Biochimica et Biophysica Acta, 643 (1981) 152—160

BBA 79199

EFFECTS OF RETINOL, FATTY ACIDS AND GLYCEROL MONOOLEATE ON THE FUSION OF CHICK EMBRYO MYOBLASTS IN VITRO

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(Received September 26th, 1980)

Key words: Cell fusion; Retinol; Fatty acid; Glycerol monooleate; Myoblast; (Chick embryo skeletal muscle)

Summary

Cell fusion of embryonic chick myoblasts has been studied in the presence of fat-soluble agents that induce erythrocytes to fuse. Retinol inhibited myoblast fusion but the cells recovered their ability to fuse within 48 h of removal of the retinol from the medium. Myristic acid, oleic acid, glycerol monooleate, linolenic acid and arachidonic acid similarly prevented fusion in myogenic cultures. By contrast, linoleic acid moderately enhanced the fusion of chick skeletal myoblasts. In addition, stearic acid, which does not fuse erythrocytes, inhibited myoblast fusion whereas the saturated, non-fusogenic fatty acid, arachidic acid, was without effect.

Introduction

The fusion of myoblasts is an important event in the normal myogenesis of skeletal muscle but, as recently remarked by Bischoff [1], there have been relatively few studies that are directly concerned with elucidating molecular events in the fusion process. Bischoff [1] also commented, however, that rearrangements in the final stage of myoblast fusion probably require the membrane to be fluid since agents that promote membrane stability tend to inhibit fusion. Cholesterol, for example, has been found to inhibit the fusion of myoblasts in vitro, and this inhibition was reversed by increasing the tem-

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perature [2]. Recent studies with fluorescent probes [3,4] have also indicated that the fusion of chick myoblasts is associated with a decrease in membrane viscosity, while inhibition of myoblast fusion by concanavalin A further indicates a possible involvement of membrane fluidity [5].

Earlier work in this laboratory showed that oleic acid induced hen erythrocytes to fuse, while stearic acid was inactive [6]. It was therefore suggested that unsaturated fatty acids and their derivatives may induce erythrocytes to fuse by increasing the proportion of hydrocarbon chains in the membrane that are in a relatively liquid state [7]. The recent observations of Prives and Shinitzky [4] are consistent with this idea since they have added fatty acids to the culture medium of chick myoblasts and found that oleic and linoleic acids facilitated fusion while stearic and elaidic acids delayed it, as also shown by the work of Horwitz et al. [8].

In the present paper we report experiments in which the effects of retinol, glycerol monooleate, five unsaturated fatty acids and two saturated (non-fusogenic) fatty acids on the fusion of chick myoblasts in vitro have been studied at concentrations that are comparable to those which induce cell fusion in other systems. The observations made, which partly differ from those of Prives and Shinitzky [4]1 show that many fat-soluble substances, e.g., retinol and glycerol monooleate that induce the fusion of hen erythrocytes, act to inhibit spontaneously occurring myoblast fusion. Since lysophosphatidylcholine [9] and Me_2SO [10] also fuse erythrocytes but inhibit the fusion of myoblasts [11,12], it seems that molecules that are fusogenic with erythrocytes generally inhibit, although perhaps not necessarily by a single mechanism, the programmed fusion of myoblasts.

Materials and Methods

Materials. Materials for cell culture, and batches of horse serum (GIBCO-Europe Limited) were selected for optimal support of cell growth and fusion. Fatty acids and glycerol monooleate were of the highest chemical purity available from Sigma (London) Chemical Company. Retinol was obtained from Roche Products Limited, and Leishman stain, in CH_3OH, was obtained from J.R. Gurr Limited (London).

Cell culture. Cells were prepared from thigh muscle of 11-day-old White Leghorn chick embryos by a mechanical disintegration technique described by Tepperman et al. [13], followed by selective plating on a glass surface to enrich the myoblast population. Suspensions of cell comprising 80—85% myoblasts (the remainder being fibroblasts) were cultured on coverslips coated with rat-tail collagen at $6.5 \cdot 10^5$ cells per 60 mm Petri dish (two coverslips in each dish) in an atmosphere of 95% O_2/5% CO_2. The cells were grown in a culture medium composed of nine parts of a 1 : 4 mixture of Medium 199 and Dulbecco's modified Eagle medium with one part of horse serum, supplemented with embryo extract (final concentration 1%), penicillin (100 units/ml) and streptomycin (50 μg/ml).

