Biochimica et Biophysica Acta 896 (1987) 19–25
Elsevier

BBA 73182

Changes in myoblast membrane order during differentiation as measured by EPR

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(Received 11 June 1986)
(Revised manuscript received 9 September 1986)

Key words: ESR; Membrane order; Myogenesis; Cell-cell recognition; Membrane fusion; Differentiation; (Chicken myoblast)

The events which make possible the characteristic fusion of the cell membranes of embryonic myoblasts are known to involve modification of the cell membrane (Hausman, R.E., Dobi, E.T., Woodford, E.J., Petrides, S., Ernst, M. and Nichols E.B. (1986) Dev. Biol. 113, 40–48). Myoblasts from chick embryos were allowed to differentiate in gyrotory aggregate culture and the order of their membranes was measured by EPR. Two spin-labels which insert at different depths into the lipid bilayer were used. Measurement with the 5-nitroxystearate label showed an increase in myoblast membrane order ($2T'_{\parallel}$) from 0–15 h of culture and again from 26–38 h of culture. Measurement with the 12-nitroxystearate label showed the 0–15 h increase in order but the second increase was greatly reduced and shifted in time. While the specific sources of these changes in membrane order cannot yet be identified, the changes observed correlated well with known events of myogenic differentiation in vitro. The initial increase in membrane order occurred while the myoblasts were recovering from the effects of trypsin dissociation and undergoing gyrotory aggregation. The second increase in membrane order occurred during the known period of prostaglandin receptor activity and increased cell-cell adhesion.

Introduction

The differentiation of embryonic myoblasts in vitro has been a useful system for the study of development. Two processes, the elaboration of specific cytoskeletal proteins which form the contractile apparatus of mature muscle [1–4] and fusion of myoblast membranes [5–10], while not fully understood, have been extensively investigated.

Myoblast fusion in vitro creates the myotube necessary for full elaboration of the characteristic proteins [11]. However, it is clear that many of the molecules specific for myogenic differentiation appear before membrane fusion [12–14]. This has led to increased interest in those aspects of myogenic differentiation which precede fusion of the myoblast cell membranes [12,15–20].

Several of these early differentiation events during myogenesis in vitro are localized at the cell surface and may play a role in the cell-cell communication necessary to coordinate both fusion and the synchronous development of contractile proteins [21]. Electron paramagnetic resonance (EPR) study of membrane bilayers with spin-labels provides a very sensitive means of detecting events at the cell membrane [22]. It has been used to detect changes in membrane fluidity during pas-

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sage of Chinese hamster ovary cells through the cell cycle [23], and to establish correlations between membrane fluidity and thermosensitivity of mammalian cells [24].

While changes in the membrane fluidity or order of some differentiating cells have been reported [25,26], EPR has not (to our knowledge) been used to investigate changes in membrane order during embryonic myogenesis. We demonstrate here dynamic changes in the membrane order of embryonic chick myoblasts. We discuss the correlation of these changes with the processes of gyrotory aggregation of the cells, binding of secreted prostaglandin by cell membrane receptors and a consequent change in myoblast adhesion. Finally, we confirm with one EPR spin-label the known increase in membrane fluidity which precedes membrane fusion.

Materials and Methods

Materials

Trypsin was obtained from Difco, soybean trypsin inhibitor and DNAase from Sigma, Dulbecco's modified Eagle's medium and penicillin/streptomycin from Gibco Europe, fetal bovine serum from Flow and potassium ferricyanide [$K_3Fe(CN)_6$] from Merck. The spin-labels, 5-nitroxystearate (2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl) and 12-nitroxystearate (2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxyl) were purchased from Aldrich. All other chemicals were obtained from Farmitalia Carlo Erba.

