a loss of cell adhesion and widening of intercellular spaces. In addition, excess vitamin A induces the production of both intracellular and extracellular 'mucous' material that is not normally present in the epidermis.

Abbreviation used: SDS, sodium dodecyl sulphate.

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This inhibitory effect of vitamin A on epidermal differentiation provides the basis for the therapeutic use of retinoids in skin conditions where keratinization is disordered (Tsambaos *et al.*, 1980). However, the mechanism by which retinoids exert such influence remains unclear.

It has been suggested that vitamin A or its metabolites may be directly involved in glycosyl-transfer reactions by acting as a carrier for sugar nucleotides in a manner similar to dolichol phosphate (Rosso *et al.*, 1975; De Luca, 1977; Adamo *et al.*, 1979). An effect on glycosylation reactions and the likely subsequent effects on the synthesis of cell-surface glycoconjugates may account for some of the reported effects of vitamin A on the surface properties of cultured cells. Cell adhesion (Jetten *et al.*, 1979), saturation density (Lotan *et al.*, 1978), proliferation and density-dependent growth rate (Lotan & Nicolson, 1977; Dion *et al.*, 1977) are all modified by vitamin A.

In a previous paper (King *et al.*, 1980) we characterized the cell-surface-associated epidermal glycoconjugates synthesized when pig skin slices were cultured in the presence of radioactive precursors. We showed that L-[^{3}H]fucose and D-[^{3}H]glucosamine were both incorporated into the same major plasma membrane-bound glycoproteins. D-[^{3}H]Glucosamine also labelled a high molecular weight peak of glycosaminoglycan-containing material that was extracellular but was not strongly bound to the plasma membrane. This well-characterized organ culture system provided us with an opportunity to study the effect of all-*trans*-retinoic acid on the synthesis of specific epidermal cell-surface glycoconjugates, and the results are presented in the present paper.

Experimental

Materials

D-[6-^{3}H]Glucosamine hydrochloride (>10 Ci/mmol), L-[1-^{14}C]fucose (>50 Ci/mol), U-^{14}C-labelled L-amino-acid mixture (>45 Ci/g-atom of carbon), $^{35}SO_4^{2-}$ and D-[1-^{14}C]galactosamine hydrochloride (>45 Ci/mol) were all obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Reagents for gel electrophoresis were from BDH. Reagents for tissue culture were obtained as described previously (King *et al.*, 1980). Proteinase type VI from *Streptomyces griseus* was from Sigma. Dispase (neutral proteinase from *Bacillus polymyxa*) was from Boehringer. Hyaluronic acid, chondroitin sulphate B and sheep testicular hyaluronidase were from Miles Laboratories, Stoke Poges SL2 4LY, U.K. Cellulose acetate sheets (Cellogel; 16 cm × 17 cm) were from Whatman. Retinoic acid was from Distillation Products, Eastman Kodak, Rochester, NY, U.S.A.

Organ culture

This was as described previously (King *et al.*, 1980). Briefly, slices of pig ear skin 1 cm square and about 0.2 mm thick were floated on 1 ml of Eagle's minimum essential growth medium containing 10% (v/v) foetal calf serum, antibiotics and D-[^{3}H]glucosamine (10 or 25 μCi/ml). For dual-labelling experiments, the medium contained L-[^{14}C]fucose (2 μCi/ml), U-^{14}C-labelled L-amino-acid mixture (1 μCi/ml), D-[^{14}C]galactosamine (4 μCi/ml) or $^{35}SO_4^{2-}$ (50 μCi/ml) in addition to 10 μCi of D-[^{3}H]glucosamine/ml. Stock solutions of all-*trans*-retinoic acid (up to 2 mM) were prepared in ethanol immediately before use and 5 μl of the appropriate concentration was added to each culture dish. Control cultures received 5 μl of ethanol only. Culture times were as given in the text and never exceeded 48 h. In some experiments human skin slices 0.5 cm square (obtained from plastic surgery procedures) were used instead of pig skin.

Separation of epidermis from dermis

For studies on the whole epidermis, skin slices were washed in phosphate-buffered saline, were treated with 1 M-$CaCl_2$ at 37°C for 1 h and the separated epidermis was solubilized with 8 M-urea/5% (w/v) SDS at 100°C (King *et al.*, 1980).

Separation of epidermis from dermis with trypsin and dispersal of epidermal cells with trypsin were as described previously (King *et al.*, 1980). Briefly, washed skin slices were treated with trypsin (0.2%, w/v) for 15 min at 37°C and the separated epidermis was trypsinized for a further 15 min. The epidermis was shaken vigorously for 1 min, the 'stratum corneum' fraction was removed and the remaining cell suspension was centrifuged (12000 ***g***) for 30 min at 4°C, yielding the 'cell pellet' fraction and the 'trypsin supernatant' fraction. These were then solubilized with 8 M-urea/5% (w/v) SDS at 100°C.

In experiments where the dermis was removed from the epidermis before culture, Dispase (0.1%, w/v, in phosphate-buffered saline, sterilized by Millipore filtration) was used for separation. Skin slices were treated for 30 min at 37°C and the epidermis was peeled away from the dermis. Under these conditions, very few cells are released from the epidermis. The separated epidermis was cultured as for whole skin slices.