The cells were cultured for 24 h in the above medium. This was then replaced by a medium containing added retinol, fatty acid, or glycerol monooleate, as appropriate (prepared as described below). The medium was sub-

sequently changed, and replaced appropriately by control or experimental medium each succeeding 24 h.

Retinol, fatty acids and glycerol monooleate. Retinol was stored and handled as described by Dingle and Lucy [14] and 10 μl of stock solution (dissolved in C_2H_5OH) was injected with a Hamilton syringe into 1 ml horse serum (to give a final concentration of 60—300 μg retinol/ml of medium). After mixing and gassing with N_2, the retinol-containing serum was deep frozen. It was used within 5 days and 9 ml serum-free culture medium was added just before use. Culture media containing fatty acids were prepared using the same procedure as for retinol. An equal volume of C_2H_5OH was added to the corresponding control medium in each experiment.

Sonicated dispersions of glycerol monooleate in 9 ml of serum-free culture medium were made, in concentrations ranging from 40 to 70 μg/ml glycerol monooleate, as described previously [6]. 1 ml of horse serum was added to 9 ml of the freshly prepared lipid dispersion and the final medium was added to the cells within 10 min of preparation.

The lipids used were systematically studied over the concentration range in which they showed no obvious toxic effects. At higher concentrations than those used here, cell damage was apparent.

Evaluation of cell fusion. Culture dishes were rinsed with two changes of phosphate-buffered saline and stained with Leishman's stain, rinsed with distilled water and allowed to dry before the cover slips were mounted on glass slides. Cell fusion was estimated by direct microscopic examination (standard WL Research Microscope, Carl Zeiss, Dagenhardt and Co. Ltd., U.K.) at a magnification of ×500. A minimum of 1000 nuclei were counted over random fields on each cover slip (4—8 cover slips per experiment) and scored as multinucleate (three or more nuclei per cell), binucleate or mononucleate cells. Percentage fusion was calculated as follows:

$$\%\text{fusion} = \frac{\text{number of nuclei within multinucleate cells} \times 100}{\text{total number of nuclei (including fibroblasts)}}$$

Student's *t*-test was used to determine the significance of the observations made.

Results

The effect of retinol on myoblast fusion

To test the effect of retinol on myoblast fusion, myogenic cultures grown in standard medium were changed at 24 h to a retinol medium containing 50—300 μg of retinol/ml medium. Retinol at concentrations greater than 60 μg/ml significantly inhibited myoblast fusion ($P = 0.0002$ for 150 μg/ml), the inhibition being directly related to the retinol concentration (Fig. 1). Increasing concentrations of retinol caused a corresponding increase in the percentage of mononucleate cells. Similar results were seen in two other experiments.

The quantity of retinol that was most effective in preventing myoblast fusion was in the range of 150—225 μg/ml medium. Significant toxicity was generally not encountered below 225 μg/ml of retinol. Furthermore, on