Myoblast cell cultures

Primary cultures were prepared from pectoral muscles of 11-day embryonic chicks by a modification of standard procedures [21]. After washing in calcium and magnesium-free Tyrode's solution, minced tissue was incubated in 0.25% trypsin in the same salt solution for 20 min at 37°C. The cells were washed twice by centrifugation with 50 mg/ml of soybean trypsin inhibitor and twice with 2 mg/ml DNAase, both in calcium and magnesium-free Tyrode's solution. Cells were resuspended in Dubecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 5000 units/ml penicillin and 5 mg/ml streptomycin to a total volume of 10 ml. Loose aggregates of cells were broken up by fire-polished Pasteur pipet, the cells counted by hemocytometer and diluted in the same medium to a final concentration of $5 \cdot 10^6$ cell/ml. This suspension of single cells was then pipeted into sterile 50 ml Erlenmeyer flasks (6 ml per flask), gassed with a mixture of 5% CO_2/95% air and the flasks were plugged. Flasks were gyrotory rotated (100 rpm, 37°C). Under these conditions only the myoblasts aggregate, the fibroblasts adhere to the flask [21]. The suspended myoblast aggregates were collected for measurement.

Microscopy

Suspensions of myoblast cells or aggregates were pipeted onto glass slides, examined and photographed with a Zeiss Ultrophot microscope. Magnifications are specified in the figure legends.

EPR measurements

Myoblasts ($40 \cdot 10^6$) were collected, centrifuged, washed three times and resuspended in phosphate-buffered saline (0.5 ml). Thorough washing was necessary to eliminate the paramagnetic signal from fetal bovine serum. The supernatant from the third phosphate-buffered saline wash was tested and gave no signal. For both 5-nitroxystearate and 12-nitroxystearate, a $3.25 \cdot 10^{-2}$ M stock solution was made in 100% ethanol and stored at −20°C until needed. 1 µl of either stock solution was added to the cell suspension, and the suspension was vortexed and rotation-incubated for 15 min at 37°C. 20 µl of a 1 M $K_3Fe(CN_6)$ solution (0.04 M) in phosphate-buffered saline was added, and the suspension was vortexed and added to capillary tubes. EPR measurement was on a temperature-controlled Varian E-4 X-band Spectrophotometer. It was operated at a microwave frequency of 9.12 GHz, at a power of 10 mW with a field modulation of 100 kHz. Temperature was monitored by digital thermometer with its thermocouple inserted into the capillary tube holder where silicon oil was added to maintain even temperature distribution. Temperature was maintained to within ±0.5 Cdeg. The data obtained were analyzed as detailed below.

Results

Spin-labels

Both spin-labels are thought to orient themselves inside the cell membrane noncovalantly (intercalate) with their longitudinal molecular axis perpendicular to the membrane surface. For 5-nitroxystearate, the location of the oxazolidine ring at position 5 of the hydrocarbon chain of the stearic acid allows the polar position of the hydrophobic tail of the lipid bilayer to be sampled. The ring motion is, therefore, related to the rotation of the adjacent segment of the molecule [27]. The structure of the spectrum obtained from myoblasts labeled with 5-nitroxystearate did not change significantly over the 48 h of experimentation; a typical spectrum is shown in Fig. 1. 12-Nitroxystearate is thought to insert deeper into the lipid bilayer [22,28,29]. Its spectra consist of at least two components [30]; a typical spectrum is shown in Fig. 2. As with 5-nitroxystearate, the structure of the 12-nitroxystearate spectra did not change significantly over the period studied. For both spin-labels, the distance between the outer hyperfine splitting ($2T_{\parallel}'$) was measured directly from the spectra and used as an indicator of membrane order. For a complete theoretical treatment of the use of spin-labels in membranes see Ref 28. We measured the $2T_{\parallel}'$ with the smallest hyperfine splitting because this distance could be more precisely measured at all time points. The signal due to the second component ($2T_{\parallel}'$ with the larger hyperfine splitting) was very small and could not be easily measured as it was sometimes masked by noise.

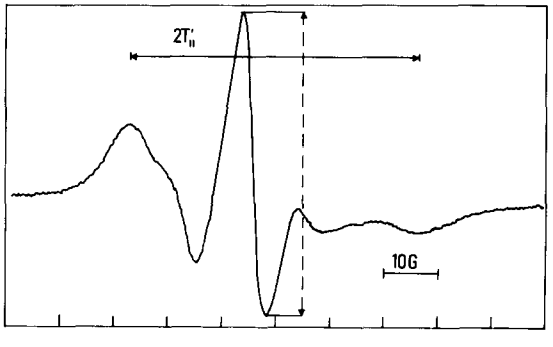

Fig. 1. Representative EPR spectrum obtained from embryonic chick myoblasts in rotation culture after labeling with 5-nitroxystearate. The external ($2T_{\parallel}'$) hyperfine resonance extrema of the T-tensor is indicated. The dotted line shows the mid-field line height. The marked scale indicates 10 G intervals.

