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Quantitation of Changes in Cell Surface Determinants During Skeletal Muscle Cell Differentiation Using Monospecific Antibody

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The differentiation of skeletal muscle is characterized by recognition, alignment, and subsequent fusion of myoblast cells at their surfaces to form large, multinucleated myotubes. Monoclonal antibodies were used to investigate antigenic changes in the cell surface membrane specific for various stages of myogenesis. Chick embryonic skeletal muscle cells were cultured in vitro to the desired stage of differentiation and then injected into BALB/c mice. Spleen cells from the immunized mice were hybridized with NS-1 or P3 8653 mouse myeloma cells. Hybrid cell clones were selected in HAT medium and screened using an indirect radioimmunoassay for the production of monoclonal antibodies specific to myogenic cell surfaces. Target cells for the radioimmunoassay included three stages of myogenesis (myoblasts, midfusion myoblasts, and myotubes) and chick lung cells as a control for polymorphic antigens. Sixty-one clones were obtained which produced antibodies specific for myogenic cells. Thirty-five of these clones were generated from mice immunized with midfusion myoblast stages of myogenesis and 26 were obtained from mice immunized with the later myotube stage of myogenesis. Quantitative measurements by RIA of myogenic determinants per cell surface area on each target cell type revealed that most of the determinants decrease during myogenesis when midfusion myoblasts are used as the immunogen. When myotube stages are used as the immunogen, more determinants increase with cell differentiation. Therefore, the most common pattern of determinant change is for them to be present at all stages of myogenesis but to vary quantitively through development. There are determinants unique to each stage of myogenesis and marked quantitative differences within a cell stage for each determinant.

Key words: skeletal muscle, cell surface, monospecific antibody, myogenesis

Myogenesis is characterized by at least two major cell–cell interactions to achieve a functional skeletal muscle cell. First is the alignment and fusion of myoblasts with one another and the second is the interaction of the multinucleated myotube with an axon from the motor neuron [1]. Undoubtedly, important interactions at the cell surface occur between myoblasts and myotubes and the

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extracellular matrix as well [2]. These interactions and changes in cell surface properties during myogenesis have been explored, but their molecular basis remains unknown [3–10]. One approach to these interactions is to identify surface determinants using monospecific antibody produced by hybridomas to developing muscle [11–14]. Described here are experiments in which hybridomas were produced from spleen cells of mice immunized with midfusion myoblasts and myotubes from embryonic chick cell cultures and quantitative measurements of changes in specific determinants that occur as myogenesis proceeds from myoblast to myotube stages.

MATERIALS AND METHODS

Cell Culture

Pectoral muscle from 12 day chick embryos was dissociated into myoblasts and cultured as previously described [15]. Target cell cultures of specific stages of myogenesis were prepared in microtiter wells (Falcon) and either fixed with glutaraldehyde or used in a radioimmunoassay without fixation. Lung cells from the same embryos were cultured under identical conditions.

Hybridization

BALB/c mice were immunized with living midfusion myoblast or myotube cell cultures according to a modification of the method of Kennett et al. [16]. Spleen cells were isolated and fused with either P-NS-1 or P3 × 63-Ag8.653 mouse myeloma cells [16]. In all experiments reported here the supernatants isolated from clones were used as a source of monospecific antibody.

Radioimmunoassay

The binding of supernatant antibodies to living or fixed cell targets was carried out according to a modification of the method of Kennett et al. [16]. The primary antibody was incubated with living cell targets (myoblasts, midfusion myoblasts, myotubes, and chick lung cells) for 2 hr at 4°C. The target cells were then washed and fixed with 0.1% glutaraldehyde and 20–30,000 cpm of ^{125}I-goat anti-mouse Fab was added for an overnight incubation at 4°C. The targets were washed and solubilized in 2 N NaOH.

The amount of secondary antibody was normalized as follows. The surface area of the myogenic cell target was determined by seriology using the method of Weibel [17]. A micrometer reticle (American Optical Corporation) with 441 line intersections was inserted into the eye piece of the microscope and superimposed on a field of cells magnified at 250×. The total point intersections falling on cell surfaces represents the fractional area of the well covered by cells. Twenty randomly selected fields for each cell target type were used. All radioimmunoassay binding data were then normalized to surface area of each microtiter well containing target cells.

RESULTS

Six fusion experiments were performed, four using spleen cells from mice immunized with midfusion stage myoblast cultures and two using myotube

cultures. The number of wells plated in the antibody-producing clones isolated is summarized in Table I. All supernatants from hybridomas were tested on four different cultured cell targets—myoblasts, midfusion myoblasts, myotubes, and chick lung cell cultures. This latter target was used to screen for tissue specificity and to screen for determinants secondary to tissue culture ingredients such as horse serum, which may bind to cells in culture. Nine percent of the clones obtained produced antibody that was specific (reacted 2–14 times greater on myogenic cell cultures than lung cell targets) to one or more myogenic cell stages.