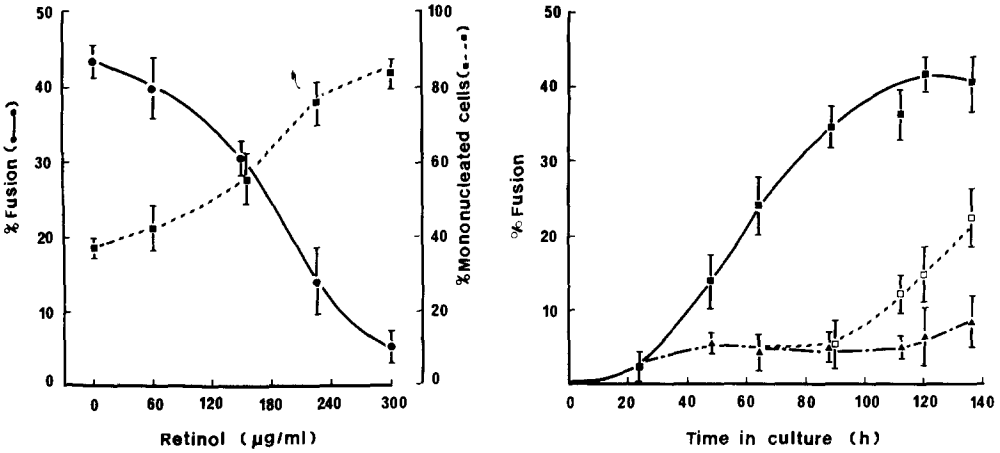

Fig. 1. Effect of retinol on myoblast fusion. 24-h cultures of chick embryo myoblasts were exposed to retinol (60—300 μg/ml) by total replacement of the culture medium with retinol-containing medium (see text). Retinol-containing medium was replaced at 24-h intervals thereafter and determinations of cell fusion were made after 88 h in culture (64 h in the presence of retinol). Control cultures were fed with a complete change of medium containing 1 μg/ml ethanol every 24 h. Values given are the mean (±S.D.) percentage fusion (●——●) or percentage mononucleate cells (including fibroblasts) (■- - - - -■) for four separate coverslips (two coverslips per culture dish).

Fig. 2. Reversible inhibition of myoblast fusion by retinol. 24-h cultures of chick embryo myoblasts were exposed to 180 μg/ml retinol for up to 112 h by the addition of a complete change of freshly prepared, retinol-containing medium every 24 h. Control cultures were similarly fed with control culture medium containing 1 μg/ml ethanol. After 64 h in culture half of the retinol-treated cultures (eight dishes) were reintroduced to control medium and continued in this for a further 72 h. Pairs of dishes from each of the three experimental groups were assessed for fusion at various intervals and the mean percentage fusion (± S.D.) was determined for the four coverslips from each pair. The values shown are for control cultures (■——■), cells grown in the presenceof 180 μg/ml retinol for 112 h (▲- - - - -▲) or cells grown in 180 μg/ml retinol for 40 h and then reintroduced to control culture conditions (□- - - - -□).

replacement of the retinol-containing culture medium with normal growth medium at 64 h, the myogenic cells recovered their ability to fuse into myotubes within 48 h (i.e., by 112 h of culture) (Fig. 2). At that time there was no decline in cell number, although by 120 h cell numbers in these cultures were significantly less than in corresponding control cultures ($P = 0.002$). Similar observations were made in two other experiments.

Inhibition of fusion by glycerol monooleate

Glycerol monooleate, which behaves like retinol in inducing the fusion of hen erythrocytes in vitro [6], similarly prevented fusion in myogenic cultures (Fig. 3). Glycerol monooleate in the range of 40—70 μg/ml inhibited the fusion of myoblasts in three experiments in which the lipid was added in serum ($P = 0.02$ to <0.001 for Fig. 3). Inhibition was more pronounced inthe cultures that were exposed to glycerol monooleate bound to horse serum than in those cultures that were exposed to glycerol monooleate in a sonicated dispersion of the same concentration. The latter only significantly inhibited at 70 μg/ml in Fig. 3 ($P = 0.018$). Glycerol monooleate was toxic above 80 μg/ml.