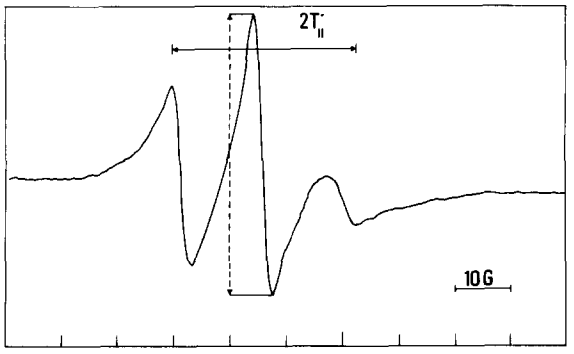

Fig. 2. Representative EPR spectrum obtained from embryonic chick myoblasts in rotation culture after labelling with 12-nitroxystearate. Parameters are marked as in Fig. 1.

The 5-nitroxystearate resonance signal obtained at each time point from the myoblasts in the presence of potassium ferricyanide remained quite stable. Measurements of the mid-field line height (Fig. 1) at intervals after introduction of the spin-label showed no decay of the signal. This observation justified the addition of potassium ferricyanide to the samples. Not only did this substance quench any free spin-labile, but (since it does not enter cells) it continuously oxidized the fatty acid spin-label which was being reduced at the cell surface [27]. The increase in signal stability thus obtained demonstrated that the spin-label was not being internalized by the cells, otherwise the ferricyanide would not be active. Thus, it is reasonably certain the the 5-nitroxystearate spin-label was probing the outer surface of the myoblast plasma membrane. In contrast, the measurements of mid-field line height from 12-nitroxystearate spectra (Fig. 2) showed a 30% decay in 1 h which was not inhibited by ferricyanide. While this decay was slow enough to allow use of the spin-label, the lack of ferricyanide protection suggested that the membrane was being probed at a deeper level.

The parameters which contribute to EPR spectra of cell membranes are not fully understood but include fluidity of phospholipids in the bilayer, local changes in charge across the membrane and molecular ordering [22]. While we have referred to

changes in $2T'_{\parallel}$ as changes in membrane order, $2T'_{\parallel}$ should be understood as an empirical measure of molecular motion in the immediate vicinity of the spin-label probe.

Kinetics of gyrotory aggregation

Single myoblasts (Fig. 3) rapidly formed large (> 100 cell) aggregates during the first 15 h of gyrotory rotation (Fig. 4). In aggregates from later stages (> 44 h), areas of smooth outer edge could be observed. These indicate the close membrane apposition and subsequent fusion characteristic of myoblasts [21].

Changes in membrane order

Investigation of membrane order over the first 48 h of differentiation in vitro with 5-nitroxystearate spin-label yielded the results shown in Fig. 5. The membrane order increased almost 8% for the first 15 h, while the single cells were undergoing gyrotory aggregation. The membrane order then decreased until 26–28 h of culture. At this time, important in myoblast membrane interactions [21], the order again began to increase with a point of maximum order reached at 38 h. The order then decreased until the end of the 48-h study.

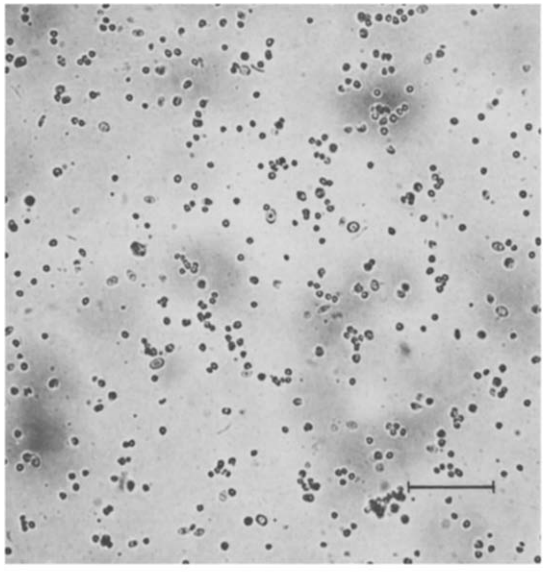

Fig. 3. Light micrograph of a suspension of 11-day embryonic chick muscle cells after 10 min of gyrotory rotation culture. Bar = 100 μm.

