REVIEW ARTICLE

The fusion of myoblasts

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Introduction

Skeletal muscle fibres are permanent multi-nucleated, non-mitotic cells. Consequently, there is considerable interest in how such a unique cell type is formed. Muscle fibres are derived from multinucleated myotubes which are themselves formed, during embryonic development, by the fusion of mononucleated myoblasts.

Myoblast fusion provides a naturally occurring process in which mechanistic proposals from the study of fusion in model fusion systems can be tested. Conversely, proposals derived from myoblast fusion studies can be profitably applied to appropriate vesicular model systems. *In vivo*, the normal development of muscle has been observed in many species. Although the transition from mono- to predominantly multi-nucleated muscle cells can be observed in sections of embryonic muscle, this is asynchronous and poses almost insurmountable difficulties for the biochemical analysis of discrete stages. Tissue culture techniques dramatically simplify this analysis. Holtzer *et al.* (1958) were the first to show that the fusion of myoblasts occurred in primary culture, and since then fusion studies have emphasized the importance of this powerful model *in vitro*. Muscle cell fusion can be studied using both primary cultures and identified cell lines. Primary cells are prepared from embryonic tissues by mechanical or enzymic disaggregation (see Konigsberg, 1978) and cultured in medium supplemented with serum (horse serum or foetal calf serum) and embryo extract. Primary muscle cells can be prepared from a variety of insect, avian and mammalian species. For fusion the most commonly used are embryonic chick or quail muscle cells. Cell lines which also undergo some stages of myogenesis, including fusion, have been isolated. The most extensively studied are the L6 and L8 lines isolated from rat muscle tissue (Yaffe, 1968; Yaffe & Saxel, 1977). Whilst several interesting reports involving the use of cell lines exist and are quoted in this Review, it should be remembered that these cells are transformed and they may not be truly representative of

a physiological situation. For example, the fusion of L6 myoblasts does not have the absolute requirement of primary cultures for embryo extract.

Some common features are observed in all systems. Myoblasts proliferate until they withdraw from the cell cycle. They align both during and after proliferation, and then fuse. Fig. 1 shows the transition from proliferating myoblasts, to aligned myoblasts, to fused myotubes. The cells that fuse are terminally differentiated, post-mitotic myoblasts which are generated from actively proliferating precursors. Myoblast fusion was last extensively reviewed by Bischoff (1978) and readers are referred to that review for background information not covered in the present article. Many reports exist in which additions have been made to the culture media that result in less fusion or a lack of fusion being observed 24h, or more, later. Such studies are, in general, uninformative about the actual process of membrane union and thus have not been quoted in this Review. Instead, I have attempted to review selectively those reports that add to our knowledge of the actual process of myoblast fusion.

Alignment and recognition

Myoblast fusion is very cell specific. Although heterotypic fusion between, for example, rabbit and rat myoblasts has been demonstrated, fusion between rat heart or kidney cells and myoblasts does not occur (see Bischoff, 1978). It is possible that the specificity of fusion is a reflection of the process of recognition and adherence of the cells during the process of alignment (see below). Alignment is the parallel apposition of the long axes of the myoblasts. The molecular factors specifying alignment are unknown. However, the guidance of myogenic cells appears to be provided by fibronectin (Chiquet *et al.*, 1981; Ehrismann *et al.*, 1981). The data of Turner *et al.* (1983) also suggest a role for fibronectin arrays in the production of branched myotubes *in vitro*, a phenomenon not observed *in vivo*.

Fig. 1. *Fusion of myoblasts in culture*

The micrographs show cells stained with Giemsa's stain (Wakelam & Pette, 1982), from a single preparation of 12-day old chick embryonic breast muscle cells, cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) horse serum and 2% (v/v) embryo extract. (a) 17 h in culture; cell division is underway and alignment is beginning. (b) 24 h in culture; the typical spindle shape of myoblasts is apparent, and extensive alignment is also apparent. (c) 43 h in culture; early fusion is underway. (d) 70 h in culture; fusion is complete, and very large multinucleated cells dominate the culture. Magnification $\times 205$.