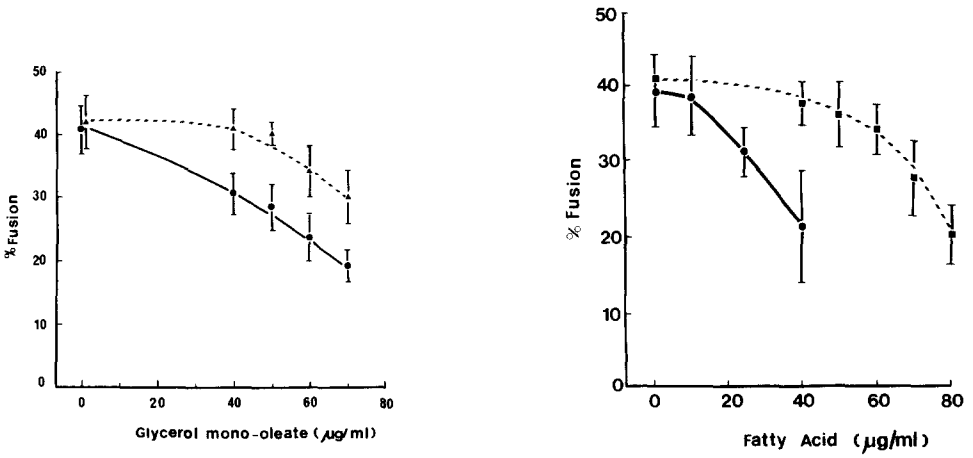

Fig. 3. Inhibition of myoblast fusion by glycerol monooleate. 24-h cultures of chick embryo myoblasts were exposed to glycerol monooleate (40—70 μg/ml), either as an ultrasonicated dispersion (▲- - - - -▲) or as an injected dispersion (●———●) (see text), replacing this with freshly prepared medium containing glycerol monooleate at 24-h intervals thereafter. Determinations of fusion were made after 88 h in culture on duplicate dishes (four coverslips) for the sonicated dispersion, or on four dishes (eight coverslips) for the injected dispersions and for the controls, and are expressed as mean percentage fusion (±S.D.).

Fig. 4. Inhibition of myoblast fusion by oleic and myristic acids. After growth for 24 h in standard medium, myoblasts were cultured for 88 h with up to 80 μg/ml of oleic acid (■———■), or up to 40 μg/ml of myristic acid (●———●), with removal of media every 24 h. For oleic acid the mean percentage of myoblast ± S.D., was determined on twelve coverslips from three separate experiments except for the 70 μg/ml value which was determined on eight coverslips. For myristic acid, the mean percentage fusion of myoblast ± S.D., was determined on fourteen, eight and ten coverslips, respectively, from two separate experiments, for 0, 25 and 40 μg/ml and on four coverslips from one experiment for 10 μg/ml.

Stearic, oleic and myristic acids

In two experiments the saturated fatty acid, stearic acid (C18 : 0), which does not fuse hen erythrocytes, significantly inhibited myoblast fusion at 10, 30 and 50 μg/ml with P values ranging from 0.026 to <0.001 (Table I). 30 μg/ml stearic acid decreased fusion at 54 h by approx. 75%. Stearic acid also caused variable cellular toxicity.

TABLE I

EFFECT OF STEARIC ACID ON MYOBLAST FUSION

After growth for 24 h in standard medium, medium containing 0—50 μg/ml of stearic acid was added to myoblast cultures, which were then grown for a total of 48—88 h with renewal of media every 24 h. The mean percent fusion of myoblasts ± S.D. determined on eight coverslips from two separate experiments for controls and for 10 μg of stearic acid/ml at 88 h. With higher concentrations of stearic acid, cellular toxicity was evident in random samples from both experiments, and only four coverslips were used to assess the myoblast fusion. Values represent % fusion (±S.D.).

Time in culture (h)	Concentration of stearic acid (μg/ml):			
	0	10	30	50
48	13 ± 2.1	—	3.0 ± 1.7	2.0 ± 1.2
54	20 ± 6.4	—	8.0 ± 1.1	7.5 ± 3.7
64	31 ± 6.8	20 ± 4.9	—	—
88	41 ± 4.4	25 ± 2.5	—	—

The unsaturated fatty acid oleic acid (C18 : 1) at a concentration of 50 μg/ml or greater significantly decreased the fusion of cultured muscle cells (P values ranging from 0.05 to <0.001) (Fig. 4). In two experiments, oleic acid was non-toxic up to 80 μg/ml, while in a third experiment it significantly decreased the number of cells at 70 μg/ml ($P = 0.01$).

The medium-chain length saturated fatty acid, myristic acid, which is also known to induce fusion in hen erythrocytes, likewise inhibited fusion of myogenic cells. Approx. 50% inhibition of fusion occurred with 40 μg/ml myristic acid at 88 h (Fig. 4). Myristic acid was toxic above 40 μg/ml.

Linolenic, arachidonic and arachidic acids

Cellular toxicity interfered with attempts to examine the effects of linolenic acid (C18 : 3) at longer times than 48 h, but at this time linolenic acid was inhibitory (Table II).