Rate of synthesis and degradation of epidermal glycosaminoglycans

To study the rate of synthesis, skin slices were cultured in the presence of D-[^{3}H]glucosamine (10 μCi/ml) with or without 10^{-5} M-all-*trans*-retinoic acid for up to 48 h without any change of medium. At appropriate times, skin slices were washed, separated with trypsin and the epidermis was further trypsinized. The 'trypsin supernatant' fraction was

then analysed by polyacrylamide-gel electrophoresis. To study the rate of degradation, skin slices were cultured for 4 or 18h in the presence of D-[^{3}H]glucosamine (10 and 25μCi/ml respectively) without any added retinoic acid. After washing in phosphate-buffered saline, pH 7.3, (sterilized by Millipore filtration) the slices were cultured in medium containing 5mM-unlabelled glucosamine, with or without 10^{-5}M-all-*trans*-retinoic acid. At appropriate times, slices were washed and trypsinized as above and the 'trypsin supernatant' fraction was analysed by polyacrylamide gel electrophoresis.

Isolation of subcellular epidermal fractions

Skin slices were cultured for 18h with D-[^{3}H]glucosamine (25μCi/ml) with or without 10^{-5}M-all-*trans*-retinoic acid, were washed in phosphate-buffered saline and each slice was added to 1g of unlabelled pig ear skin. The tissue was minced, homogenized in 5mM-Tris/HCl (pH 7.5)/0.25M sucrose, filtered through nylon bolting cloth and the filtered homogenate was fractionated by differential centrifugation at $376\,g_{av.}$ and $113\,700\,g_{av.}$. The $113\,700\,g_{av.}$ pellet was further fractionated by centrifugation on discontinuous sucrose gradients as described previously (King *et al.*, 1980).

Polyacrylamide-gel electrophoresis

Samples in 8M-urea/5% (w/v) SDS/10mM-2-mercaptoethanol or 2% (w/v) SDS/10mM-2-mercaptoethanol were analysed by electrophoresis on 7% (w/v) polyacrylamide gels (King *et al.*, 1980) with the modified buffer system of Ugel *et al.* (1971). Gels were washed in 10% (w/v) trichloroacetic acid, sliced into 2mm sections using a BioRad model 190 gel slicer and were dissolved in H_2O_2 (100vol.)/conc. NH_3 (19:1, v/v) as described previously (King *et al.*, 1980).

Cellulose acetate electrophoresis

Skin slices were labelled with D-[^{3}H]glucosamine (10μCi/ml) and D-[^{14}C]galactosamine for 18h with or without 10^{-5}M-all-*trans*-retinoic acid and were washed and treated with trypsin. The 'trypsin supernatant' fraction was then treated with proteinase type VI (1mg/ml) for 18h at 60°C. Samples of hyaluronic acid and chondroitin sulphate B (100μg of each/ml) were added as carrier followed by an equal volume of 10% (w/v) trichloroacetic acid. After 1h at 0°C, precipitated protein was removed by low-speed centrifugation. The supernatant was mixed with 3vol. of 5% (w/v) potassium acetate in ethanol and was allowed to stand overnight at 4°C. Precipitated glycosaminoglycans were collected by low-speed centrifugation, washed in 2ml each of ethanol, ethanol/diethyl ether (1:1, by vol.) and diethyl ether and were dissolved in a small volume of water (50–100μl). Samples (up to 10μl) were analysed by electrophoresis on cellulose acetate in 0.05M-phosphate buffer, pH 7.2, for 1h with a constant current of 30mA. The sheets were stained with 1% (w/v) Alcian Blue in 1:1 (v/v) ethanol/0.05M-acetate buffer, pH 5.8, for 10min (Breen *et al.*, 1976). Background staining was removed by several washes in acetic acid/ethanol/water (1:2:17, by vol.). Each sample lane was then cut into 1cm strips which were dissolved in 1ml of 80% (v/v) acetic acid by leaving overnight at 15°–20°C.

Protein and radioactivity measurements

Protein was measured by the Hartree (1972) modification of the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as standard.

Radioactivity was measured by liquid-scintillation counting with PCS scintillation fluid (Hopkin and Williams, Romford, Essex RM1 1HA, U.K.).

Histological examination of cultured skin slices

Skin slices cultured with or without 10^{-5}M-all-*trans*-retinoic acid were prepared for light-microscope histology by conventional procedures. Sections were stained with Haematoxylin and Eosin (Drury & Wallington, 1967) or for carbohydrate by the periodate/Schiff/Alcian Blue procedure (Culling, 1963). Tissue sections were treated with Diastase to eliminate the possibility of positive reactions due to glycogen staining.

