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CELL FUSION, HAEMOLYSIS AND MITOCHONDRIAL SWELLING INDUCED BY RETINOL AND DERIVATIVES

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Summary

A comparative study has been made of the abilities of retinol and its derivatives to induce cell fusion and haemolysis of hen erythrocytes and to cause swelling of rat liver mitochondria. Retinol, retinaldehyde, α-retinoic acid, iso-13-retinol and to a lesser extent retinyl acetate were active in all three systems. Iso-13-retinoic acid was extremely membranolytic but did not produce stable fused cells. By contrast retinoic acid, its cyclopentyl derivative RO8-7699, and the long chain fatty acid esters of retinol, viz. the oleate, linoleate and palmitate esters, were neither fusogenic nor haemolytic, nor did they affect mitochondria.

Introduction

Retinol (vitamin A alcohol) is a surface active molecule [1,2] that is one of a number of lipophilic compounds previously shown to be capable of causing cells to fuse [3—6], of which only glycerol mono-oleate has been studied in detail [3]. Vitamin A influences secretory events both in vivo [7] and in vitro [8—12], and several of the actions of excess of retinol have been attributed to its effects on membranes [2,13]. There is considerable current interest in the developmental changes induced in epithelial tissues [14,15] by retinoids (synthetic derivatives of retinol), and in the possible use of retinoids as chemotherapeutic agents for treating epithelial neoplasia [16,17]. Some comparisons of the activities of naturally-occurring and synthetic derivatives of retinol on cell and membrane systems have been made [1,18—22] and in the present paper we have extended this approach by examining a group of derivatives of retinol for their ability to induce the fusion of hen erythrocytes. Fusogenic

properties have also been compared with haemolysis, and with the ability of the substances studied to swell rat liver mitochondria in vitro.

Materials and Methods

Preparation of cells. Hen erythrocytes were collected and prepared on each day of use as described previously [3], and resuspended in modified Eagle's basal salt solution (MEM-Dextran), buffered at pH 5.6, which contained 80 g/l Dextran 60C (Sigma Chemical Company Limited, London, England).

Retinoids. Retinol, retinoic acid and retinyl acetate were from Roche Products Limited (Welwyn Garden City, Herts, England), retinyl palmitate and retinaldehyde were from the Sigma Chemical Company Ltd., retinyl oleate, retinyl linoleate, iso-13-retinol, iso-13-retinoic acid, α-retinoic acid and a dimethylacetylcyclopentanyl derivative of retinoic acid (RO8-7699) were the generous gifts of Dr. Dewitt S. Goodman [18].

The retinoids were dissolved in absolute ethanol and stored at a concentration of 35 or 350 mM at 4°C, under nitrogen in the dark [20]. They were diluted in ethanol for each day's experiment, and characterised by their ultra violet spectra and extinction coefficients [23,24] just prior to use.

Fusion and haemolysis. Fusion and haemolysis were produced by adding aqueous dispersions of the retinoids to hen erythrocytes, pre-warmed to 37°C, in the ratio of 1.0 : 1.6 (cell suspension to retinol dispersion) to give a final cell suspension of 3×10^8 cells/ml, and final concentrations of the retinoids of 87.5—1400 μM. Dispersions of retinoids were prepared by injecting 25—100 μl of their solutions (35 mM) in ethanol into MEM-Dextran, at room temperature using a Hamilton syringe. The aqueous dispersions were added to the cell within 10 s of their preparation to minimise oxidation of the retinoids. The concentration of ethanol in the final suspension was between 0.5 and 2% (v/v). Cell fusion was monitored by continual observation using a phase-contrast microscope (Zeiss Standard WL).

Following treatment with fusogenic retinoids, cells rounded and only after a distinct interval were binucleate and multinucleate cells observed. Once fusion had started it proceeded quite rapidly. The distinct lag period before fusion began was readily and reproducibly measured and has been called the time to fusion (t_f) [3]. Haemolysis of the erythrocytes was determined by measuring the absorbance of haemoglobin as cyanomethaemoglobin at 540 nm in the supernatant obtained by centrifuging the incubation mixture, at least 10 min after the addition of 0.2 ml of this supernatant to 4.8 ml of a solution containing $NaHCO_3$ (1 g/l), $K_3Fe(CN)_6$ (0.2 g/l) and KCN (0.2 g/l) [25]. Haemolysis was expressed as a percentage of the total haemoglobin measured in uncentrifuged samples, treated with 1% (v/v) Triton X100 for 15 min at 37°C.

Mitochondrial swelling. Rat liver mitochondria were prepared in sucrose-Tris, pH 7.4 [21] and incubated in the presence of the retinoids (35 μM). Mitochondrial swelling was determined from the change in absorbance at 520 nm as described earlier [21].

