Vol. 126, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 16, 1985 Pages 313-319

EFFECT OF MONENSIN ON MYOBLAST FUSION*

Halina Den

Department of Anatomy and Cell Biology and H. Houston
Merritt Clinical Research Center, Columbia University,
New York, NY 10032

Received November 20, 1984

SUMMARY: Monensin, at a concentration of 0.5-10 μM, completely (100%) and reversibly inhibits fusion of embryonic chick myoblasts in vitro. At the same time, monensin administration leads to a marked accumulation of glycopeptides inside the cells and a decrease of those secreted into the medium. Chromatography of the intracellularly retained glycopeptides on Con A-Sepharose shows that the increase is most pronounced in the high-mannose fraction. Mild proteolysis of cells labeled with [2-^{3}H]mannose releases less radioactivity from the surface of monensin-treated than from control cells, although the amount of total radioactivity is almost four times higher than in the control cells. Since it has now been established that monensin interferes with the intracellular transport of newly synthesized glycoproteins it is assumed that its inhibitory effect is the result of the inability of glycoprotein(s) essential for myoblast fusion to reach the cell surface.

Ever since experiments with mixed cell populations demonstrated that myoblasts recognize each other and fuse only with other myoblasts (1,2), the existence of a cell-cell recognition or specific adhesion step in the process of myoblast fusion in vitro has been taken for granted. More recently, however, this concept was verified by experiments (3) showing that the fusion process occurs through several discrete steps, starting with cell recognition and adhesion.

It has been shown that in many instances carbohydrate-containing molecules function as adhesion factors. Interactions between surface glycoconjugates and specific molecules such as surface lectins (4,5) or surface glycosyltransferases (reviewed in ref. 6) mediate a wide range of cell-cell recognition events. The involvement of glycoconjugates in the cell recognition and/or adhesion step of the

* This investigation was supported by National Science Foundation Grant NSF PCM-82-08375 and by a grant from Muscular Dystrophy Association.

The abbreviations used are: MEM, minimal essential medium, Endo H, endo-β-N-acetylglucosaminidase.

0006-291X/85 $1.50

myoblast fusion process has been a subject of numerous studies, but no clear answer has yet emerged.

For several years, the β-D-galactosyl-specific developmentally regulated lectin was believed to mediate the recognition step in myoblast fusion. However, recent results (7,8) clearly indicate that this lectin has no function in myoblast fusion.

It has been shown that myoblast fusion in both rat (9) and quail (10) is inhibited by tunicamycin, an inhibitor of N-glycosylation. This finding suggests a role for glycoproteins in myoblast fusion. However, it has been claimed that the requirement for the carbohydrate portion of glycoproteins is only indirect, through stabilization of the protein moiety against proteolytic digestion (10).

Some time ago, we presented evidence that concanavalin A (Con A) inhibits myoblast fusion (11). Since then, several reports have emphasized the importance of glycoproteins with affinity for Con A in muscle differentiation. Measurements of the lateral mobility and topographical distribution of Con A receptors have revealed dramatic changes during the fusion period (12). More recently, using a genetic approach, two laboratories experimenting with Con A-resistant mutants of L6 myogenic cells have presented evidence linking Con A resistance of these cells with the inability to form multinucleated myotubes (13-15). The Con A resistant variants bound significantly less Con A than their parental populations, indicating that they had reduced levels of surface glycoproteins of the high mannose type. In one class of the Con A-resistant mutants, loss of a single Con A-binding protein having MW of 40K was correlated with the inability of these cells to differentiate (15).

In the present report, we describe the effect of monensin on chick myoblast fusion. At a concentration of 0.5-10μM this ionophore completely and reversibly inhibits the fusion process. Our results indicate that this inhibition may be the result of the well documented interference of monensin with the migration of nascent glycoproteins to the cell surface (16-20), and thus indirectly implicate glycoproteins in the process of myoblast fusion.

MATERIALS AND METHODS

Materials

Con-A Sepharose and Sephadex G-25 were purchased from Pharmacia. D-[2-^{3}H] mannose (10-20 Ci/mmole) is a product of Ammersham. Pronase was purchased from Calbiochem, monensin from Sigma.