Results

Whole epidermis

In the first series of experiments we examined the effect of increasing concentrations of all-*trans*-retinoic acid on the total synthesis of epidermal glycoconjugates in pig skin slices cultured for 18h. Skin slices were cultured in the presence of D-[^{3}H]glucosamine to label total glycoconjugates together with $^{35}SO_4^{2-}$ to specifically label sulphated glycosaminoglycans, L-[^{14}C]fucose to label glycoproteins and ^{14}C-labelled L-amino acid mixture to label proteins (Fig. 1). Epidermis was separated from dermis by using 1M-$CaCl_2$ and was solubilized by using 8M-urea/5% (w/v) SDS at 100°C. all-*trans*-Retinoic acid at concentrations greater than 10^{-7}M caused a significant increase in the incorporation of D-[^{3}H]glucosamine into the solubilized epidermis. The total incorporation of $^{35}SO_4^{2-}$, L-[^{14}C]fucose and ^{14}C-labelled L-amino acid mixture into the epidermis was not significantly affected at any of the all-*trans*-retinoic acid concentrations used.

To determine the effect of all-*trans*-retinoic acid on the labelling of individual epidermal components, samples of solubilized epidermis were fractionated

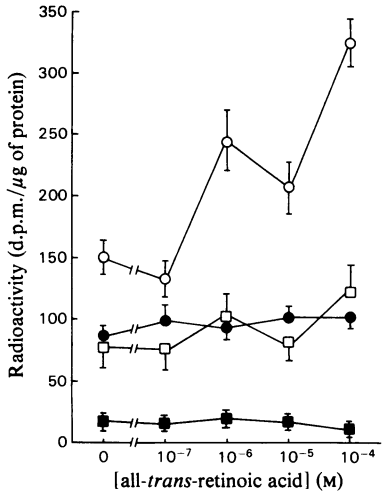

Fig. 1. *Effect of all-trans-retinoic acid on the incorporation of* D-[^{3}H]*glucosamine,* $^{35}SO_4^{2-}$, L-[^{14}C]*fucose and U-*^{14}*C-labelled* L*-amino acids into pig epidermis*
Pig skin slices were cultured for 18h in the presence of D-[^{3}H]glucosamine (10μCi/ml) together with $^{35}SO_4^{2-}$ (50μCi/ml), L-[^{14}C]fucose (2μCi/ml) or U-^{14}C-labelled L-amino acids (1μCi/ml) with increasing concentrations of all-*trans*-retinoic acid. The epidermis was separated from dermis with 1M-$CaCl_2$ and was extracted with 8M-urea/5% (w/v) SDS at 100°C. The radioactivity and protein content of the extracts was then determined. Each point represents the mean ± S.E.M. of three separate skin slices. ○, D-[^{3}H]glucosamine; ●, $^{35}SO_4^{2-}$; ■, L-[^{14}C]fucose; □, U-^{14}C-labelled L-amino acids.

by electrophoresis in 7% (w/v) polyacrylamide gels (Fig. 2). As we found previously in 42h cultures (King *et al.*, 1980), the major peak in control epidermis labelled with D-[^{3}H]glucosamine remained close to the top of the gel (Figs. 2*a*–2*c*). The incorporation of D-[^{3}H]glucosamine into this epidermal peak was increased several-fold in all-*trans*-retinoic acid-treated skin slices (Figs. 2*d*–2*f*). The labelling of this peak with $^{35}SO_4^{2-}$ (Fig. 2*d*) and L-[^{14}C]fucose (Fig. 2*e*) was slightly increased relative to the controls (Figs. 2*a* and 2*b*). all-*trans*-Retinoic acid had little effect on the labelling of other epidermal components with D-[^{3}H]glucosamine, $^{35}SO_4^{2-}$, L-[^{14}C]fucose or U-^{14}C-labelled L-amino acids.

Histological examination of pig skin slices cultured for 18h revealed that the migration of epidermal cells around the edges of the explant (epiboly) was unaltered in the presence of 10^{-5}M-all-*trans*-retinoic acid. Intercellular spaces were considerably widened. Periodate/Schiff/Alcian Blue-stained sections of control skin showed a faint positive reaction around the surface of cells in the living layers of control epidermis. A considerably stronger positive reaction was detected in the epidermis of all-*trans*-retinoic acid-treated skin slices. The increased staining was not uniform throughout the epidermis, but appeared to be localized in certain areas. This localization could not be correlated with any specific epidermal structures. In general, increased periodate/Schiff/Alcian Blue staining was more prominent near the edges of the explant and in the upper living layers of the epidermis.

Table 1. *Effect of all-trans-retinoic acid on the labelling of extracellular epidermal glycosaminoglycans in cultured human skin slices*
Slices of human skin (0.5cm × 0.5cm × 0.2mm thick) were cultured in the presence of D-[^{3}H]-glucosamine (10μCi/ml) and increasing concentrations of all-*trans*-retinoic acid for 18h. After washing, the epidermis was separated from the dermis with trypsin, was further trypsinized and the 'trypsin supernatant' fraction was analysed by electrophoresis in 7% (w/v) polyacrylamide gels which were sliced into 2mm sections. The values show the total radioactivity in the glycosaminoglycan peak (means of three separate skin slices ± S.E.M.).

