The study of the development of muscle is of interest not only to meat producers but also to developmental biologists, membrane biochemists, and molecular biologists, among others. Developing muscle is becoming quite popular among basic scientists not only because of its importance in health and nutrition but also because of certain technical aspects: Muscle cells are relatively easy to isolate and maintain in cell culture where the processes of development can be readily manipulated. In addition, the dramatic morphological changes that occur upon muscle development provide a useful visible marker for differentiation.

The preparation of the cultures is simple: Pectoral muscles are dissected from 11- or 12-day chick embryos. The muscles are minced into fragments of about 1 or 2 mm, and the muscle cells are dislodged from the fragments either by incubation with proteases, collagenase, or by mechanical disruption. The cells are then plated in plastic petri dishes at an appropriate density so that they will undergo several cell divisions before they become crowded. The cells that are isolated in this manner are called myoblasts.

For the purposes of this paper muscle development will be considered as beginning with the myoblast. One can obtain these from a variety of animals: mammals, birds, reptiles and insects have all been used as sources of myoblasts. The most common animals used are chick embryos and newborn mice or rats. Since work in my laboratory primarily involves chick muscle I'll confine my remarks to the chick system but most aspects of muscle differentiation are common to both mammalian and avian systems. Myoblasts are proliferative cells that have a rather distinctive appearance. They are bipolar and slightly rounded which allows them to be distinguished from mesenchymal cells, undifferentiated cells from which myoblasts are derived, and from fibroblasts, connective tissue cells that always contaminate the muscle cultures to some extent. After a few rounds of cell division in culture the myoblasts stop dividing, align themselves in strings, and fuse to form the multinucleated cells called myotubes. The myotubes are enlarged by further fusion with myoblasts, and the synthesis of proteins characteristic of differentiated muscle is initiated. After a short time the myotubes usually contract spontaneously.

While the myoblasts are morphologically differentiated from other cells, they are not functionally differentiated. Differentiation normally begins after cell fusion. The protein content of the cell then becomes substantially different from its precursors in that proteins characteristic of striated muscle are synthesized. These include the muscle isoforms of the contractile proteins such as actin, myosin, and tropomyosin (Allen, et al., 1979). These contractile proteins are also quite abundant in non-muscle tissue but the isoforms found in non-muscle tissue are different from the isoforms in striated muscle and can be distinguished by gel electrophoresis. Other differentiated proteins include the muscle isoforms of creatine kinase (Lough and Bischoff, 1977) and adenylate cyclase (Prives and Patterson, 1974) and the cell surface proteins involved in reception of nerve impulses, the acetylcholine receptor and acetylcholinesterase (Prives and Patterson, 1974).

The media in which the muscle cells are cultured always contain a defined component and an undefined component. The defined component is a commercial tissue culture medium that contains amino acids, carbohydrates, salts, and vitamins. The undefined component of the medium usually consists of either horse serum plus embryo extract, which is a soluble fraction from homogenized chicken embryos, or fetal-calf serum. Many investigators have tried to fractionate the sera to ascertain what factors are needed by the cultured cells. The answers are not all in yet, but it appears that a number of factors are needed, particularly the peptide hormones such as insulin and epidermal growth factor. Antibiotics and anti-fungal agents are also added to the culture media.

The goal of studying muscle development in cell culture is the elucidation of molecular events involved in differentiation. To do this one has to be able to measure quantitatively myoblast fusion and the synthesis of muscle-specific proteins. Figure 1 is a generalized drawing that shows the kind of data that are found in many of the papers in the muscle literature. Myoblast fusion is measured after fixing the cells and staining the nuclei; the percent of total nuclei that are in myotubes is then counted. With the chick cultures fusion usually begins after about two days in culture and it is relatively synchronous. The methods used for following protein synthesis depend on the protein of interest. Enzymatic activity is measured for appropriate enzymes, the contractile proteins are usually measured after addition of a radioactive amino acid to the culture media.
Muscle cell fusion is a very specific process and it has long been postulated that cell surface proteins are required for this specificity. Along these lines, it is known that protein synthesis must be occurring in pre-fusion myoblasts in order for fusion to occur. If protein synthesis is inhibited with cycloheximide, fusion does not take place.

A certain lipid composition is presumably required for myoblast fusion. This has been studied by adding to the culture medium certain unusual fatty acids and sterols which the cells can incorporate into membrane lipids. It appears that the rate of myoblast fusion can be accelerated or decreased by substantial alterations in the membrane lipid composition.