With low concentrations of arachidonic acid and longer culture times, or a higher concentration of acid and a shorter culture time, significant inhibition of fusion was seen (Table II) without significant cell damage. By contrast, arachidic acid (C20 : 0) generally exerted little effect upon myoblast fusion, causing a small inhibition only in one case out of seven (Table II). Arachidic acid was nontoxic up to 70 μg/ml but it caused the accumulation of cytoplasmic fat droplets within myoblasts: these droplets were increased in size as the concentration of arachidic acid was increased.

TABLE II

EFFECTS OF LINOLENIC, ARACHIDONIC AND ARACHIDIC ACIDS UPON MYOBLAST FUSION

After growth for 24 h in standard medium, myoblasts were cultured for up to 88 h with 10—70 μg/ml of the fatty acids, with renewal of media every 24 h. Then mean percent fusion of myoblasts ± S.D., was determined on four coverslips. The significance of the inhibition of fusion is shown in the right hand column. n.s., not significant.

Culture conditions	Agent (μg/ml)	% Fusion (±S.D.)	P *
48 h, control		31.9 ± 2.3	
48 h	arachidonic acid (70)	14.6 ± 1.0	0.0001
48 h	linolenic acid (70)	16.8 ± 2.6	0.0004
64 h, control		38.0 ± 2.1	
64 h	arachidic acid (70)	30.3 ± 1.2	0.01
64 h, control		35.1 ± 2.3	
64 h	arachidic acid (70)	35.7 ± 4.1	n.s.
88 h, control		36.5 ± 3.1	
88 h	arachidic acid (30)	32.6 ± 3.2	n.s.
88 h	arachidic acid (50)	31.1 ± 3.0	n.s.
88 h	arachidic acid (70)	32.7 ± 2.2	n.s.
88 h, control		37.3 ± 1.8	
88 h	arachidic acid (30)	35.2 ± 2.8	n.s.
88 h	arachidic acid (50)	39.5 ± 3.9	n.s.
88 h	arachidonic acid (10)	30.4 ± 2.6	0.004
88 h	arachidonic acid (30)	27.9 ± 5.1	0.01

* The probability that the agent did not significantly decrease myoblast fusion.

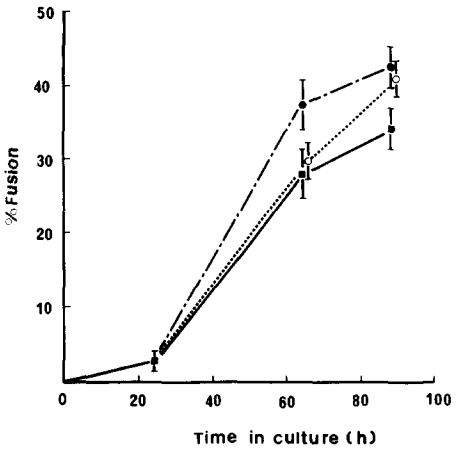

Fig. 5. Stimulation of myoblast fusion by linoleic acid. After growth for 24 h in standard medium, myoblasts were cultured for up to 88 h with 40 or 50 μg/ml of linoleic acid, with renewal of media every 24 h. Pairs of Petri dishes were taken at 64 and 88 h to determine the mean percentage fusion of the myoblasts ± S.D., counting cells on four coverslips. ■———■, control; ○- - - - -○, 40 μg/ml of linoleic acid, and ●- - - - -●, 50 μg/ml of linoleic acid.

Linoleic acid

To test the effect of linoleic acid (C18 : 2) on the fusion, myogenic cultures were supplemented with 50 μg/ml linoleic acid. In two experiments linoleic acid significantly enhanced fusion at 64 and 88 h (P values ranging from 0.01 to 0.004) (Fig. 5). In two further experiments, 30 μg/ml and 40 μg/ml of linoleic acid also significantly stimulated fusion at 88 h ($P = 0.017$ and 0.00025). The cell number per field in linoleic acid-supplemented cultures was never significantly different from controls, indicating no alteration in cell division in these cultures. Toxicity with linoleic acid was only observed above 50 μg/ml.

Discussion

The report that lysophosphatidylcholine, which can fuse a number of different cell types [9], acts to inhibit the spontaneous fusion of rat myoblasts in culture [11] has led us to examine the effects of retinol and a series of fusogenic and nonfusogenic fatty acids or esters on a similar myoblast culture system.