Concn. of all-*trans*-retinoic acid (M)	10^{-3} × Radioactivity in glycosaminoglycan peak (d.p.m.)
0 (control)	6.9 ± 1.4
10^{-7}	14.9 ± 5.9
10^{-6}	17.6 ± 4.6
10^{-5}	13.1 ± 2.3
10^{-4}	5.7 ± 0.7

Effect of trypsin

When D-[^{3}H]glucosamine-labelled epidermis was treated with trypsin and shaken, the epidermal cells were released from the stratum corneum, which was mainly unlabelled (Figs. 3*a* and 3*d*). Centrifugation of the suspension of dispersed epidermal cells resulted in a 'cell pellet' fraction which was depleted of the high-molecular weight material labelled with D-[^{3}H]glucosamine (Figs. 3*b* and 3*e*). Most of this material was recovered in the 'trypsin supernatant fraction (Figs. 3*c* and 3*f*). Its distribution was similar in control and all-*trans*-retinoic acid-treated epidermis. About 75–85% was released by trypsin and was therefore extracellular. The labelled material in this extracellular peak was degraded by testicular hyaluronoglycosidase in both control and all-*trans*-retinoic acid-treated epidermis (results not shown). Thus it consisted entirely of glycosaminoglycans rather than glycoproteins in both cases.

all-*trans*-Retinoic acid also stimulated the incorporation of D-[^{3}H]glucosamine into extracellular epidermal glycosaminoglycans when human, rather than pig, skin slices were cultured for 18h (Table 1).

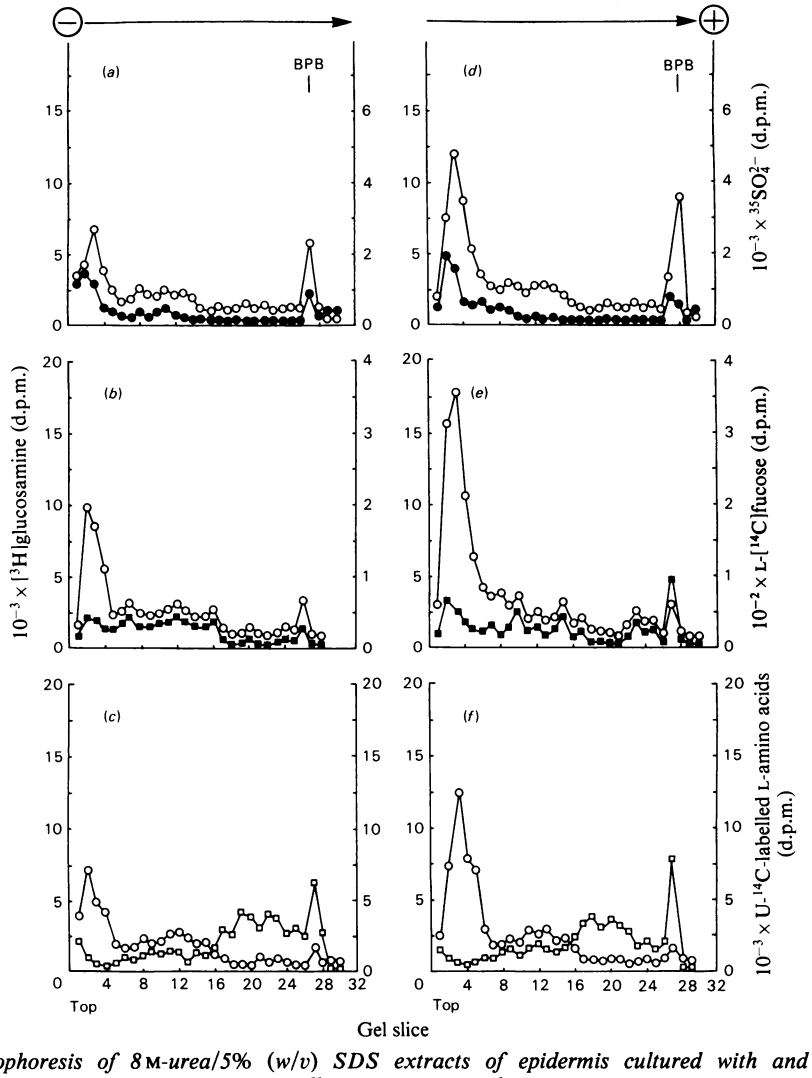

Fig. 2. *Gel electrophoresis of 8* M*-urea/5% (w/v) SDS extracts of epidermis cultured with and without* 10^{-5} M*-all-trans-retinoic acid*

The solubilized epidermis from skin slices cultured without (*a*, *b* and *c*) or with (*d*, *e* and *f*) 10^{-5} M-all-*trans*-retinoic acid as described in the legend to Fig. 1 was analysed by electrophoresis on 7% (w/v) polyacrylamide gels which were washed, frozen, sliced and dissolved as described in the Experimental section. Samples containing 750 μg of epidermal protein were applied to each gel. The minor differences in D-[^{3}H]glucosamine labelling patterns seen between sets (*a*) and (*d*), (*b*) and (*e*), and (*c*) and (*f*) arise from the fact that each set of double-labelling experiments was performed at different times. The arrow indicates the direction of electrophoretic migration. BPB indicates the position of the Bromophenol Blue tracking dye. O, D-[^{3}H]Glucosamine; ●, $^{35}SO_4^{2-}$; ■, L-[^{14}C]fucose; □, U-^{14}C-labelled L-amino acids.