Results

Cell fusion

From Table I it can be seen that the compounds studied may be divided into

TABLE I

TIME TO FUSION (t_f) OF HEN ERYTHROCYTES INCUBATED WITH RETINOL AND DERIVATIVES

Values for the time to fusion (t_f) in min (±S.D.) obtained for hen erythrocytes (3×10^8 cells/ml) incubated at 37°C with aqueous dispersions of retinol and derivatives in MEM-Dextran (pH 5.6). The figures in brackets refer to the number of independent experiments that were performed; both values obtained are given for experiments done in duplicate.

Compound	Concentration				
	87.5 μM	175 μM	350 μM	700 μM	1400 μM
Retinol	36.0 ± 1.4 (8)	12.5 ± 0.4 (26)	4.0 ± 0.2 (40)	2.5 ± 0.2 (19)	1.5 (1)
α-Retinoic acid	—	15.0 9.0 (2)	5.0 3.5 (2)	2.0 3.0 (2)	—
Retinaldehyde	26.5 ± 1.4 (4)	11.0 ± 0.5 (10)	7.0 ± 0.4 (10)	5.0 ± 0.3 (9)	—
Iso-13-retinol	—	25.0 (1)	7.0 (1)	4.5 (1)	2.0 2.0 (2)
Retinyl acetate	—	52.0 (1)	26.0 ± 0.7 (7)	18.5 ± 4.5 (4)	8.0 ± 0.8 (4)
Retinyl oleate	—	—	No fusion (1) 120 (1)	No fusion (2) 120 (1)	No fusion (2) 60 (1)
Retinyl linoleate	—	No fusion (1)	No fusion (1)	No fusion (1)	No fusion (2) 20 h (1)
Retinoic acid	—	No fusion (2)	No fusion (1) 46 h (1)	No fusion (5)	—
Retinyl palmitate	—	No fusion (5)	No fusion (6)	No fusion (7)	—
Iso-13-retinoic acid	No fusion (3)	No fusion (3) 120 (1)	No fusion (3) 12.5 (1)	5.5 (1)	—

three categories based on their ability to cause the fusion of hen erythrocytes. Retinol, α-retinoic acid, retinaldehyde and iso-13-retinol all induced comparably rapid cell fusion particularly when compared at a concentration of 350 μM. Retinyl acetate was also an effective fusogen although it acted relatively slowly.

Secondly, retinyl oleate, retinyl linoleate and retinoic acid were virtually non-fusogenic, producing only one or two bi- or tri-nucleate cells after several hours of incubation at higher concentrations. Finally, retinyl palmitate and RO8-7699 were non-fusogenic, although there was insufficient material available to allow compound RO8-7699 to be used at a concentration greater than 175 μM.

The behaviour of iso-13-retinoic acid differed from that of the other retinoids. Although the cells began to fuse rapidly when they were treated with this compound, they also lysed rapidly so that there was no accumulation of multi-nucleated cells in the suspension. This compound thus exhibited a similar fusogenic behaviour to that of lysophosphatidylcholine [4].

Haemolysis

In general, a marked ability to cause cell fusion was accompanied by a considerable haemolytic acitivity as may be seen from Fig. 1. Of the five compounds that were effective fusogens, retinol, α-retinoic acid and iso-13-retinol gave lysis that was similar to that produced by oleic acid and glycerol mono-oleate [3]. Interestingly, retinaldehyde was markedly less haemolytic

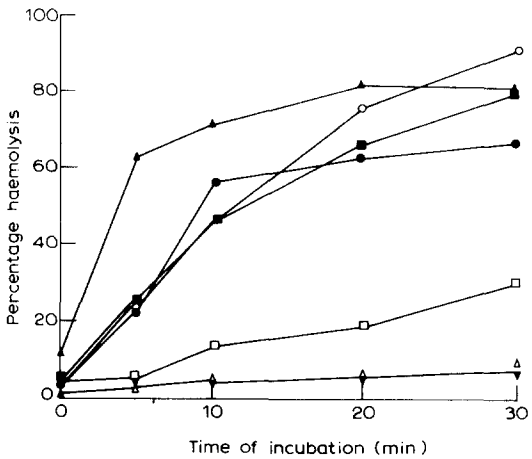

Fig. 1. A comparison of the percentage haemolysis produced by 350 μM retinol and derivatives. The percentage haemolysis induced in 3×10^8 hen erythrocytes/ml incubated at 37°C with aqueous dispersions (350 μM) of retinol (○), α-retinoic acid (●), retinaldehyde (□), iso-13-retinol (■), retinyl acetate (△), iso-13-retinoic acid (▲) or with retinyl palmitate, retinyl oleate, retinyl linoleate, retinoic acid, or with an ethanol control (2% v/v)) (▼), in MEM-Dextran (pH 5.6).