Fusion occurs following alignment. The timing of this is dependent upon medium composition and cell density (see Linkhart *et al.*, 1981). This could be due either to the production of a fusion-stimulating factor or to the depletion of growth-promoting factors, and it is pertinent that myoblasts withdraw from the cell cycle before fusion. In general, the fusion of chick primary myoblast cultures begins after about 42 h and lasts for about 18 h (Fig. 2). Several models have been proposed for the sequence of events. One proposal is that, upon contact, fusion directly occurs between apposed regions of membrane. An alternative view is that fusion results from a sequence of separate recognition and adhesion events. The available evidence, mainly the work of Horwitz and his colleagues, strongly supports the latter view. This group has utilized a suspension fusion system which yields multinucleated myoballs as opposed to the normal myotubes upon fusion. Early studies used myoblasts grown for 51 h in a low- Ca^{2+} , fusion-impermissive, culture medium (Knudsen & Horwitz, 1977, 1978). The cells are harvested by EDTA treatment, resuspended in fresh medium and gently agitated at 37°C. In the absence of Ca^{2+} the cells fail to aggregate. In the presence of 1.6 mM- Ca^{2+} the cells aggregate within a few minutes. The formed aggregates are made up of myoblasts and exclude fibroblasts. Under these conditions the strength of the aggregates increases with time. For the first 20 min aggregates can be dispersed by removing Ca^{2+} with EDTA. Between 30 and 60 min the aggregates are dissociable with trypsin but not with EDTA. After this time the aggregates are undissociable and by 2 h have become recognisably multinucleated single cells (Knudsen & Horwitz, 1977).

These observations suggest a sequence of distinct stages in the fusion process. Recognition, the first stage, is followed by adhesion. This is defined as the stage where the aggregates are resistant to dispersion in EDTA-containing medium, but are still sensitive to trypsin. The next stage, characterized by trypsin resistance, probably reflects the onset of membrane union. Alternatively, a stage of irreversible adhesion could precede membrane union.

Support for the occurrence of distinct stages is provided by the effects of chemical agents and other manipulations upon myoblast interactions in suspension. The initial formation of aggregates is sensitive to the concentration of Ca^{2+} and has the same dependence on pH, temperature and age of culture as fusion in monolayer cultures (Knudsen & Horwitz, 1977). It is not clear if these factors additionally influence other stages. Myoblasts treated with trypsin, glutaraldehyde, energy poisons, or grown with inhibitors of sterol synthesis

neither aggregate nor fuse (Knudsen & Horwitz, 1978; Cornell *et al.*, 1980). The uniqueness of the adhesive interactions is shown by inhibition of fusion by 20 mM- Mg^{2+} or concanavalin A whereas aggregation is not inhibited (Knudsen & Horwitz, 1978). Significantly, such inhibited cells are not resistant to EDTA dispersion. Inhibition of fusion by cytochalasin b or colchicine is characterized by aggregated cells that are resistant to dispersion by EDTA but not by trypsin (Knudsen & Horwitz, 1978). Cells grown under culture conditions that result in enrichment of the membrane lipids with elaidate are also resistant to EDTA but not trypsin dispersion. It is probably at this point in the fusion process that a fluid membrane is required (see below). Similar experiments can also be done with myoblasts grown in suspension throughout their culture life, and Neff & Horwitz (1982) have utilized such cultures to develop a rapid fusion assay.

The process of irreversible adhesion thus appears to be obligatory for fusion, though it is not clear if it should be regarded as an event that is distinct from fusion or as a part of the process. It is interesting in this respect that structures which may correspond to gap junctions have been observed, and using electro-physiological techniques, myoblasts were identified that were electrically coupled but not fused. Ultrastructural examination showed areas of close membrane apposition similar to gap junctions in all such cells (Rash & Fambrough, 1972). In support of this observation, freeze-fracture studies of 19-day foetal rat muscle have suggested that such junctions also occur *in vivo* (Rash & Staehelin, 1974).

Fusion kinetics

Metal ions and fusion

Like many other fusion systems, myoblast fusion is Ca^{2+} -dependent. Shainberg *et al.* (1969) decreased the medium Ca^{2+} concentration from 1.4 mM to 0.27 mM and observed normal proliferation but an inhibition of fusion of rat myoblasts. Restoration of the normal Ca^{2+} concentration resulted in fusion. Lowering the medium Ca^{2+} concentration has both specific and general effects. General inhibition of muscle cell differentiation has been observed upon Ca^{2+} depletion (Patterson & Strohman, 1972; Easton & Reich, 1972). Myoblasts do, however, divide, align and achieve fusion-competence when cultured at a medium Ca^{2+} concentration as low as 0.1 μM . The addition of 1.4 mM- Ca^{2+} after 50 h to such cells results in rapid fusion (Fig. 2), and this procedure has proved to be of great use in the examination of fusion as a synchronous process. Studies using such a procedure have shown the optimum Ca^{2+}