When the epidermis was separated from the dermis before, rather than after, culture, the incorporation of D-[^{3}H]glucosamine into the glycosaminoglycan peak was severely and specifically decreased (King & Tabiowo, 1980). However, all-*trans*-retinoic acid stimulated the incorporation of D-[^{3}H]glucosamine into extracellular epidermal glycosaminoglycans even in the absence of dermis, when the control levels of incorporation were much decreased compared with whole skin (Table 2). Thus the primary effect of retinoic acid was on the epidermis rather than the dermis.

Rate of synthesis and degradation of extracellular epidermal glycosaminoglycans

The time course for the incorporation of D-

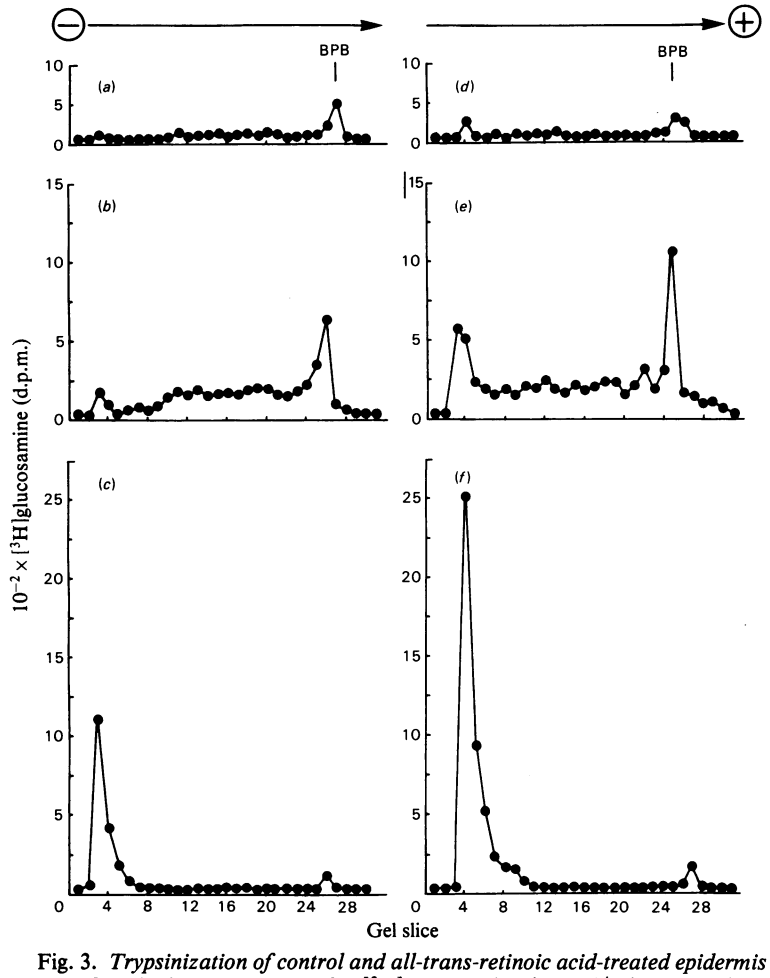

Fig. 3. *Trypsinization of control and all-trans-retinoic acid-treated epidermis*

Skin slices were cultured for 18h in the presence of D-[^{3}H]glucosamine (10μCi/ml) without (*a*, *b* and *c*) or with (*d*, *e* and *f*) 10^{-5}M-all-*trans*-retinoic acid. After washing, the epidermis was separated from dermis with trypsin and the epidermis was further trypsinized as described in the text. The three resulting epidermal fractions: stratum corneum (*a* and *d*), cell pellet (*b* and *e*) and trypsin supernatant (*c* and *f*) contained 13%, 44% and 43% respectively of the total radioactivity in control and 6%, 41% and 53% in 10^{-5}M-all-*trans*-retinoic acid-treated epidermis. One-tenth of each fraction was analysed by electrophoresis in 7% (w/v) polyacrylamide gels.

Table 2. *Effect of all-trans-retinoic acid on the labelling of extracellular epidermal glycosmainoglycans in cultured epidermis and cultured skin*

Pig skin slices were separated by using Dispase either before (cultured epidermis) or after (cultured skin) organ culture in the presence of D-[^{3}H]glucosamine for 18h. The medium also contained 10^{-5}M-all-*trans*-retinoic acid, or ethanol in the controls. The epidermis was trypsinized and the 'trypsin supernatant' was analysed by electrophoresis on 7% (w/v) polyacrylamide gels. The total radioactivity in the glycosaminoglycan peak was determined after slicing the gels. The values are means for three separate tissue samples ± S.E.M.

	$10^{-3} \times$ Radioactivity in glycosaminoglycan peak (d.p.m.)	
Tissue ...	Cultured epidermis	Cultured skin
Addition		
None (control)	2.1 ± 0.6	40.0 ± 6.3
10^{-5}M-all-*trans*-Retinoic acid	14.8 ± 2.4	89.0 ± 18.7

[^{3}H]glucosamine into extracellular epidermal glycosaminoglycans is shown in Fig. 4. Increased labelling was apparent after 4–8h and continued up to 48h in culture.

