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Lateral motion of fluorescent molecules embedded into cell membranes of clonal myogenic cells, L6, changes upon cell maturation

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The lateral motion of fluorescent molecules embedded into cell membranes of myogenic cell line, L6, was measured. The motion of S-F-ConA became faster at cell fusion stage, and became slower after fusion. On the other hand, the motion of lipid analog, F18, was not changed at cell fusion stage. However, after fusion when myotubes were formed, the motion of F18 became slower. At cell fusion stage, there was a large variation in the motion of S-F-ConA. This means that at this stage the properties of myoblasts change drastically and rapidly.

Lateral motion; Fluorescence photobleaching recovery; Cell fusion; Maturation; (Myogenic cell)

1. INTRODUCTION

Muscle cells exhibit prominent changes upon their maturation. At an early stage, they are mononuclear cells which are called myoblasts. As they mature, they aggregate and fuse with each other resulting in the formation of large multinuclear cells which are called myotubes. One of the myogenic cell lines, L6, isolated by Yaffe [1], also exhibits changes similar to muscle cells. For this reason, L6 cells are widely used as a model for muscle cells. With morphological changes mentioned above [2], L6 cells exhibit molecular and physiological changes upon maturation as muscle cells do. For example, an increase in the content of myosin heavy chain [3], an increase in enzymatic activities [4], appearance of acetylcholine receptors followed by their clustering [5], generation of action potentials [6], localization of binding sites of concanavalin A [7] and formation of functional synapses with neurons [8,9] have been observed.

Since the onset of cell fusion is primarily an event on the cell surface, properties of molecules of cell membranes must play an essential role in this event. One of them is the motion of molecules within membranes. Indeed, it is reported that close apposition and destabilization of bilayer are required for lipid vesicle fusion [10,11]. On the other hand, it is known that the dynamic properties of membranes change upon cell differentiation. Fluidity of cell membranes of neuronal cells changes upon outgrowth of neurites [12–14], and the activity of Na^+ channels seems to be affected by composition and motion of lipids [15–17]. Therefore, there are very good reasons to expect that the dynamic properties of plasma membranes also play an important role in cell maturation, especially in cell fusion of muscle and L6 cells.

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Abbreviations: S-F-ConA, succinyl concanavalin A conjugated with fluorescein isothiocyanate; F-ConA, concanavalin A conjugated with fluorescein isothiocyanate; F18, 5-(octadecylthiocalbamoylamino)fluorescein; FPR, fluorescence photobleaching recovery; PBS, phosphate buffered saline

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In this report we show that lateral motion of molecules in cell membranes changes drastically in the course of maturation of L6 cells.

2. MATERIALS AND METHODS

2.1. *Cell culture*

L6 cells were plated onto cover slips in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% newborn calf serum. The cells were cultured at 37°C in 10% CO_2 and 90% air atmosphere.

2.2. *Fluorescent probes*

A fluorescent analog of fatty acid, F18 was synthesized as in [18]. Concanavalin A was conjugated with fluorescein isothiocyanate as in [7]. Briefly, ConA (Sigma) (10–50 mg) was mixed with fluorescein isothiocyanate (Sigma) (0.38 mg) on celite (3.8 mg) and incubated in PBS containing 0.1 M glucose at room temperature for 15 min with gentle stirring. The mixture was freed from celite by centrifugation. The supernatant was freed of excess dye by passing through a Sephadex G-25 column with PBS as an eluting solution. Native conjugates were separated from denatured ones by passing through a Sephadex G-100 column with PBS as the first and 0.1 M glucose in PBS as the second eluting solution. S-F-ConA was obtained from F-ConA as in [19].

2.3. *Labelling of cells*

Cells grown on cover slips were washed twice with PBS. The washed cells were incubated in PBS containing 10 μM F18 for 3 min at 37°C or in PBS containing 100 μg/ml S-F-ConA for 15 min at 37°C. Then the cover slips were washed 4 times with PBS. The cover slips were set in a temperature-controlled quartz container and were placed on a stage of a microscope.

2.4. *Measurements*

Lateral diffusion of fluorescent molecules was measured by fluorescence photobleaching recovery. The instrumental description and analysis are shown in [13]. The diameter of the bleached area was 2.8 μm using a 100× objective and a pinhole. In order to reduce the spontaneous motion of cells and internalization of labelled molecules, 10 mM NaN_3 was added.