Fig. 2. Time course of myoblast fusion

This shows the extent of fusion under differing culture conditions. Fusion is scored as a percentage of total nuclei present in cells containing three or more nuclei (see Wakelam & Pette, 1982). □, Myoblasts cultured in medium containing 1.4 mM-Ca^{2+} ; ▲, cells cultured in medium containing $0.1\text{ }\mu\text{M-Ca}^{2+}$; ○, cells cultured in the presence of $0.1\text{ }\mu\text{M-Ca}^{2+}$ for 50h, and then in the presence of 1.4 mM-Ca^{2+} .

Fig. 3. Ca^{2+} -dependence of fusion

Myoblasts were cultured as in Fig. 1 except that the concentration of Ca^{2+} was varied (as in Wakelam, 1983). Fusion was scored as in Fig. 2 after 60h in culture.

concentration for fusion to be 1.4 mM (van der Bosch *et al.*, 1972; Schudt & Pette, 1975). The effect of the Ca^{2+} concentration in the medium upon fusion is shown in Fig. 3.

The requirement for Ca^{2+} is specific. Other ions such as Mg^{2+} , Zn^{2+} , Mn^{2+} , Ba^{2+} , Cu^{2+} , Cd^{2+} , La^{3+} and Li^{+} cannot substitute and indeed at higher concentrations inhibit fusion. The exception is Sr^{2+} which at 2.4 mM can partially substitute for Ca^{2+} (Schudt *et al.*, 1973). The role of Ca^{2+} is certainly multiple though it is clear that Ca^{2+} entry precedes fusion (David *et al.*, 1981). These authors also showed that the Ca^{2+} ionophore, A23187,

could induce precocious fusion but only in the 9h period before the onset of fusion in control cultures. The addition of A23187 to fusing cultures has no effect on the fusion rate at any medium Ca^{2+} concentration (Schudt & Pette, 1975). Ca^{2+} thus has both intra- and extracellular roles in myoblast fusion. Contrary to simple expectations that pre-aligned cells cultured for 50h at a medium Ca^{2+} concentration of $0.1\text{ }\mu\text{M}$ should immediately fuse upon Ca^{2+} addition, the kinetics observed are sufficiently complex to suggest that multiple processes may be involved. The kinetics of the fusion process have been studied by Neff *et al.* (1984) who

used two complementary fusion assays. One, designed to measure the onset of membrane continuity, involved the transfer of a fluorescent lipid from labelled to unlabelled cells. The second was the previously described suspension assay (Knudsen & Horwitz, 1977). Both assays showed that significant membrane continuity occurred within 20–30 min of fusion initiation but that multinucleate morphology did not appear for at least an additional 1 h. The time taken for membrane continuity to occur indicates that some change must take place in the membrane structure of myoblasts to permit fusion. Research into myoblast fusion has thus attempted to find out what is unique about fusion-competent myoblasts and to see if any changes occur in the cell upon the initiation of fusion.

Surface biochemistry of myoblasts during fusion

Proteins

An involvement of surface proteins, especially glycoproteins, in the fusion of myoblasts has been suggested because concanavalin A, a lectin that binds to surface glycoproteins, reversibly blocks fusion (Den *et al.*, 1975; Sandra *et al.*, 1977; Burnstein & Shainberg, 1979). Only the tetrameric form of the lectin is effective and thus the inhibition probably depends on the cross-linking of surface glycoproteins. Concanavalin A is also cytotoxic to muscle cells upon continued exposure. Cell lines have, therefore, been isolated which are resistant to the cytotoxic effects of concanavalin A (Parfett *et al.*, 1981; Cates *et al.*, 1984). The resistant cell lines are defective in glycoprotein biosynthesis and, significantly, do not fuse. This implies that mannosylated glycoproteins may be involved in myoblast fusion. Cates *et al.* (1984) have isolated two classes of concanavalin A-resistant, non-fusible L6 mutants. In one, a selective reduction in the binding of ^{125}I -concanavalin A was shown to be due to the absence of a single polypeptide of M_r 46 000. Significantly, somatic hybrids produced by complementation not only regained the capacity to produce the glycoprotein but also the ability to fuse. Glycoprotein involvement is further implicated by the observation that tunicamycin, an inhibitor of protein glycosylation, inhibits myoblast fusion (Gilfix & Sanwal, 1980). This inhibition was partially reversed when proteinase inhibitors were added with tunicamycin (Olden *et al.*, 1981). Olden *et al.* (1981) suggest that glycoproteins partially mediate myoblast fusion but consider the role of carbohydrate to be indirect, stabilizing proteins against proteolysis.