It was considered important to determine whether all-*trans*-retinoic acid affected the rate of degradation of extracellular epidermal glycosaminoglycans. Skin slices were labelled for 4h or 18h, were washed in phosphate-buffered saline and were then placed on fresh medium containing an excess of unlabelled glucosamine with or without 10^{-5}M-all-*trans*-retinoic acid. The rate of decrease in the labelling of the extracellular epidermal glycosaminoglycan peak in skin slices chased after 4h (Fig. 5*a*) or 18h in culture (Fig. 5*b*) was not significantly affected by all-*trans*-retinoic acid.

Characterization of extracellular epidermal glycosaminoglycans

Electrophoresis on cellulose acetate at pH 7.2 indicated that hyaluronic acid accounted for 80–90% of the total extracellular epidermal glycosaminoglycan labelled by D-[^{3}H]glucosamine in both control (Fig. 6*a*) and all-*trans*-retinoic acid-treated skin slices (Fig. 6*b*). An identical pattern of glycosaminoglycan labelling was obtained when D-[^{14}C]galactosamine was used as the radioactive precursor (Fig. 6). Thus the predominance of labelled hyaluronic acid did not reflect preferential incorporation of D-[^{3}H]glucosamine into glucosaminoglycans rather than into galactosaminoglycans.

Plasma membrane glycoproteins

To determine the effect of all-*trans*-retinoic acid on the synthesis of epidermal plasma membrane

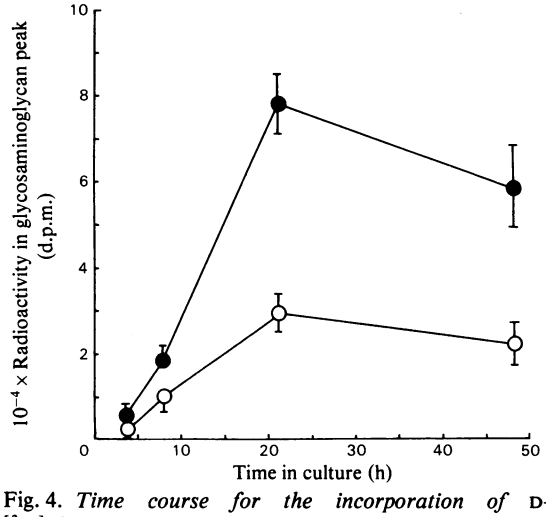

Fig. 4. *Time course for the incorporation of* D-[^{3}*H*]*glucosamine into extracellular glycosaminoglycans in control and all-trans-retinoic acid-treated epidermis*

Skin slices were cultured without (○) or with (●) 10^{-5}M-all-*trans*-retinoic acid for the indicated time, were washed, separated with trypsin and the epidermis was further trypsinized. The 'trypsin supernatant' obtained after centrifuging the dispersed epidermal cells was subjected to electrophoresis in 7% (w/v) polyacrylamide gels. The total radioactivity in the glycosaminoglycan peak was determined after slicing the gels. Values are the means ± S.E.M. for three separate skin slices.

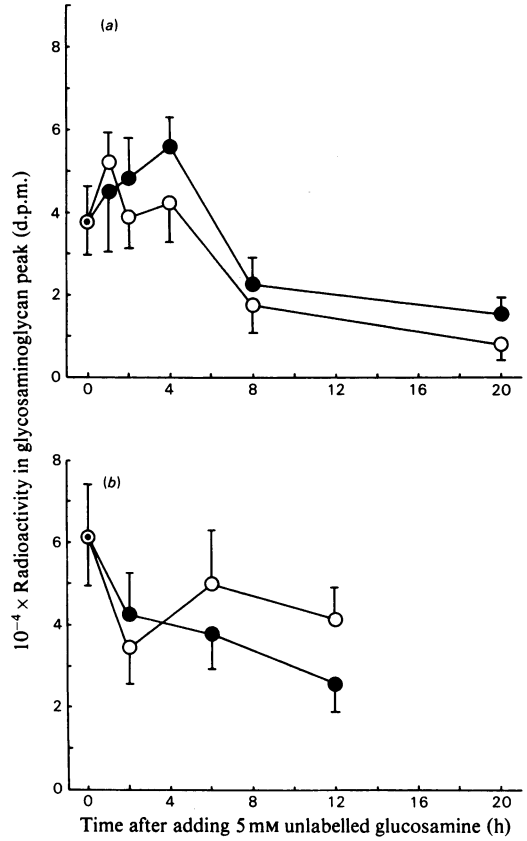

Fig. 5. *Rate of degradation of extracellular glycosaminoglycans in control and* 10^{-5}M-*all-trans-retinoic acid-treated epidermis*

Skin slices were cultured for (*a*) 4h with D-[^{3}H]glucosamine (25μCi/ml) and (*b*) 18h with D-[^{3}H]glucosamine (10μCi/ml). After washing in sterile phosphate-buffered saline, the slices were transferred to fresh medium containing 5mM-unlabelled glucosamine without (○) or with (●) 10^{-5}M-all-*trans*-retinoic acid. At appropriate times slices were removed, washed, separated with trypsin and the epidermis was further trypsinized. The radioactivity in the glycosaminoglycan peak was determined after polyacrylamide-gel electrophoresis of the 'trypsin supernatant' fraction. Values are the means ± S.E.M. of three separate skin slices.