Each of the myogenic cell specific antibodies (61 supernatants) was then classified as to its specificity for the three stages of myogenesis (Table II). The number of determinants for each antibody on myoblasts, midfusion myoblasts, and myotube culture targets was quantitated under conditions where the primary monospecific antibody was saturating. The amount of secondary antibody bound was normalized to cell surface area present in each of the cell targets assayed. Therefore, the data reported here are a quantitative measurement of determinants per square millimeter of surface area of various stages of myogenesis.

It is clear from Table II that when the antigen used to immunize the donor mouse is at an early stage of myogenesis (midfusion myoblasts), a higher percentage of the clones obtained produce antibodies to determinants most prevalent on the myoblast stage of myogenesis. When myotubes are used as the immunogen, more clones produce antibodies to myotube stages or to determinants prevalent at later stages of myogenesis. Fifty of the antibodies reacted to some degree with all three types of myogenic targets. However, there were 11 antibodies that reacted with only one or two myogenic targets (Table III).

TABLE I. Summary of Six Cell Fusions to Produce Hybridomas to Myogenic Cells

	Midfusion myoblast immunogens	Myotube immunogens
Wells plated	1920	1101
Clonal growth	348	337
Antibody producing clones	118	137
Myogenic specific clones	35	26

TABLE II. Characterization of Monospecific Antibodies to Myogenic Cells

	Number of antibodies (%)			
Targets with greatest number of determinants/mm² surface area[a]	Midfusion immunogens		Myotube immunogens	
Myoblasts	19	(54)	7	(27)
Midfusion myoblasts	3	(9)	3	(12)
Myotubes	12	(34)	14	(54)
Midfusion myoblasts and myotubes	1	(3)	2	(8)

[a]The binding of each antibody to living cell targets is recorded according to the target which has the highest binding per surface area of target cell. Most antibodies (58) bind to more than one target (see Figures 1 and 2 and Table III).

TABLE III. Monospecific Antibodies That Bind to Only One or Two Cell Stages in Myogenesis

	Midfusion immunogens									Myotube immunogens	
Target cell type	cpm bound/mm²									cpm bound/mm²	
Antibody	A	B	C	D	E	F	G	H	I	J	K
Myoblasts	51	55	53	0	0	0	0	0	0	0	0
Midfusion myoblasts	0	0	29	29	91	39	18	0	0	8	10
Myotubes	0	0	0	0	49	56	26	47	43	33	25

To better visualize changes in determinants relative to developmental stages, all quantitative measurements of binding were converted to percentage change in binding as myogenesis proceeded from the myoblast through the midfusion to the myotube stage of development (Fig. 1). The data are plotted as relative changes (either as an increase or as a decrease) compared to the myoblast stage. Only those antibodies reactive with all stages are plotted. When midfusion antigens were used to immunize mice, 85% of the determinants detected decreased from the myoblast to the midfusion stage. About one-quarter of the detected determinants (6/26) decreased at midfusion and increased above myoblast levels by myotube stages. Fifteen percent (4/26) of the detected determinants rose throughout myogenesis with the myotube stage being the highest in all but one instance.

In Figure 2 are plotted the quantitative changes in determinants relative to the myoblast stage of development when the antigenic source used to immunize the mice was a myotube culture. Again, most determinants (67%) decreased as myoblasts entered the midfusion stage. There were eight determinants (31%) that decreased at midfusion but rose at myotube stages. However, the most evident aspect of the developmental pattern is that more than 66% of the detected determinants were increased at myotube stages and that the magnitude of some of these changes was very large.

DISCUSSION

In these studies myogenesis was arbitrarily divided into three developmental stages—myoblasts, a replicating single cell population; midfusion, a myoblast population in which the cells have aligned themselves and are in the process of cell fusion or have partially fused: and the myotube stage, where large multinucleated cells are present. The hope was that hybridomas could be used to detect antigens unique to specific stages. About 5% of the antibodies detected were reactive with only one of the myogenic stages. The importance of these determinants for developmental events remains to be analyzed.

The most common finding for determinants detected by antibodies developed in mice immunized with midfusion stage myogenic cells was a quantitative decrease in the determinants as development proceeded. In particular, the greatest quantitative change occurred when the midfusion stage was reached with nearly 85% of the detected determinants decreasing at this time. However, this was a transient decrease because in several instances (23%) these determinants would increase again as myotubes developed. The most striking finding in the