The synthesis of several membrane proteins has been shown to increase during myogenesis and to

decline after fusion. As a result of these observations it has been claimed that certain proteins are involved in the fusion process. The best characterized of these is fibronectin. Walsh & Phillips (1981), using several surface-labelling methods, concluded that synthesis of fibronectin increases upon cell fusion and declines during further myotube differentiation. This protein may, however, be involved only in adhesion and alignment (as noted above). In avian primary cultures, electronectin, a β -D-galactoside-binding protein, is found to increase transiently before fusion (Gartner & Podleski, 1975; Podleski & Greenberg, 1980). Soluble electronectin activity is regulated by a second protein, myonectin, which also undergoes a transient increase before fusion. Importantly an electronectin-like β -D-galactoside-binding protein has been isolated from chick embryonic muscle (Den & Malinzak, 1977; Nowak *et al.*, 1977; MacBride & Przybylski, 1980). It has been claimed that this endogenous lectin is involved in fusion, since the β -D-galactoside-binding protein has been shown to inhibit fusion in aligned myoblasts (Gartner & Podleski, 1975; MacBride & Przybylski, 1980). This role has been disputed, however (Den *et al.*, 1976; Kaufman & Lawless, 1980), and Den & Chin (1981) have been unable to detect the lectin on the myoblast cell surface. The involvement of the lectin in fusion, although potentially interesting, thus remains unclear. Changes in other myoblast surface proteins have also been observed. Cates & Holland (1978) reported both increased synthesis and accumulation of a protein of apparent M_r of 70 000, concomitant with the onset of myoblast fusion. The relative amount of a cell surface protein of M_r 200 000–250 000 has been found to increase during the fusion of the L6 myoblast cell line, but was not found in a non-fusion variant (M3A). Instead an M_r 90 000 protein was observed which was not found in normal L6 cells (Yoshioka & Suroka, 1983). Senechal *et al.* (1982) examined changes in phosphorylated plasma membrane proteins. Developmentally regulated changes were found to take place in four phosphoproteins (apparent M_r values 165 000, 105 000, 60 000 and 45 000). It is not clear from the results, however, if these phosphorylations are causal or are a consequence of fusion.

An alternative approach to the study of membrane proteins in myoblast fusion is the identification of unique surface antigens, particularly by using monoclonal antibodies. Monoclonal antibodies have been raised by immunizing with whole muscle cells or muscle membranes prepared from different stages in development. In addition, antibodies raised against the surfaces of other cell types have been found to be reactive with muscle cell surface antigens. Immunofluorescence analy-

ses of cells *in vitro* at different stages of development have revealed that distinct quantitative and topographic changes of some cell surface antigens accompany fusion. Changes in antigenic expression have been observed by many workers. Walsh *et al.* (1984), using primary human muscle cell cultures, have defined stages of muscle differentiation *in vitro*. Interestingly, one surface antigen (24.1D5) is found only on pre-fusion myoblasts and not on myotubes or fibroblasts. Kaufman & Foster (1984) immunized with the L8E63 cell line and identified 40 monoclonal antibodies, which fall into five discrete temporal classes, presumably defining different stages of differentiation. Of particular interest are antibodies that mark the aligned and the fusing cells. In addition to quantitative changes, alterations in the surface distribution of the antigens were observed. An example of this is the H58 antigen, which appears to undergo a transient loss of accessibility to antibody during fusion. Whilst monoclonal antibody studies have provided much information concerning myoblast membrane structure, it is to be hoped that the identity of these developmentally regulated antigens will soon be determined. A role for these proteins or glycoproteins in the fusion process could then be more easily examined.