The action of retinol on chick embryo myoblasts in vitro closely resembles that of lysophosphatidylcholine since it not only inhibits myoblast fusion but its action is reversible. It is of interest that phorbol-12-myristate-13-acetate [15] and diazepam (Valium®, Roche) also reversibly inhibit the fusion of myoblast [16], whilst sodium butyrate delays fusion for 2—3 days [17]. None of these agents would appear, however, to induce cell fusion in other cell types.

The inhibitory action of lysophosphatidylcholine on myoblast fusion has been suggested to be due to an inhibition of the production of cells that are capable of fusion [1] or to an interference with the metabolic turnover

of membrane phospholipids [18]. However, lysophosphatidylcholine also inhibits the calcium-induced fusion of plasma membrane vesicles isolated from cultured myoblasts [19] in which system a metabolic effect would seem unlikely. It has previously been shown that retinol penetrates the plasma membranes of hen erythrocytes under conditions that cause cell fusion, and it also enters vesicles of phosphatidylcholine [20]. Conceivably then, both retinol and lysophosphatidylcholine may inhibit the fusion of myoblast membranes either by direct actions on membrane structure, and/or by metabolic effects.

The inhibitory effects of oleic, linolenic and arachidonic acids that were observed in the present work were unexpected because it would be anticipated that these unsaturated fatty acids would act to increase membrane fluidity and hence facilitate myoblast fusion. Prives and Shinitzky [4] reported that membrane viscosity decreased sharply during the first day of culture, and that the minimum microviscosity corresponded with the onset of myoblast fusion. However, as pointed out by Bischoff [1], the initial decrease in membrane viscosity could have resulted from damage to the cells that arose during tissue degradation. Our observation on the inhibitory effect of oleic acid differs from that of Prives and Shinitzky [4] who found that this fatty acid facilitated fusion. Horwitz et al. [8,21] observed a slightly enhanced extent of fusion in oleate-enriched chick myoblasts, while cells enriched in elaidate or C19 : 0 fatty acid showed a marked decrease in fusion. Our work with oleic acid is more consistent with that of Boland et al. [22], who found that the growth and differentiation of chick myoblast cultures was poor in medium containing foetal calf serum (which has 33% of its lipid as palmitate and 31% as oleate) by comparison with cells in a medium containing horse serum (17% palmitate and 10% oleate).

The moderate stimulation of myoblast fusion by linoleic acid seen in our study agrees with the finding of Prives and Shinitzky [4] and is consistent with the report of Boland et al. [22] that myoblasts differentiate better in the presence of horse serum, which contained a high concentration of linoleate (48%), by comparison with foetal calf serum (5% linoleate). Cells that differentiate into myotubes in media containing horse serum increase their content of linoleate during cultivation [22,23].

The inhibition of myoblast fusion observed in the present work not only with stearic acid but also with the short-chain myristic acid, and with the unsaturated acids oleic (and its ester), linolenic and arachidonic, indicates that membrane fluidity is apparently not a major factor at issue. Conceivably these fatty acids may exert their inhibitory effect via their incorporation into membrane phospholipids, but some other more specific action may perhaps be involved. In this connection it is of interest that Anderson and Jaworski [24] found unsaturated fatty acids and lysophosphatidylcholine to inhibit the activity of a preparation of adenylate cyclase from fibroblasts. Increasing unsaturation in the fatty acids increased their inhibitory effect but saturated fatty acids, varying in chain length from 8 to 20 carbon atoms, did not inhibit. This finding nevertheless raises the possibility that the inhibition of myoblast fusion by fatty acids reported here may be mediated by the inhibition of adenylate cyclase in view of the work of Zalin and Montague [25] which indicated that the onset of fusion in myoblast cultures is controlled by the

concentration of intracellular cyclic AMP. It may be significant that, of all the fatty acids we have studied, only linoleic acid stimulated myoblast fusion. Linoleate is a precursor of prostaglandin E_1 and there is evidence that this prostaglandin may be involved in myoblast fusion [26].

Acknowledgements

This work was supported by the award of a British Council Scholarship to S.N. A.R.F. thanks the Medical Research Council for an intercalated B.Sc. course award.

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