Recovery fraction, f, and lateral diffusion coefficient, D, are determined using the following equations:

$$f = (I_{inf} - I_{bl})/(I_{ini} - I_{bl})$$

$$D = 0.22w^2/t_{1/2}$$

I_{ini}, I_{bl} and I_{inf} are fluorescence intensities before bleaching, just after bleaching and a long time after bleaching when recovery is saturated, respectively; w and $t_{1/2}$ are the radius of the bleached area and the half recovery time, respectively.

3. RESULTS

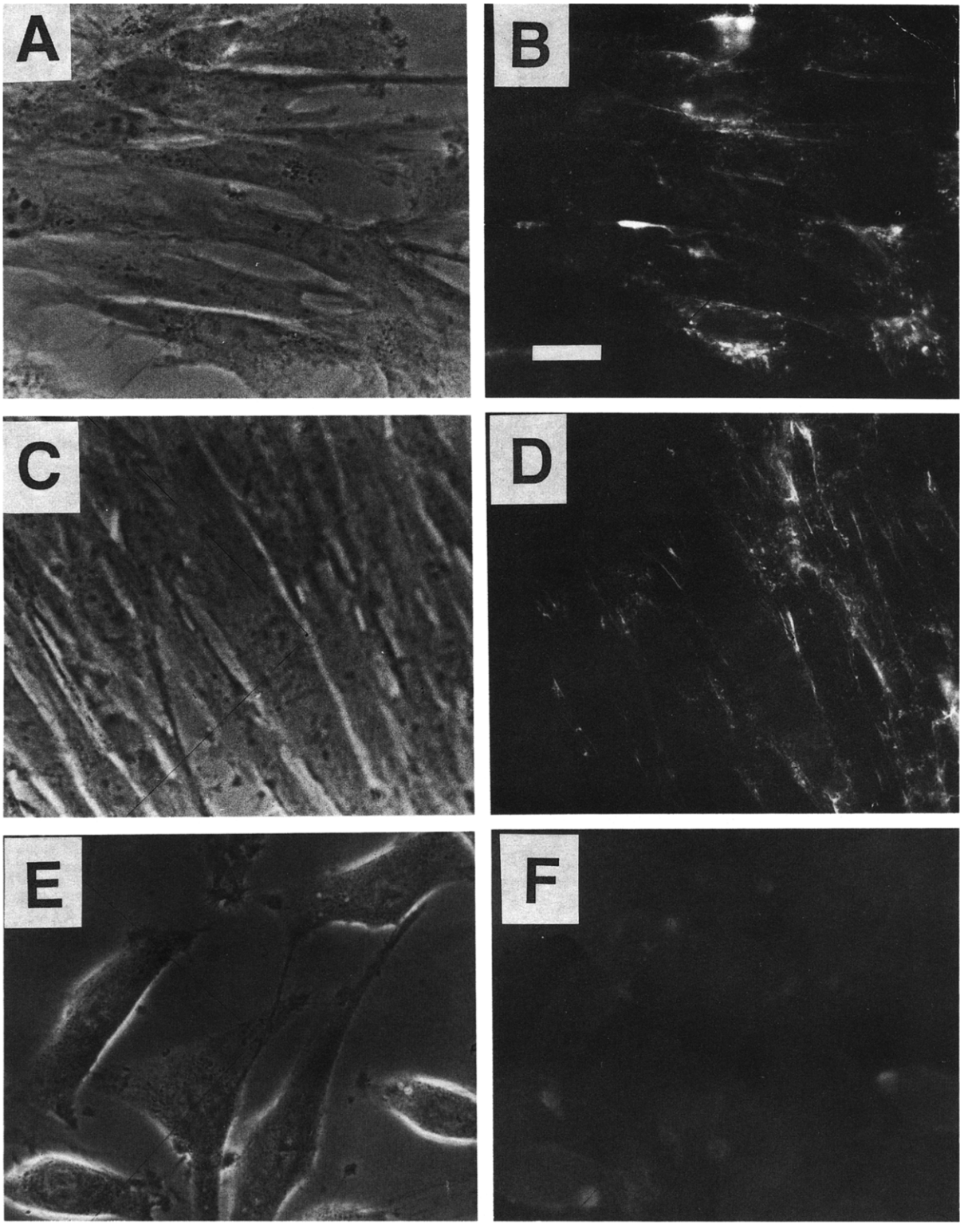
Fig.1 shows the phase and fluorescence microscope images of L6 cells labelled with S-F-ConA (A–D) and F18 (E,F). Distribution of S-F-ConA was heterogeneous in all stages. In myoblasts, continuous faintly labelled regions along the cell peripheries were seen, and the entire cell surfaces were more faintly and homogeneously labelled. Besides, patches in cell surfaces as well as around the nuclei were seen (B). As the cells matured and fusion proceeded, only the peripheral regions (boundary of cells and substrate) were strongly labelled (D). This was similar to the results in [7]. On the other hand, labelling with F18 was homogeneous except for a few bright patches (F). This pattern was seen both in cells in the fusion stage and in myotubes (not shown).

