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RETINOIDS INCREASE THE INCORPORATION OF D-[^{3}H]GALACTOSE INTO EPIDERMAL GLYCOPROTEINS.

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All-trans retinoic acid increased the incorporation of D-[^{3}H]galactose into particulate and soluble glycoproteins in the epidermis of cultured pig skin slices nearly two-fold. Increased incorporation of D-[^{3}H]galactose was not blocked by tunicamycin. This effect was specific for D-[^{3}H]galactose since the incorporation of D-[^{3}H]glucosamine and L-[^{14}C]leucine into epidermal glycoproteins was unaffected by all-trans retinoic acid. All-trans retinoic acid and 13-cis retinoic acid had quantitatively similar effects on D-[^{3}H]galactose incorporation. All-trans retinyl acetate and an aromatic retinoic acid analogue ('Etretinate') were less effective. SDS polyacrylamide gel electrophoresis and fluorography showed increased incorporation of D-[^{3}H]galactose into all epidermal glycoproteins in the presence of all-trans retinoic acid. There was no evidence for synthesis of new glycoproteins such as mucins.

Although it is not yet clear how retinoids exert their influence over epithelial differentiation, an effect on the synthesis of glycoconjugates is thought to be involved (1,2). All-trans retinyl acetate has been shown to increase the incorporation of radioactive sugars into total glycopeptides of cultured mouse keratinocytes (3,4,5) although it was not clear whether the synthesis of specific glycoproteins was altered. We have previously used an explant culture system to study the biosynthesis of complex carbohydrates in pig ear epidermis (6). Retinoids increased the incorporation of D-[^{3}H]glucosamine into epidermal glycosaminoglycans, mainly hyaluronic acid (7), by up to three-fold (8,9) but had little effect on the labelling of epidermal glycoproteins with this sugar. We now show that retinoids increase the incorporation of D-[^{3}H]galactose into epidermal glycoproteins under conditions where their labelling with glucosamine and leucine is unaffected. Altered glycosylation of epidermal glycoproteins as

Abbreviation: SDS - sodium dodecyl sulphate

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well as increased synthesis of glycosaminoglycans may therefore be important for the biological activity of retinoids in this tissue.

MATERIALS AND METHODS

Materials. D-[1-^{3}H]galactose (10 Ci/mmol). D-[6-^{3}H]glucosamine hydrochloride (25 Ci/mmol) and, L-[U-^{14}C]leucine (330 mCi/mmol) were from Amersham International. All-trans retinoic acid (type XX) and all-trans retinyl acetate (type I) were from Sigma. 13-cis retinoic acid and an aromatic retinoic acid analogue [ethyl all-trans-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8 nonatetraenoate or 'Etretinate'] were obtained from Roche Products Ltd., Welwyn Garden City, Hertfordshire, U.K., courtesy of Dr. A.J. Miller. Tunicamycin was a gift from Professor G. Tamura, Department of Agricultural Chemistry, University of Tokyo. Dispase was from Boehringer. Sheep testicular hyaluronidase was from Miles Laboratories.

Explant culture. This was essentially as described previously using 1 cm square slices of pig ear skin (6) floated for 18 hr on 1 ml of Eagles minimum essential growth medium containing 10% (by vol.) foetal calf serum and 50 μCi/ml D-[^{3}H]galactose or 50 μCi/ml D-[^{3}H]glucosamine. In some dual-labelling experiments L-[^{14}C]leucine (15 μCi/ml) was included with the labelled sugars. Freshly-prepared retinoids (2 x 10^{-3}m) were added to a final concentration of 10^{-5}M. Control cultures received the same volume (5 μl/ml) of ethanol. In some experiments tunicamycin (2 mg/ml in 25mM NaOH) or 25mM NaOH (1 μl/ml) was added to the culture medium (10). Epidermis was separated from dermis after treating washed labelled skin slices with 1M $CaCl_2$ (1 hr at 37°C) or 0.1% (w/v) Dispase in phosphate-buffered saline (30 min at 37°C). In the latter case epidermis was washed four times with phosphate-buffered saline at 0 to 4°C to remove the enzyme.

Whole epidermal glycoproteins. $CaCl_2$ separated epidermis from each labelled skin slice was minced and homogenized for 1 min in 10 ml of 10mM Tris, HCl pH 7.5, filtered through nylon bolting cloth (hole size 0.13 x 0.13 mm) and was mixed with an equal volume of 10% (w/v) trichloroacetic acid at 0°C for 30 min. The precipitate was resuspended in a minimum of 1M KOH and aliquots were taken for measurement of protein (11) and radioactivity.