Culture Conditions

The culture conditions for cells grown in high-Ca^{++} medium to permit nonsynchronous fusion have been described in detail previously, as have the culture conditions for cells made to fuse synchronously by growth in EGTA-containing medium, then changed to high-Ca^{++} medium (11).

Preparation of [2-^{3}H] Mannose-Labeled Glycopeptides From Cultured Myoblasts

About 16 h after plating (at $3x10^6$ cells per 100mm dish), before onset of fusion (which commences about 20 h after plating), growth medium (MEM containing 15% horse serum and 5% chicken embryo extract) was replaced with labeling medium (glucose-free MEM with 10% dialyzed horse serum) and after 15 min with 5 ml of labeling medium containing 0.2 mCi of [2-^{3}H] mannose per ml. After 15 min pulse the cells were washed with cold growth medium which was also used for the subsequent 4-h chase. Monensin (10 μM) was added at the initiation of the pulse and its presence was maintained during the chase. The 4-h duration of the chase was chosen because that period is much in excess of the maturation time reported for the secretory and membrane proteins studies (18,22,23). At the end of the chase period, the cells were washed thoroughly with cold PBS, scraped off the dishes with a rubber policeman, and centrifuged. One ml of 0.1 M Tris-HCl, pH 8, containing 20 mM $CaCl_2$ and 10 mg pronase was added to each pellet and the sample was incubated overnight at 56°. The digests were then boiled for 3 min, clarified by centrifugation, and desalted by passing through a Sephadex G-25 column (1 x 50 cm) in 7% propanol (21).

RESULTS AND DISCUSSION

The effect of monensin on myoblast fusion after release from EGTA fusion-block is shown in Table 1. The inhibitory effect of the ionophore was noticeable at the concentration of 0.1 μM; at 0.5-10 μM, the inhibition of fusion was complete

Table I

Synchronous Fusion of Chick Myoblasts in the Presence of Monensin

Monensin Concentration μM	Fusion %	Inhibition %
None	82	0
0.1	72	12
0.3	16	80
0.5-10	---	100

Fusion-blocked cells were grown in the presence of 1.85 mM EGTA. After 50 h in culture, the EGTA-containing medium was replaced with high-Ca^{++} medium alone or with monensin; the cultures were then incubated for 4-6 additional h. Nuclei were counted in randomly selected fields of Giemsa-stained cultures, using an ocular counting grid. The degree (percent) of fusion was determined by counting the nuclei within myotubes of three or more nuclei and dividing by the total number of nuclei.

Table II

Distribution of [^{3}H] Mannose-Labeled Glycopeptides

Inhibitor	Cells cpm x 10^{-3}	Chase Medium cpm x 10^{-3}
None	49	230
Monensin (10 μM)[a]	177	98
NH_4Cl (30mM)[a]	42	262
Chloroquine (10 μM)[a]	35	214

At the end of the chase period, the chase medium was decanted and the washed cells were scraped from the dishes. The cell pellets were digested with pronase and the [^{3}H]-labeled glycopeptides were separated from residual [2-^{3}H] mannose by Sephadex G-25 essentially as described in ref. (21). To determine the amount of protein-associated radioactivity secreted by the cells an aliquot of the chase medium was passed through a Sephadex G-25 column. The excluded material was digested with pronase (this was necessary because of a non-specific adsorption of residual [^{3}H] mannose to serum proteins) and passed again through a Sephadex G-25 column.

a. Added at the beginning of the labeling period and present during the chase.

(100%). The cells tolerated the drug well. Slight vacuolization of myoblasts was observed by phase microscopy, but no cell detachment occurred during the 4-6h of experiment, even at the highest concentration of 10 μM. Furthermore, the inhibition could be reversed by frequent changes of growth medium. Essentially the same results were obtained when monensin was added to cultures with normal growth medium (no EGTA) before the onset of fusion.