TABLE II

MITOCHONDRIAL SWELLING, CELL FUSION AND HAEMOLYSIS PRODUCED BY RETINOL AND DERIVATIVES

Swelling of rat liver mitochondria incubated at 37°C, in 0.4 M sucrose-0.05 M Tris (pH 7.4), induced by 35 μM retinol and derivatives was measured as described in the text (data represent the relative changes in A_{520nm} in two independent experiments, each performed in triplicate). Cell fusion, expressed as the reciprocal of the time of fusion ($1/t_f$ min^{-1}) and percentage haemolysis produced in hen erythrocytes (3×10^8 cells/ml) by 350 μM retinol or derivatives are also presented (mean values ± S.D., taken from Table I and Fig. 1, respectively). (n), number of independent experiments; n.t., not tested; no fusion, no fusion detected within 90 min of incubation.

Compound	Mitochondrial swelling (relative change in A_{520nm} per min)		Cell fusion $1/t_f$ (min^{-1})		Percentage haemolysis	
Retinol	100%	(2)	0.250 ± 0.110	(40)	92.0 ± 3.0	(9)
α-Retinoic acid	n.t.		0.200; 0.285	(2)	67.0	(1)
Retinaldehyde	n.t.		0.143 ± 0.041	(10)	27.0; 35.0	(2)
Iso-13-retinol	50%; 60%	(2)	0.143	(1)	81.0 ± 5.3	(3)
Retinyl acetate	12.5%; 23.2%	(2)	0.038 ± 0.007	(7)	8; 9	(2)
Retinyl oleate	2.5%; 0%	(2)	no fusion	(2)	6.0	(1)
Retinyl linoleate	10%; 0%	(2)	no fusion	(1)	9; 8	(2)
Retinoic acid	20%; 30%	(2)	no fusion	(2)	7.5%; 7.5% **	(2)
Retinyl palmitate	10%; 0%	(2)	no fusion	(6)	6.5; 5.5	(2)
Iso-13-retinoic acid	60%; 40.7%	(2)	0.080	(1)	79.5 ± 3.9	(3)
RO8-7699	5%; 9.3%	(2)	no fusion *	(2)	8.0 ± 3.1 *	(6)
Control (1—2% (v/v) ethanol)	5%; 1%	(2)	no fusion	(3)	4.5 ± 1.2	(3)

* RO8-7699 tested at 175 μM.

** Sonicated retinoic acid.

whilst being equally fusogenic. Consistent with the observations made with the light microscope, iso-13-retinoic acid induced the most rapid haemolysis.

The remaining compounds in Table I were barely haemolytic, including the relatively effective fusogen retinyl acetate.

Mitochondrial swelling

Table II shows the changes in absorbance of light at 520 nm, relative to that produced by retinol, given by the various retinoids acting on mitochondrial suspensions. This table also compares mitochondrial swelling with fusion and haemolysis, and it may be seen that fusogenic ability is generally paralleled by a haemolytic perturbation of erythrocyte membranes.

Discussion

From the data reported here it is apparent that the active fusogenic forms of vitamin A are those having a polar end group, such as retinol, retinaldehyde, the 13-*cis* isomer of retinol, and α-retinoic acid. The non-fusogenic derivatives of vitamin A are the longer-chain fatty acid esters of retinol and of retinoic acid. The short-chain ester, retinyl acetate, is a weak fusogen.

Similar relative activities of these compounds have been reported in other cell-free natural or artificial membrane systems such as in their penetration into a phosphatidylcholine-cholesterol monolayer [1] and in the disruption of isolated lysosomes [8,9]. When retinoids were added to cells in culture, however, retinoic acid, retinyl esters and the cyclopentyl derivative of retinoic acid, RO8-7699 were found to have equal or sometimes even greater activity than retinol itself, both in altering membranes, as is the case when retinoids stimulate the secretion of lysosomal enzymes from cultured cartilage [14,18], and in the more complex situation where retinoids are found to affect differentiation in epithelial tissues [20,21]. Presumably retinyl esters are hydrolysed in cells to the more active retinol in these longer term experiments.

Retinoic acid may be ineffective in altering isolated membranes because of its insolubility, for it was found to be virtually impossible to produce a homogenous aqueous dispersion of retinoic acid by injection of ethanolic solutions into MEM-Dextran. Only after sonication did the dispersion resemble that given by the other derivatives of vitamin A and even then retinoic acid was capable of producing only one or two fused cells after many hours of incubation. Although it had some activity in producing mitochondrial swelling, it was virtually nonhaemolytic. The concentrations of retinoids required to effect the release of lysosomal enzymes in cultured cartilage [14,18] are lower than those for activity on isolated membranes, but at lower concentrations retinoic acid and RO8-7699 may perhaps be presented to cells in a form that is more effective on their membranes.

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