Hydrolysis and Chromatography. Previous studies have established the validity of using D-[^{3}H]glucosamine to label epidermal glycoconjugates (6). To check for possible randomization of labelling with D-[^{3}H]galactose, the trichloroacetic acid-insoluble fraction from $CaCl_2$-separated, D[^{3}H]galactose-labelled epidermis, was dialysed against H_2O, freeze-dried and then hydrolysed in 1M H_2SO_4 at 100°C *in vacuo* for 4 hr. A neutral sugar fraction, prepared from the hydrolysate by ion-exchange chromatography (12) was examined by paper chromatography in ethyl acetate/pyridine/H_2O (10:4:3). Standard sugars were detected by $AgNO_3$ staining. Chromatograms were cut into 1 cm strips which were eluted with water and the radioactivity in the eluate was determined.

Subcellular fractionation. When specific radioactivities (dpm/mg protein) were to be measured in subcellular fractions, Dispase-separated epidermis was mixed with unlabelled carrier tissue, homogenized in 0.25M sucrose/10 mM Tris, HCl pH 7.5, filtered and was fractionated by differential centrifugation at 376 g_{av} and 113700 g_{av} (6). In some experiments the particulate fraction (113700 g_{av} pellet) was further fractionated by sucrose density gradient centrifugation and a plasma membrane-enriched fraction banding at the 0.5 M/1.0 M sucrose interface was isolated (6).

When subcellular fractions of labelled epidermis were to be examined by SDS polyacrylamide gel electrophoresis, $CaCl_2$-separated epidermis from labelled skin slices was minced, homogenized in 10 mM Tris HCl pH 7.5 and filtered as above. The particulate fraction (113700 g_{av} pellet) was extracted with 2% (w/v) SDS, 10% (w/v) glycerol, 0.01% (w/v) bromophenol blue at 100°C for 5 min

while the soluble fraction (113700 g_{av} supernatant) was dialysed against 10 mM Tris HCl pH 7.5/0.25 mM phenylmethane sulphonyl fluoride at 4°C and freeze dried before extraction in SDS sample buffer. In some experiments, aliquots of the soluble fraction were treated with hyaluronidase (0.1 mg/ml) for 1 h at 37°C before solubilization with SDS. Samples were reduced with 5% 2-mercapto-ethanol and analysed on 7% (w/v) polyacrylamide slab gels (13). Gels were fixed in 25% (w/v) propan-2-ol/10% (v/v) acetic acid before fluorography (14,15).

RESULTS

Whole epidermis. All-trans retinoic acid in ethanol increased the incorporation of D-[^{3}H]galactose into the trichloroacetic acid-insoluble fraction of epidermal homogenates nearly two-fold compared to ethanol alone (Table 1). Tunicamycin (2 μg/ml) in 25 mM NaOH reduced the total epidermal incorporation of D-[^{3}H]galactose by about 50% compared to the solvent control. However, even in the presence of tunicamycin, all-trans retinoic acid was still found to increase D-[^{3}H]galactose incorporation into the epidermis nearly two-fold (Table 1). Thus the observed effect of all-trans retinoic acid was unlikely to be due to an effect on the formation of dolichol-linked sugar intermediates.

Paper chromatography of neutral sugar fractions from control (Fig. 1a) and all-trans retinoic acid-treated epidermis (Fig. 1b) showed that at least 75% of the radioactivity in epidermal glycoproteins was still present as D-[^{3}H]galactose.

Table 1. Effect of all-trans retinoic acid and tunicamycin on incorporation of D-[^{3}H]galactose into epidermal glycoproteins

Addition to Medium	Incorporation of D-[^{3}H]galactose into epidermal homogenates. dpm x 10^{-3}/mg protein	experimental / solvent control
Ethanol	60.4 ± 9.6	1.88
Ethanol + retinoic acid	113.6 ± 10.0	
NaOH	101.6 ± 16.4	0.48
NaOH + tunicamycin	49.2 ± 8.8	
Ethanol + NaOH	65.2 ± 10.4	0.83
Ethanol + NaOH + retinoic acid + tunicamycin	54.0 ± 6.0	

Skin slices were labelled with D-[^{3}H]galactose in the presence of all-trans retinoic acid in ethanol (10^{-5}M) and/or tunicamycin in 25mM NaOH (2 μg/ml). Control cultures received ethanol and/or NaOH. After separation with 1M $CaCl_2$ the epidermis was homogenized and treated with trichloroacetic acid as described in the Methods section. Protein and radioactivity were measured in the acid insoluble fraction. Each value is the mean ± S.E.M. of nine skin slices examined in three separate experiments.