Does the motion of these molecules change with the maturation of L6 cells? We measured the lateral motion of S-F-ConA by FPR. The homogeneously labelled surface region near the center of cells where the number of patches was small was chosen (fig.2).

Significant points in fig.2 are as follows. (i) The lateral motion of S-F-ConA at the fusion stage was enhanced, and after the completion of fusion, the motion was suppressed. (ii) Among myotubes, the older ones showed slower motion. (iii) At the fusion stage, the data fluctuated significantly. This is not due to instrumental errors because these were reproducible. The individuality of each cell seems to be responsible although classification from morphology was difficult. This individuality was suppressed in myotubes. (iv) Recovery fraction did not change from day 5 to 11 and upon formation of myotubes.

Next, we measured the motion of fluorescent lipid analog, F18 (fig.3). Compared to the lateral motion of S-F-ConA, that of F18 was faster by 2 orders of magnitude, and was rather insensitive to cell maturation. However, on myotubes, the motion of F18 was slower than that on myoblasts even

Fig.1. Phase and fluorescence images of L6 cells labelled with S-F-ConA or F18. A, B, C, D, L6 cells labelled with S-F-ConA. Cells were cultured for 6 days (A, B) and for 9 days (C, D). Bar = 20 μm. E, F, L6 cells labelled with F18. Cells were cultured for 6 days.


A
B
C
D
E
F

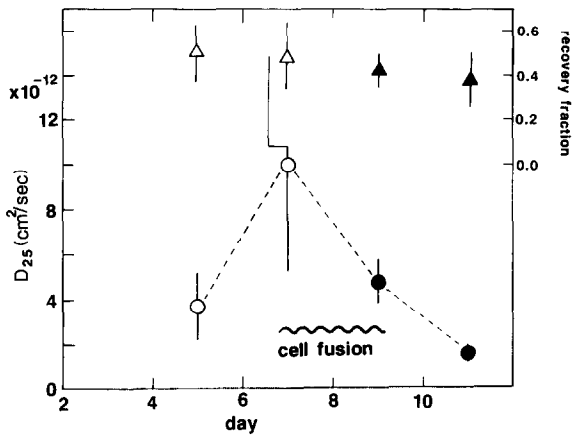


Fig.2. Dependence of lateral diffusion coefficient of S-F-ConA labelled to L6 cells on cell maturation. D_{25} is lateral diffusion coefficient at 25°C. Abscissa, culture day; triangles, recovery fraction; circles, lateral diffusion coefficient; open symbols, myoblasts; closed symbols, myotubes. About 10 cells were measured for each point. Temperature was 25°C. As indicated, cell fusion occurred from day 8.

at the same culture day. This tendency was similar to the motion of S-F-ConA. Therefore, it can be concluded that the motion of some molecules in lipid phase of cell membranes of myotubes of L6 is slowed compared with that of myoblasts. Here

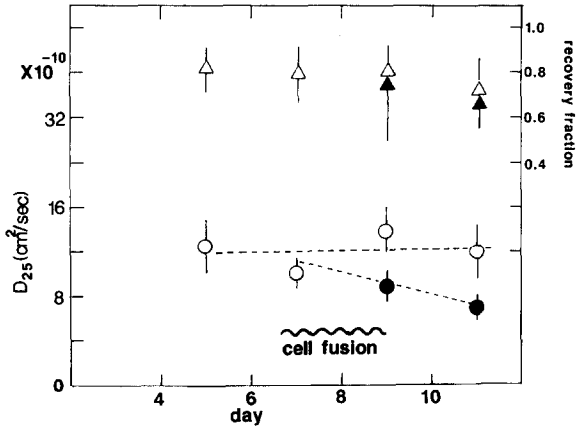


Fig.3. Dependence of lateral diffusion coefficient of F18 labelled to L6 cells on cell maturation. D_{25} is lateral diffusion coefficient at 25°C. Abscissa, culture day: triangles, recovery fraction; circles, lateral diffusion coefficient; open symbols, myoblasts; closed symbols, myotubes. About 10 cells were measured for each point. Temperature was 25°C. As indicated, cell fusion occurred from day 8.

again, recovery fraction did not change from day 5 to 11 and upon formation of myotubes.