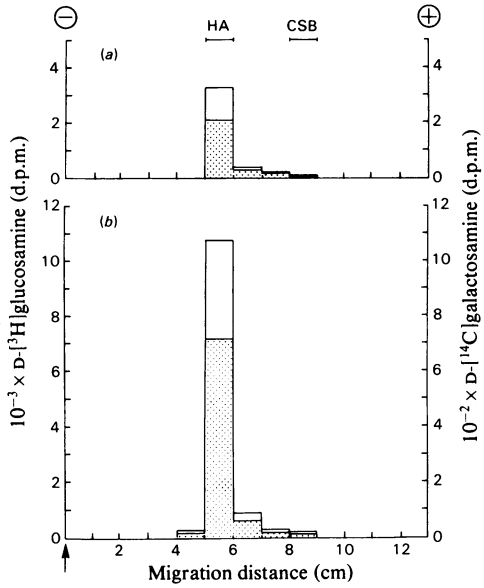

Fig. 6. *Cellulose acetate electrophoresis of extracellular glycosaminoglycans from control and all-trans-retinoic acid-treated epidermis*

Skin slices were cultured (*a*) without or (*b*) with 10^{-5} M-all-*trans*-retinoic acid in the presence of D-[^{3}H]glucosamine (10 μCi/ml) and D-[^{14}C]-galactosamine (4 μCi/ml) for 18 h. After trypsinization and cell dispersal, the 'trypsin supernatant' fraction was extensively degraded with Pronase (1 mg/ml). After adding carrier glycosaminoglycans, the trichloroacetic acid-soluble material was precipitated with ethanol, washed as described in the Experimental section and equivalent sample volumes were applied to cellulose acetate sheets (16 cm × 17 cm). Electrophoresis was carried out at 30 mA for 1 h. The sheets were stained with Alcian Blue and were cut into 1 cm sections which were dissolved in 80% (w/v) acetic acid before measurement of radioactivity. Open area, D-[^{3}H]glucosamine; shaded area, D-[^{14}C]galactosamine. HA and CSB indicate the position of hyaluronic acid and chondroitin sulphate B standards.

glycoproteins, D-[^{3}H]glucosamine-labelled skin slices were mixed with unlabelled skin as carrier, homogenized and the filtered homogenate was fractionated by differential centrifugation. The crude membrane fraction (113 700 $g_{av.}$ pellet) was further fractionated by sucrose-density gradient centrifugation (Table 3). The labelling of the plasma membrane fraction which banded at the 0.5 M/1.0 M-sucrose interface was not affected in all-*trans*-retinoic acid-treated skin slices. Polyacrylamide-gel electrophoresis indicated that the labelling of the major glycoproteins in the plasma membrane (apparent mol.wt. range 150000–70000) was unaffected by all-*trans*-retinoic acid. An apparent increase in the relative labelling of lower apparent molecular weight minor glycoproteins was occasionally observed in the presence of all-*trans*-retinoic acid. However, these changes were not consistent either in the degree of alteration or in apparent molecular weight.

Discussion

Several studies have shown that retinoids affect cell surface properties. In fibroblasts, retinoids altered cell morphology, increased cell–substratum adhesion and induced density-dependent inhibition of growth (Dion *et al.*, 1977; Lotan & Nicolson, 1977; Jetten *et al.*, 1979). The synthesis of glycolipids and sulphated glycosaminoglycans was increased and the profile of surface-exposed proteins was altered (Patt *et al.*, 1978; Jetten *et al.*, 1979). Synthesis of receptors for epidermal growth factor was increased (Jetten, 1980). There are few comparable studies on epithelial cells, despite the fact that epithelia are target tissues for retinoids *in vivo*. Using an established cell line derived from intestinal epithelium, Shapiro & Poon (1979) showed that retinoic acid also affected cell morphology and growth rate. However, unlike fibroblasts, cell adhesion was decreased, as was the synthesis of sulphated glycosaminoglycans. No changes could be detected in the surface glycoprotein receptors for concanavalin A.

In the epidermis, evidence for retinoid-induced surface changes has come mainly from ultrastructural studies. In retinoic acid-treated epidermis the number of desmosomes was decreased, intercellular spaces were widened and some surface membranes formed new microvilli on which a filamentous surface coat was observed (Barnett & Szabo, 1973). Proliferation of the Golgi and endoplasmic reticulum, together with histochemical evidence, indicated increased glycoprotein synthesis (Yuspa & Harris, 1974). In support of this, it was shown that retinyl acetate stimulated the incorporation of radioactive sugars into glycopeptides isolated from cultured epidermal cells after extensive proteolytic digestion (DeLuca & Yuspa, 1974; Adamo *et al.*, 1979). However, the relationship of these glycopeptides to the complex carbohydrates of the cell surface was not explored. Retinyl acetate was also found to stimulate the incorporation of $^{35}SO_4^{2-}$ into sulphated glycosaminoglycans synthesized and secreted by epidermal cells in culture (Shapiro & Poon, 1978).