The role of cell surface carbohydrates in myoblast fusion and protein synthesis is not yet clear. Cell surface oligosaccharides are known to be involved in some differentiating systems and some evidence indicates that oligosaccharides are likely to be involved in muscle differentiation as well. The muscle system is turning out to be very popular for molecular biologists as a system to study regulation of gene expression. In the near future a lot of work will probably appear that is directed toward an understanding of what factors turn off the expression of undifferentiated genes, such as for non-muscle actin, and what turns on the expression of the muscle-specific genes. Currently, workers are isolating messenger RNA’s for the muscle proteins, cloning DNA that is complementary to the messenger RNA, then using the cloned DNA as a tool to answer specific questions about the regulation of gene expression.

It should be noted that not all the myoblasts fuse into myotubes, either in culture or in vivo. The unfused myoblasts in vivo remain associated with the periphery of bundles of muscle fibers, where they are termed satellite cells. These cells are responsible for muscle regeneration after injury. The nuclei within the muscle fibers that resulted from myoblast fusion can no longer divide; all nuclear division that takes place after injury results from stimulation of the dormant myoblasts or satellite cells to divide.

As indicated before, there has been considerable interest in the role of cell surface lipids in myoblast fusion. We are interested in what regulates the levels of certain lipids in cell membranes, and we were intrigued by the observation that treating bacterial cultures with a certain bacterial phospholipase C prevents membrane fusion (Nameroff et al., 1973). The phospholipase treatment allows the cells to line up as if they are undergoing normal processes of cell recognition and adhesion, but the myoblasts do not fuse.

The extent of inhibition depends on the amount of enzyme added to the culture. At the minimum level of enzyme needed to prevent fusion, there is quite a bit of phospholipase in the cell culture. The ability of myoblasts to survive this treatment is a surprise, because this particular enzyme is quite destructive to the major membrane lipid, phosphatidylcholine. Figure 2 show the enzymatic reaction catalyzed by phospholipase C. The enzyme converts phosphatidylcholine to diacylglycerol and phosphocholine. From what we know about the properties of these compounds, the membranes should be destroyed by this treatment, but the myoblasts are not. Is it possible that the lipids are not destroyed because access of the enzyme to the cell membrane is prevented by components of an extracellular coat? Possibly, but we do
EFFECTS OF PHOSPHOLIPASE C ON SOME CULTURED CELLS

Figure 2. Effects of Phospholipase C on Some Cultured Cells.

know that at least some of the enzyme can get to the surface because we can demonstrate breakdown of cellular lipid, (Kent, 1979). In the presence of phospholipase C about 50% of the labeled phosphatidylcholine was degraded after 16 hours with the enzyme while the lipid in untreated cells was not degraded. The myoblasts, however, contain as much phosphatidylcholine as untreated cells, indicating that the rate of synthesis of phosphatidylcholine must be increased to compensate for the increased rate of degradation.

The rate of synthesis of phosphatidylcholine can be measured by following the rate of incorporation of lipid precursors into that lipid (Kent, 1979). The rate of synthesis of phosphatidylcholine is three to four-fold higher in cultures treated with phospholipase C. We are now using this system to answer several questions. One question concerns the detection of membrane damage: How do the cells know that the surface membrane is being degraded and that the rate of synthesis of phosphatidylcholine should be increased? What enzyme in the pathway of synthesis of phosphatidylcholine is responsible for increasing the rate of synthesis of that lipid?

We are also interested in the different types of response we have observed when different types of cells have been treated with the enzymes (Figure 2). For instance, if we add the enzyme to cultures of chick embryo fibroblasts, the cells do respond by making more phosphatidylcholine. However, they also make much higher levels of triglycerides, while the myoblasts do not. If the enzyme is added to older cultures in which differentiation has already taken place, the myotubes are destroyed. We would like to know what factors are regulating the different abilities of these cells to survive the phospholipase treatment and in the different responses in lipid metabolism that the enzyme invokes.

Figure 3 shows one reason why we are so interested in the system just described. Our results indicate that the muscle cells have a way of repairing the surface membrane, although we do not yet know what that mechanism is. There is some evidence to indicate one of the earliest observable defects in Duchenne’s muscular dystrophy is damage to the surface membranes. A number of researchers believe that the destruction of muscle may be due to the inability of Duchenne muscle to repair the surface damage. Since we know absolutely nothing about the ability of muscle or any other cell to repair its surface membrane, this appears to be a promising model system that can be used for further studies in this area.

References