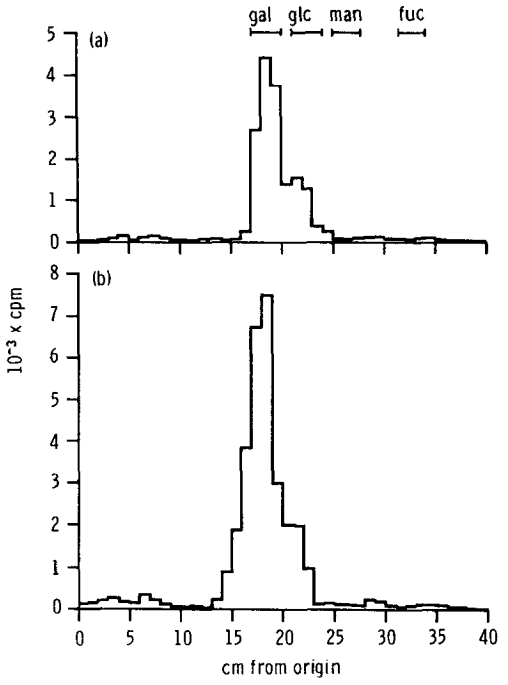

Fig. 1. Paper chromatography of hydrolysates from D-[^{3}H]galactose-labelled epidermis. The neutral sugar fraction from hydrolysates of acid-insoluble components of control (a) and all-trans retinoic acid-treated epidermis (b) was examined by paper chromatography in ethyl acetate/pyridine/H_2O (10:4:3, by vol) for 20 hr as described in the Methods section.

Subcellular fractions. All-trans retinoic acid increased the incorporation of D-[^{3}H]galactose into acid-insoluble components of both particulate and soluble fractions to a similar extent (Table 2). The increase in D-[^{3}H]galactose labelling caused by all-trans retinoic acid in these subcellular fractions from Dispase-separated epidermis was comparable with that found in $CaCl_2$-separated epidermal homogenates (Table 1). Increased incorporation of D-[^{3}H]galactose was also observed in plasma membrane-enriched vesicles isolated from the total particulate fraction by sucrose density gradient centrifugation (Table 2). The effect of all-trans retinoic acid was specific for D-[^{3}H]galactose since the incorporation of D-[^{3}H]glucosamine and L-[^{14}C]leucine into acid-insoluble components was little affected (Table 2).

Quantitatively similar effects on D-[^{3}H]galactose incorporation were observed with all-trans retinoic acid and 13-cis retinoic acid (Table 2). Although all-trans retinyl acetate and the aromatic retinoic acid analogue also increased

Table 2. Effect of four retinoids on incorporation of ^{3}H-labelled sugars into subcellular fractions of epidermis.

	Labelling of subcellular fraction from retinoid-treated epidermis relative to control		
	[^{3}H]gal (6)	[^{3}H]glcN (3)	[^{14}C]leu (3)
Particulate fraction			
all-trans retinoic acid	2.1 ± 0.2	1.1 ± 0.1	1.2 ± 0.1
all-trans retinyl acetate	1.5 ± 0.2	1.1 ± 0.1	1.0 ± 0.05
13-cis retinoic acid	1.9 ± 0.3	0.8 ± 0.1	1.1 ± 0.1
aromatic retinoid	1.8 ± 0.3	0.8 ± 0.1	0.9 ± 0.1
Plasma membrane-enriched fraction			
all-trans retinoic acid	1.7	1.1	1.2
all-trans retinyl acetate	1.3	1.3	1.1
13-cis retinoic acid	1.9	0.9	1.0
aromatic retinoid	1.4	0.9	0.9
Soluble fraction			
all-trans retinoic acid	2.0 ± 0.1	1.0 ± 0.1	1.2 ± 0.1
all-trans retinyl acetate	1.4 ± 0.2	1.1 ± 0.2	1.1 ± 0.05
13-cis retinoic acid	2.3 ± 0.5	0.9 ± 0.05	1.1 ± 0.05
aromatic retinoid	1.7 ± 0.2	0.8 ± 0.05	0.8 ± 0.1

Skin slices were cultured with the appropriate ^{3}H-labelled sugar or with [^{14}C]leucine for 18 h in the absence or presence of each retinoid (10^{-5}M). After separation with Dispase and dilution with unlabelled carrier tissue, the epidermis was homogenized, filtered and fractionated into particulate and soluble fractions by differential centrifugation at 113700 g_{av} as described in the Methods section. The 113700 g_{av} pellets were pooled from several experiments and were further fractionated by sucrose density gradient centrifugation to yield a plasma membrane-enriched fraction. Specific radioactivities (dpm/mg protein) were determined for each fraction after precipitation with trichloroacetic acid. The results are expressed as specific radioactivity of each subcellular fraction from retinoid-treated epidermis divided by that of the same fraction from control epidermis. Values are the means for the number of skin slices shown in parentheses ± S.E.M.

incorporation of D-[^{3}H]galactose into particulate and soluble fractions of the epidermis, these retinoids were generally less effective than all-trans retinoic acid and 13-cis retinoic acid.