To analyze the effect of the ionophore at the molecular level, the changes in the distribution of N-linked oligosaccharides caused by monensin administration were examined. It is evident from Table II that administration of monensin to the cultures caused retention of radioactivity in the cells at the expense of the secreted fraction. This intracellular accumulation of N-linked oligosaccharides is consistent with the well known interference of the carboxylic ionophores with the intracellular pathway in the maturation of plasma membranes and secretory proteins (16-20, 23). Other inhibitors of myoblast fusion examined do not affect the distribution of N-linked oligosaccharides. This is of particular interest in the case of two lysosomotropic amines, NH_4Cl and chloroquine, which inhibit the fusion process at the concentrations tested (personal observation) (Table II; see also Fig. 1). Since carboxylic ionophores and lysosomotropic amines exert similar effects on those aspects of vesicular traffic that are attributable to the alkalin-

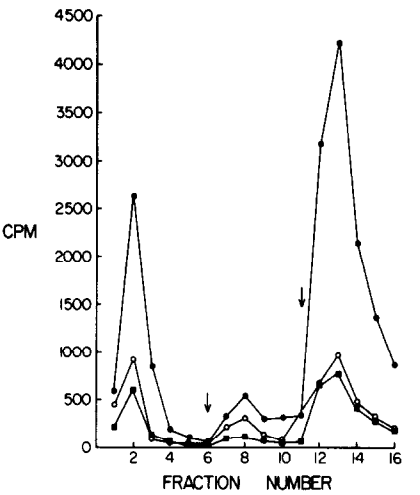

Figure 1: Chromatography of [^{3}H] mannose-labeled glycopeptides on ConA-Sepharose. Aliquots of the labeled glycopeptides extracted from the cells as described above were chromatographed on ConA-Sepharose columns (0.7x3 cm) essentially according to ref. (21). The samples were eluted sequentially with 10 mM α-methylglucoside and 500 mM α-methylglucoside at 56°. The arrows in the graphs indicate the points at which the composition of the eluate was changed. Fraction size was 1.5 ml. Control (○), 10 μM monensin (●), 10 μM chloroquine (■). It is now established (27) that ConA-Sepharose binds biantennary complex-type N-linked oligosaccharides that can be eluted with 10 mM α-Methylglucoside (Fraction 2), as well as high-mannose-and certain hybrid-type oligosaccharides that can be eluted with 500 mM α-methylglucoside (Fraction 3), Tri-and tetra-antennary complex-type N-linked oligosaccharides, as well as biantennary complex-type oligosaccharides with "bisecting" N-acetylglucosamine residue pass through the column (Fraction 1).

ization of lysosomal components (24,25), the difference in their effects on myoblast fusion may provide an additional argument for the independence of the lysosomal and secretory routings (25).

To determine if monensin affected all glycoproteins or only selected ones, the cell glycopeptides were chromatographed on Con A-Sepharose (21). Fig. 1 demonstrates changes in the profile of the N-linked oligosaccharides induced by monensin. Although the amount of radioactivity increased in all three fractions, the increase was clearly most pronounced in the high-mannose fraction.

The actual stage at which monensin blocks intracellular transport is not clear. Morphological evidence suggest that blocking occurs within the Golgi organelle, causing it to become distended and swollen (26). However, the passage of some glycoproteins is blocked at the Endo H-sensitive stage whereas the terminal glycosylation of others has been completed (18,25), indicating that the glycoproteins have different sites of arrest. The fact that the amount of radioactivity

Table III

Removal of [^{3}H] Mannose-Labeled Surface Material by Pronase Treatment of Intact Cells

Cultures	Radioactivity released cmp x 10^{-3}
Experiment 1:	
control	39
monensin-treated (10μM)	30
Experiment 2:	
control	44
monensin-treated (10μM)	28

At the end of the chase period, the cultures were washed repeatedly with PBS, then treated with pronase in PBS (100 μg/ml) at room temperature for one minute in Experiment 1 and for two minutes in Experiment 2. The pronase-released material was decanted, centrifuged to remove detached cells, and counted.

was increased in all the fractions when [^{3}H]-labeled glycopeptides from monensin-treated cells were fractionated on Con A-Sepharose (Fig. 1) suggests that the monensin block was between the trans-Golgi, where complex oligosaccharides are constructed, and the cell surface.