In the present study we examined the effect of all-*trans*-retinoic acid on the synthesis specifically of surface-associated carbohydrates in the epidermis of organ-cultured skin slices. The major effect was an

Table 3. *Effect of all-trans-retinoic acid on the labelling of epidermal subcellular fractions*

Pig skin slices were cultured for 18h without or with 10^{-5}M-all-*trans*-retinoic acid in the presence of D-[^{3}H]-glucosamine (25μCi/ml). Each slice was mixed with 1g of unlabelled pig skin, homogenized and the homogenate was fractionated by differential centrifugation at 376$g_{av.}$ and 113700$g_{av.}$ as described in the Experimental section. The 113700$g_{av.}$ pellet was further fractionated by centrifugation on sucrose-density gradients. The values are means for three different skin slices ± S.E.M.

	Specific radioactivity (d.p.m./μg of protein)	
Addition ...	None	10^{-5}M-all-*trans*-
Fraction	(control)	Retinoic acid
Homogenate	37 ± 2.5	46 ± 1.0
376$g_{av.}$ pellet	20 ± 1.0	28 ± 1.2
113700$g_{av.}$ pellet	88 ± 9.2	62 ± 6.0
0.5/1.0M-sucrose	140 ± 6.1	138 ± 6.4
1.0/1.5M-sucrose	101 ± 6.6	86 ± 10.0
1.5/2.0M-sucrose	57 ± 4.1	51 ± 3.9
113700$g_{av.}$ supernatant	57 ± 5.2	75 ± 4.6

increase in the incorporation of D-[^{3}H]glucosamine into epidermal glycosaminoglycans (Fig. 2). Their distribution in the epidermis was unchanged, with most of the newly synthesized glycosaminoglycan released by trypsin and therefore extracellular (Fig. 3). The increased labelling of extracellular epidermal glycosaminoglycans in all-*trans*-retinoic acid-treated epidermis was due to increased synthesis since 'chase' experiments indicated that their rate of degradation was unaffected (Fig. 5).

Our results indicate that increased glycosaminoglycan synthesis may be the first response of the epidermis to excess retinoic acid. Increased glycosaminoglycan synthesis was observed under conditions where incorporation of ^{14}C-labelled amino acids (reflecting synthesis mainly of tonofilament polypeptides) was unaffected (Figs. 1 and 2). The synthesis of epidermal plasma-membrane glycoproteins was also unaffected (Table 3). all-*trans*-Retinoic acid had no effect on the epidermal incorporation of L-[^{14}C]fucose under the conditions used here (Fig. 1), suggesting that there was no stimulation of fucose-containing mucous glycoprotein synthesis. Thus the effect on epidermal glycosaminoglycan synthesis precedes the major effects observed in previous ultrastructural studies, i.e. inhibition of tonofilament synthesis, inhibition of desmosome formation and induction of mucin synthesis.

It is known that the dermis influences the differentiation pathway of the epidermis (Billingham & Silvers, 1967). McLoughlin (1961) has shown that gizzard mesenchyme can induce mucous metaplasia in overlying chick epidermis. For these reasons it has been suggested that the effect of retinoids on the epidermis may occur at the level of dermal–epidermal interaction (Hardy, 1974). Our results do not support this view. Although the dermis is required for the complete synthesis of glycosaminoglycans in the epidermis (King & Tabiowo, 1980), stimulation of their synthesis by retinoic acid was observed even in the absence of the dermis (Table 2). It is likely therefore that retinoids act directly on epidermal cells rather than via their interaction with the dermis.

Hyaluronic acid was the major glycosaminoglycan synthesized by both control and all-*trans*-retinoic acid-treated epidermis in organ culture (Fig. 6). It accounted for 80–90% of the total labelled glycosaminoglycan compared with values of 50–60% obtained by chemical analyses of adult epidermis that had not been cultured (Mier & Wood, 1969). Thus the rate of synthesis of hyaluronic acid in the epidermis of organ-cultured skin slices is probably rather higher than in adult skin *in vivo*. In this respect the organ culture system appears to resemble foetal skin, where the hyaluronic acid concentration is also very high and decreases during foetal development (Breen *et al.*, 1970).

The mechanism by which retinoids alter epithelial differentiation is not known. However, three different levels of action have been proposed. Firstly, they may act at the level of membrane organization (Roels *et al.*, 1969) causing labilization of lysosomes and increased degradation of cellular structures (Lazarus *et al.*, 1975). Secondly, they may act at the level of gycosylation reactions by their postulated role in glycosyl-transfer reactions (Wolf *et al.*, 1979). Thirdly, they may act at the nuclear level. A retinoic acid-binding protein has been identified (Sani & Hill, 1974) which may enter the nucleus (Sani & Donovan, 1979) and act in a manner analogous to that of the steroid receptors (Chytil & Ong, 1978). The increased synthesis of epidermal glycosaminoglycans observed in the present study with all-*trans*-retinoic acid is unlikely to

be due to an effect on lysosomes. The rate of degradation of these components was not affected (Fig. 5). It is not possible to distinguish between an effect on glycosylation reactions or on gene expression. However, the rapid effect of all-*trans*-retinoic acid on glycosaminoglycan synthesis suggests that the former possibility is more likely.

Increased epidermal glycosaminoglycan synthesis was observed in human as well as pig skin treated with all-*trans*-retinoic acid (Table 1) indicating a similar mechanism in both cases. The present results therefore have important implications for understanding the mode of action of natural and synthetic retinoids in the treatment of disorders of keratinization.

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