SDS polyacrylamide gel electrophoresis. Particulate and soluble fractions from $CaCl_2$-separated epidermis were examined by SDS polyacrylamide gel electrophoresis and fluorography (Fig. 2) to determine whether specific glycoproteins were affected by all-trans retinoic acid. The labelled band in the stacking gel (most prominent in the soluble fraction) was degraded by testicular hyaluronidase (results not shown), suggesting that it consisted mainly of hyaluronic acid. The

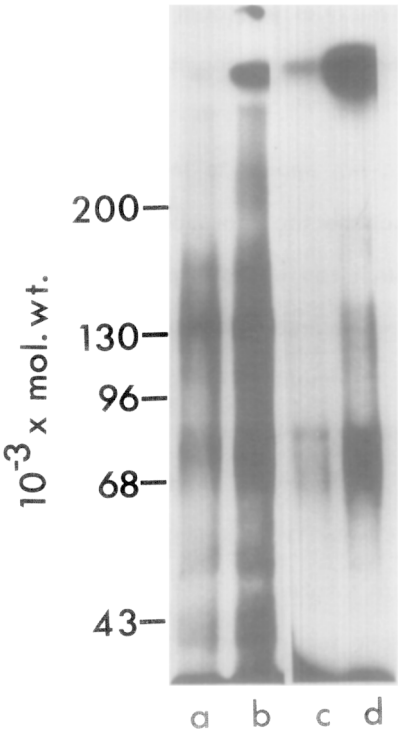

Fig. 2. SDS polyacrylamide gel electrophoresis of particulate and soluble fractions from control and all-trans retinoic acid-treated epidermis. Particulate (a,b) and soluble (c,d) fractions of $CaCl_2$-separated epidermis from control (a,c) and all-trans retinoic acid-treated (b,d) skin slices labelled with D-[^{3}H]galactose were solubilized and examined by SDS polyacrylamide gel electrophoresis and fluorography. 50 μg of protein was applied to each track. Similar results were obtained in four separate experiments.

incorporation of D-[^{3}H]galactose into this material was increased by all-trans retinoic acid confirming our previous findings of an effect of epidermal hyaluronic acid synthesis (8,9). In the resolving gel the incorporation of D-[^{3}H]galactose into all particulate and soluble components was increased to a similar extent, suggesting that the labelling of all epidermal glycopoteins was increased similarly. No new labelled glycoproteins were detected in all-trans retinoic acid-treated epidermis.

DISCUSSION

Despite the increased periodic acid-Schiffs staining observed in retinoic acid-treated pig epidermis (8) there was no evidence in the present work for retinoid-induced synthesis of mucins. All-trans retinoic acid increased the incorporation of D-[^{3}H]galactose into a band of high mol. wt. material in the stacking gel

(Fig. 2). However, this material was completely degraded by hyaluronidase, confirming our previous finding that it consisted of glycosaminoglycans rather than mucous glycoproteins (8).

Although retinoids did not appear to induce the synthesis of novel glycoproteins they did affect the incorporation of D-[^{3}H]galactose into all the glycoproteins normally synthesized by adult pig epidermis in vitro. The incorporation of D-[^{3}H]mannose into particulate and soluble glycoproteins was also increased by retinoid treatment (I.A. King, unpublished observation). However, their labelling with D-[^{3}H]glucosamine and [^{14}C]leucine (Table 2) was not significantly affected. Tunicamycin did not block the effects of retinoids on D-[^{3}H]galactose labelling (Table 1), suggesting that an effect on dolichol-linked sugar intermediates was not involved. There are several possible explanations for increased D-[^{3}H]galactose incorporation into epidermal glycoproteins. A direct or indirect role for galactosyl derivatives of retinoids (18) in glycoprotein synthesis such as suggested for mannosyl retinyl phosphate (1,5,17) cannot be ruled out. An effect on galactosyl transferase activity as found in rat tracheal epithelium (18) is another possibility. A third possible explanation is that retinoid treatment affects the size of UDP-galactose pools in the epidermis. Although the reason for increased incorporation of D-[^{3}H]galactose is not yet clear it seems likely from the present results that retinoids may have an effect on the glycosylation of epidermal glycoproteins in vitro. Nemanic et al (19) observed altered lectin staining in the epidermis of retinoid-treated mice that was consistent with altered glycosylation of epidermal glycoconjugates in vivo.

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