The inhibition of myoblast fusion by monensin may be the result of a failure of the glycoproteins necessary for fusion to reach the cell surface. Although this possibility is presently under investigation in our laboratory, the results of a preliminary experiment, shown in Table III, seem consistent with this idea. At the end of the chase period, the [^{3}H]-labeled cell surface material was partly removed by mild proteolysis. The amount of radioactivity released from the surface of monensin-treated myoblasts was significantly reduced in spite of the fact that they contained almost four times more total radioactivity than did the corresponding control (Table II).

ACKNOWLEDGMENT

The help, interest, and many useful suggestions from Dr. C.A. Gabel are greatly appreciated.

REFERENCES

1. Yaffe, D., and Feldman, M. (1965) Develop. Biol., 11, 300-317.
2. Okazaki, K., and Holtzer, R. (1965) J. Histochem. and Cytochem. 13, 726-739.
3. Knudsen, K.A., and Horwitz, A.F. (1977) Dev. Biol. 58, 328-338.
4. Vacquier, V., and Moy, G. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 2456-2460.
5. Grabel, L.B., Rosen, S.D., and Martin, G.R. Cell, 17, 477-484.

6. Pierce, M.E., Turley, E.A., and Roth, S. (1980) Int. Rev. Cytol. 65, 1-47.
7. Den, H., and Chin, J.H. (1981) J. Biol. Chem. 256, 8069-8073.
8. Kaufman, S.J., and Lawless (1980) Differentiation 16, 41-48.
9. Gilfix, B.M., and Sanwal, B.D. (1980) Biochem. Biophys. Res. Commun. 96, 1184-1191.
10. Olden, K., Law, J., Hunter, V.A., Romain, R. and Parent, J.B. (1981) J. Cell Biol. 88, 199-204.
11. Den, H., Malinzak, D.A., Keating, H.J. and Rosenberg, A. (1975) J. Cell Biol. 67, 826-834.
12. Fernandez, S.M. and Herman, B.A. (1982) in Muscle Development: Molecular and Cellular Control (Pearson, M.L., and Epstein, H.F., eds.) pp. 319-327, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. Parfett, C.L., Jamieson, J.C., and Wright, J.A. (1981) Exp. Cell Res. 136, 1-14.
14. Parfett, C.L., Jamieson, J.C., and Wright, J.A. (1983) Exp. Cell Res. 144, 405-415.
15. Cates, G.A., Brickenden, A.M., and Sanwal, B.D. (1984) J. Biol. Chem. 259, 2646-2650.
16. Tartakoff, A.M. and Vassalli, P. (1977) J. Exp. Med. 146, 1332-1345.
17. Uchida, N., Smilowitz, H., Tanzer, M.L. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 1868-1872.
18. Strous, G.J.A.M. and Lodish, H.F. (1980) Cell 22, 709-717.
19. Rotundo, R.L. and Fambrough, D.M. (1980) Cell 22, 595-602.
20. Green, R. and Shields, D. (1984) J. Cell Biol. 99, 97-104.
21. Reitman, M.L., Townbridge, I.S., and Kornfeld, S. (1980) J. Biol. Chem. 25, 9900-9906.
22. Fitting, T., and Kabat, D. (1982) J. Biol. Chem. 257, 14011-14017.
23. Lodish, H.F., Kong, N., Snider, M. and Strous, G.J.A.M. (1983) Nature (Lond.) 304, 80-83.
24. Basu, S.K., Goldstein, J.L., Anderson, R.G.W. and Brown, M.S. (1981) Cell 24, 493-502.
25. Tartakoff, A.M. (1983) Cell 32, 1026-1028.
26. Somylo, A.P., Garfield, R.E., Chacko, S., and Somylo, A.V. (1975) J. Cell Biol. 66, 425-443.
27. Cummings, R.D., and Kornfeld, S. (1982) J. Biol. Chem. 257, 11235-11240.