4. DISCUSSION

In the present study, we used S-F-ConA. As a probe for membrane properties, S-F-ConA is superior to F-ConA in that the former has a weaker activity to aggregate surface molecules and does not inhibit the myoblast fusion [20]. The labelling patterns of S-F-ConA were almost identical to those of F-ConA [7]. The difference was that, in myoblasts in confluent state and also in myotubes, the entire cell surface was faintly fluorescent. This difference may arise from the fact that, using Ar^+ laser, finer images were obtained due to reduced background.

In this report, it was shown that the dynamic behavior of cell membranes of the myogenic cell line, L6, changed in a similar fashion with its morphological differentiation. We measured the motion of fluorescent molecules at the center of cells, where no intense fluorescence of S-F-ConA was observed. The present results showed that even in the region where binding sites of S-F-ConA were not largely clustered, the motion differed depending on cell states. Even in this region, recovery fractions were only 0.4 to 0.5 (see figs 2, 3). This means that a considerable fraction of S-F-ConA binding sites was immobilized. This immobilization may have occurred from anchoring and/or microclustering of S-F-ConA binding sites. (Here, microcluster means a cluster composed of 2 to several tens of molecules and not recognized as a patch.) Clustering of more than 10 S-F-ConA binding sites decreases lateral diffusion coefficient to less than 10–12 cm^2/s. Motion slower than 10^{-12} cm^2/s is out of the detection limit of FPR, and the motion is regarded as immobile. Indeed, at the region where intense fluorescence of S-F-ConA was observed, the motion of S-F-ConA was almost inhibited (not shown).

The diffusion coefficient of S-F-ConA obtained in the present study was similar to that of membrane proteins [21,22] and that of F18 was similar to those of lipids in membranes [23,24]. Therefore, it is plausible that S-F-ConA binding sites are membrane proteins and the motion of F18 represents that of lipids.

The first important point in the present study is

that at the stage of fusion, the motion of S-F-ConA became faster. However, the motion of F18 was not changed, and recovery fraction did not change either in S-F-ConA or in F18 (see figs 2, 3). These results indicate that at fusion, the motion of some specific but not all, molecules is activated, and releasing of anchored or clustered molecules does not occur.

Prives and Shinitzky reported that membrane fluidity increased just before fusion of muscle cells [25]. Their results apparently disagree with the data of F18 shown here (no change in diffusion coefficient just before fusion as shown in fig.3). This apparent disagreement may be explained as follows. (i) Prives and Shinitzky used 1,6-diphenyl-1,3,5-hexatriene and measured fluorescence anisotropy. Fluorescence anisotropy shows rotational or wobbling motion. 1,6-Diphenyl-1,3,5-hexatriene is a neutral fluorescent molecule and is considered to be located at the hydrophobic region of membranes such as acyl chains of lipids. On the other hand, F18 has a negative charge at the head position and its head is supposed to be located at the hydrophilic region. Therefore, the following is considered. Just before fusion, intermolecular spacing in the hydrophobic region increases or molecular motion of the acyl chain is activated, and the hydrophilic region does not change. (ii) Both 1,6-diphenyl-1,3,5-hexatriene and F18 are trapped in temporal cages (cages which open and close temporarily). The existence of the cage was suggested in erythrocyte membranes [26]. Just before fusion, fluidity and/or size of the cages increase while time constants of opening and closing do not change. This results in increasing the freedom of the rotational or wobbling motion while there is little change in lateral motion. (Molecules have to escape from the cages in order to move laterally, and this step is rate limiting.)

At present we do not know which explanation is correct. Simultaneous measurement of rotational and lateral motion of molecules in the same cell will give the answer.

The second important point is that at fusion stage, dispersion of the data was obtained (see fig.2). This may arise from the fact that at this stage, the difference in the properties of individual cells is enhanced. From the morphological observation, it was difficult to detect this difference in a quantitative manner. Within the cells measured, cells in various stages, such as long before, just before and partly in fusion stages, may be included.

The third important point is that, in myotubes, the motion of both S-F-ConA and F18 became slow. And among myotubes, the old ones showed more pronounced suppression of motion. These results are identical to those reported by Prives and Shinitzky [25]. Since the clustering of S-F-ConA binding sites at the center of myotubes was not observed and recovery fraction did not change, this suppression is not due to formation of clusters of S-F-ConA binding sites.