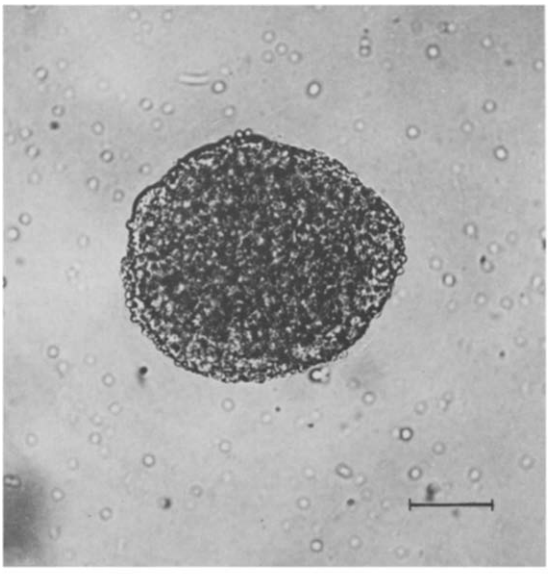

Fig. 4. Light micrograph of an aggregate of 11-day embryonic chick myoblasts and remaining single cells after 24 h of gyrotory rotation culture. The cells remaining single are virtually depleted of myoblasts. Bar = 100 μm.

Probing deeper into the membrane bilayer with 12-nitroxystearate spin-label yielded a different picture (Fig. 6). The increase in membrane order associated with the period of gyrotory cell aggregation remained but that associated with subse-

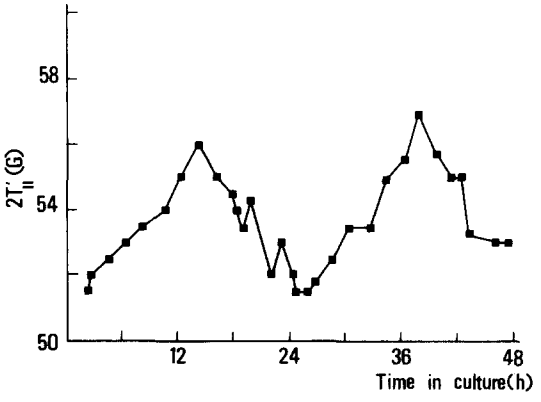

Fig. 5. Myoblast membrane order as represented by $2T'_{\parallel}$ using the 5-nitroxystearate spin-label plotted as a function of time in culture. The plot represents measurements from five separate experiments with overlapping time points. At each data point, the average value is shown. For each experiment, five measurements of $2T'_{\parallel}$ were taken per time-point. The curve was obtained by connecting the experimental points.

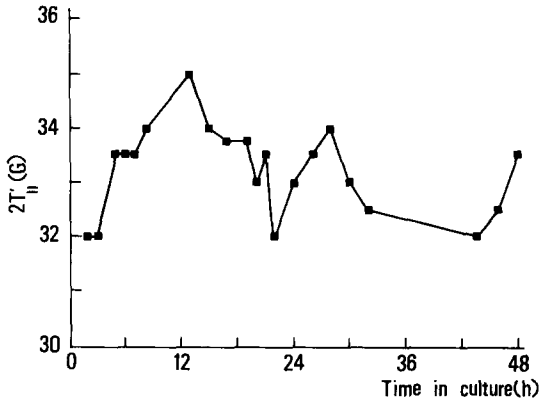

Fig. 6. Myoblast membrane order as represented by $2T'_{\parallel}$ using the 12-nitroxystearate spin-label plotted as a function of time in culture. The plot was obtained as described in Fig. 5.

quent myoblast membrane interactions was greatly reduced and shifted in time.