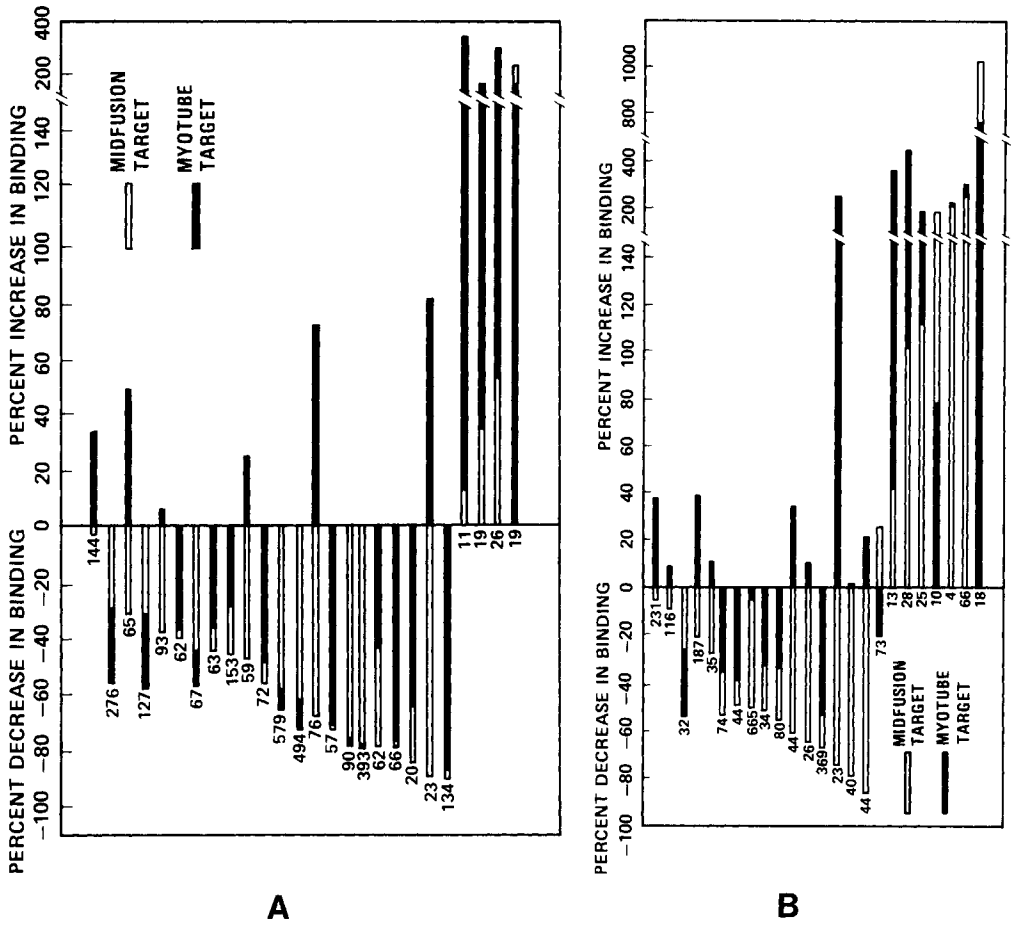

Fig. 1. Binding of hybridoma supernatants to myoblasts, midfusion myoblasts, and myotube cells. Data are plotted as relative change (either an increase or a decrease) compared to the myoblast stage of development (0 binding). Only those antibodies reactive with all stages are plotted. A) is for hybridomas produced from mice immunized with midfusion cells. B) is for hybridomas from mice immunized with myotube stage cells. The numbers below each column indicate the cpm of ^{125}I-Fab bound per mm^2 of surface area of myoblast cells.

midfusion myoblast clones was that of the 26 antibodies studied (Fig. 1) 62% of the determinants were most prevalent on myoblasts during the process of cell differentiation. When myotubes were used as the immunogen, a different, quantitative pattern of detected determinants was found during differentiation (Fig. 2). Here, the most striking finding was that most of the detected determinants increased as cells progressed from the myoblast to the myotube stage (67% of determinants). There were also six examples of a particular determinant behaving in a discordant fashion in development, ie, decreasing at one stage and increasing at another. In all but one of these instances the decrease was at the midfusion stage and the increase was at the myotube stage.

Not only is there a pattern to the type of determinant detected when the antigenic source is different, but also there are quantitative differences in the amount of many determinants at specific stages (see Figs. 1 and 2). For example, as shown in Figure 1 there is a 20-fold range in the quantity of determinants on the myoblast stage itself. It is recognized that there is the possibility that several of these clones could produce antibodies that are to the same determinants. Therefore, each data point in Figures 1 and 2 might not be a distinctly different determinant. That the antibodies used here are not to the same determinant or to a relatively small number is suggested by our finding that there is a wide range in the amounts of determinant detected on each target cell type. With 61 antibodies, it is not possible to do competitive binding assays with each antibody against every other antibody on three cell targets. But of the 16 antibodies that were tested in competitive binding assays, none competed for the same determinant.

These data indicate that there are surface determinants specific to myogenic cells that distinguish them from other embryonic cells (lung for example). Most determinants are not unique to specific stages of myogenesis even though they are specific to myogenic cells. Most determinants tend to change quantitatively during myogenesis, though there are some determinants unique to specific stages. The fact that the stage of myogenic culture used to immunize the mice (midfusion myoblasts or myotubes) influences the type of clonal populations of antibody producing cells obtained supports the conclusion that there are surface changes characteristic of different stages of myogenesis.

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