At present it is not known which mechanisms are involved and which meanings are included in these phenomena. In the process of fusion of liposomes, it is reported that disorder in bilayer structure is generated [10,11]. This disorder may promote the motion of some molecules.

In neuronal cells, the motion of fluorescent molecules embedded in cell membranes becomes slower as the cells mature [12–14]. Together with the present results, this may be a general tendency that, as cells mature or get old, the motion of molecules in cell membranes is restricted. In neuronal cells, the decrease in fibronectin receptor density occurs in parallel with the decrease in lateral motion of F18 [27]. This kind of change in molecular level may also be expected in L6.

REFERENCES

[1] Yaffe, D. (1968) Proc. Natl. Acad. Sci. USA 61, 477–483.
[2] Lewis, M.R. (1915) Am. J. Physiol. 1, 153–161.
[3] Benoff, S. and Nadal-Ginard, B. (1980) J. Mol. Biol. 140, 283–298.
[4] Schubert, D., Harris, A.J., Devine, C.E. and Heinemann, S. (1974) J. Cell. Biol. 61, 398–413.
[5] Schubert, D., Harris, A.J., Heinemann, S., Kidokoro, Y., Patrik, J. and Steinbach, J.H. (1973) in: Current Topics in Neurobiology (Sato, G. ed.) Tissue Culture of the Nervous System 1, pp.55–86, Plenum, New York.
[6] Spitzer, N.C. (1979) Annu. Rev. Neurosci. 2, 363–397.
[7] Seto-Ohshima, A., Wakayama, N. and Amano, T. (1981) Cell Struct. Funct. 6, 245–243.
[8] Kidokoro, Y. and Heinemann, S. (1974) Nature 252, 593–594.
[9] Kidokoro, Y., Heinemann, S., Schubert, D., Brandt, B.L. and Klier, F.G. (1976) Cold Spring Harb. Symp. Quant. Biol. 40, 373–388.
[10] Nir, S., Bentz, J., Wilschut, J. and Duzgunes, N. (1983) Prog. Surf. Sci. 13, 1–124.

[11] Wilschut, J. and Hoekstra, D. (1984) Trends Biochem. Sci. 9, 479–483.
[12] Kawasaki, Y., Wakayama, N., Koike, T., Kawai, M. and Amano, T. (1978) Biochim. Biophys. Acta 509, 440–449.
[13] Kawasaki, Y. and Amano, T. (1982) FEBS Lett. 140, 117–120.
[14] Horie, H., Kawasaki, Y. and Takenaka, T. (1986) Brain Res. 377, 246–250.
[15] Takenaka, T., Horie, H. and Kawasaki, Y. (1983) J. Neurobiol. 14, 574.
[16] Takenaka, T., Horie, H., Hori, H. and Kawasaki, Y. (1986) Biomed. Res. 7, suppl., 49–51.
[17] Horie, H., Kawasaki, Y. and Takenaka, T. (1987) Brain Res. 411, 298–303.
[18] Keller, P.M., Person, S. and Snipes, W. (1977) J. Cell Sci. 28, 167–177.
[19] Gunther, A.R., Wang, J.L., Yahara, I., Cunningham, B.A. and Edelman, G.M. (1973) Proc. Natl. Acad. Sci. USA 70, 1012–1016.
[20] Sandra, A., Leon, M.A. and Przybylski, R.J. (1977) J. Cell Sci. 28, 251–272.
[21] Schlessinger, J., Koppel, D.E., Axelrod, D., Jacobson, K., Webb, W.W. and Elson, E.L. (1976) Proc. Natl. Acad. Sci. USA 73, 2409–2413.
[22] Axelrod, D., Ravdin, P., Koppel, D.E., Schlessinger, J., Webb, W.W., Elson, E.L. and Podleski, T.R. (1976) Proc. Natl. Acad. Sci. USA 73, 4594–4598.
[23] Wu, En-S., Jacobson, K. and Papahadjopoulos, D. (1977) Biochemistry 16, 3936–3941.
[23] Rubenstein, J.T.R., Smith, B.A. and McConnell, H.M. (1979) Proc. Natl. Acad. Sci. USA 76, 15–18.
[25] Prives, J. and Shinitzky, M. (1977) Nature 268, 761–763.
[26] Tsuji, A. and Ohnishi, S. (1986) Biochemistry 25, 6133–6139.
[27] Kawasaki, Y., Horie, H. and Takenaka, T. (1987) Brain Res. 397, 185–188.