Discussion

The magnitude of the changes in $2T'_{\parallel}$ seen in this study are greater than those observed in most other complex cellular systems [23,24,26]. This is particularly significant since the changes in EPR spectra observed here are the result of gyrotory-mediated cell aggregation and myogenic differentiation in vitro, not the result of any direct experimental modification of the cell membrane. As noted earlier, the spectra of 12-nitroxystearate appears to consist of at least two spectral components. These may be due to lipid–protein interactions [30] or arise from a characteristic distribution of the spin-label between plasma and intracellular membranes. The similar changes in membrane order seen over the first 15 h of culture with the 5-nitroxystearate and 12-nitroxystearate spin-labels and their known degrees of insertion into the lipid bilayer [22,28,29] give us confidence that the value of $2T'_{\parallel}$ with the smallest hyperfine splitting of the deeper-inerting spin-label is not due to insertion of the label primarily into intracellular membranes. Thus, we are observing molecular order changes in the myoblast plasma membrane. This conclusion is further supported by the ability (reported here) of ferricyanide to enhance the 5-nitroxystearate signal and by work currently in progress.

Most investigations of the structure of embryonic myoblast cell membranes have focused on membrane fusion. This occurs between the membranes of closely apposed myoblasts beginning at 40 h in our culture system and continuing through 60 h [21]. Thus, the period of culture from 38–48 h is that immediately preceding membrane fusion for most myoblasts in the aggregates. Prives and Shinitzky [6] and Herman and Fernandez [7], using fluorescence spectroscopy, have both reported an increase in membrane fluidity during the period immediately preceding myoblast fusion. Our results with the 5-nitroxystearate spin-label, showing a decrease of four Gauss in $2T'_{\parallel}$ from 38–48 h of culture confirm their findings. However, our results with the deeper-inserting spin-label show no decrease in membrane order over this period of differentiation.

Our primary interest is in the earlier events of myogenic differentiation, specifically those which occur at the myoblast cell surface. There are three discrete events which occur over the period of differentiation investigated here. They are gyrotory aggregation, binding of prostaglandin to a cell membrane receptor, and the change in cell-cell adhesion called recognition [21]. The latter two events are necessary for subsequent fusion and differentiation [31]. Throughout this period of culture, the myoblast aggregates also synthesize and secrete prostaglandins [21] which are known to affect membrane fluidity [22].

The initial increase in membrane order from 0 to 15 h of culture seen with both spin-labels correlates with two events unique to aggregate culture: recovery of the cell membranes from the effects of trypsin [32] and gyrotory aggregation of the myoblasts [21]. Because fibroblasts are gradually being selected out of the cell suspension [21], the composition of the EPR sample is also changing. The similar observation with both labels suggests that this change in membrane order occurs at both the outer surface and in the interior of the lipid bilayer, and perhaps is associated with the events of gyrotory aggregation and replacement of cell surface proteins.

The subsequent increase in membranes order (from 28 to 38 h of culture) appears to be associated with the surface of the lipid bilayer. It is seen only with the 5-nitroxystearate spin-label. This

change in order occurs at the time when prostaglandin begins to bind to a specific membrane receptor on the myoblast membrane [21]. This causes a dramatic change in cell-cell adhesion [31] resulting in the close apposition of myoblast membranes which is so difficult to distinguish from actual membrane fusion [10,21].

Membrane order (as measured by $2T'_{\parallel}$) can be influenced by several processes simultaneously. These may contribute to the final spectra to differing extents. Furthermore, spin-label spectra obtained from fatty acid probes are sensitive to pH. Thus, the spectral changes observed may reflect contributions from local changes in membrane charge. These charge differences would themselves be significant in that they might directly affect cell-cell interactions. We have suggested some processes which are likely to contribute to the 0–15-h and 28–38-h increases in membrane order seen in this study. We cannot yet attribute these changes in order to specific processes such as gyrotory aggregation, especially since the myoblast membrane is unlikely to be homogeneous and different regions may contribute different signals [30]. However, the differences observed in membrane order between the two spin-labels from 28 to 38 h suggest that the factors affecting membrane order during this period of myoblast differentiation are different from those which contribute to the 0–15-h increase in order.

The finding by EPR of a change in myoblast membrane order which is associated with prostaglandin receptor activation and myoblast recognition and adhesion raises the possibility of understanding the biophysics of these myoblast membrane differentiation events. Toward this end, we are currently investigating the changes in membrane order after manipulation by probes for prostaglandin receptors and by inhibitors of prostaglandin metabolism.

Acknowledgments

We thank Mr. Giacomo Monteleone for expert photography and Dr. Barbara Caccia for mathematical assistance. This work was partially supported by Basic Research Grant 1-859 from the March of Dimes Birth Defects Foundation.

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