Another approach is to examine variations in iodination patterns of surface proteins. Moss *et al.* (1978) and Pauw & David (1979) utilized lactoperoxidase-catalysed iodination of surface proteins. Decreased amounts of high- M_r proteins and higher amounts of low- M_r proteins were found during fusion. Couch & Strittmatter (1983) proposed that this is attributable to limited proteolysis during fusion itself. They have also observed that inhibition of metalloendoproteinases appears to block fusion. This observation raises the possibility that myoblast fusion may be controlled by a 'fusion-specific' proteinase. A role for soluble proteolytic enzymes in fusion processes is suggested by recent observations that limited proteolysis can initiate erythrocyte fusion (Lucy, 1984a). Lucy (1984b) has also proposed that hydrophobic polypeptides produced by proteolysis may induce membrane fusion. Couch & Strittmatter (1984) have now shown that the specific blockers of myoblast fusion that inhibit proteinases act on a cytosolic protein with an apparent M_r of 80000. It is not clear if this identified proteinase is activated by Ca^{2+} , but a Ca^{2+} -activated neutral proteinase has been reported to appear in myoblasts at around the time of fusion (Kaur & Sanwal, 1981), and the two enzymes might be identical. Thus, it is possible that the known entry of Ca^{2+} into myoblasts before fusion (David *et al.*, 1981) could be a signal for specific proteolysis and thus for the induction of fusion.

Lipids

Examinations of the phospholipid, cholesterol and fatty acid contents of the plasma membranes of muscle cells in culture did not show any changes that correlate with the transitions from myoblasts, through fusion-competent myoblasts, to myotubes (Kent *et al.*, 1974; Boland *et al.*, 1977). Despite this, observations concerning the asymmetry of plasma membrane phospholipids may be important in relation to fusion mechanisms. Sessions & Horwitz (1981, 1983) examined two aminophospholipids, phosphatidylethanolamine and phosphatidylserine, in embryonic chick and quail myoblasts, in chick embryonic fibroblasts and in L6 myoblasts. Labelling studies with two different, non-penetrating, amidating reagents produced vastly differing labelling plateaux. These results suggest that in chick and quail myoblasts 65% of the phosphatidylethanolamine and 45% of the phosphatidylserine are externally disposed. This compares with values of 35% and 20% respectively in fibroblasts and 22% and 30% respectively in L6 myoblasts. There is thus a 2–3-fold increase in lipids known to be fusogenic in artificial systems on the external leaflet of the myoblast as opposed to the fibroblast plasma membrane. The lower occurrence of the two lipids on the outer leaflet in L6 myoblasts may be relevant to the much slower rate of fusion of these cells compared to primary myoblasts. However, whilst the large quantities of externally disposed aminophospholipids may be important in myoblast fusion they do not in themselves confer fusion competence since they are also found in fusion-incompetent cells.

Fusing myoblasts have a specific ganglioside pattern. Ganglioside GD_{1a} increases 3–4-fold during L6 fusion, falling to pre-fusion levels after fusion (Whitley *et al.*, 1976). These changes were not observed in non-fusing mutants. The GD_{1a} ganglioside has been shown to be fusogenic in other systems (Maggio *et al.*, 1978, 1981). It is not clear, however, if generation of this ganglioside occurs during the onset of fusion or is part of the modification of the myoblast membrane leading to fusion competence. The generation of GD_{1a} has also not been demonstrated in primary cells.

In an attempt to examine which lipids may be critical to fusion, lipid metabolism has been experimentally modified. The most extensively examined lipid changes concern cholesterol. From the data of Kent *et al.* (1974) a cholesterol content of $0.33 \mu\text{mol/mg}$ of protein in the plasma membrane can be calculated. Addition of cholesterol (1 mg/ml) to the medium 4 h before fusion onset inhibits fusion (van der Bosch *et al.*, 1973); dipalmitoylphosphatidylcholine (0.3 mg/ml) has the same effect. However, in a more detailed

examination of the role of cholesterol, Cornell *et al.* (1980) showed that inhibition of cholesterol synthesis inhibited myoblast fusion. This effect is, however, probably upon the aggregation step (see earlier) and not on membrane union itself. Lowry & Horwitz (1982) have been shown that inhibition of cholesterol synthesis does not alter the incorporation of protein into the plasma membrane.

Enzymic modifications of the plasma membrane phospholipids have also been shown to affect fusion. Addition of purified phospholipase C (0.5 mg/ml) to the culture medium completely inhibits fusion in a reversible manner without affecting cell proliferation (Nameroff *et al.*, 1973; Schudt & Pette, 1976). Phospholipase A had the same effect (Schudt & Pette, 1976) and addition of lysolecithin itself to the culture medium also produced inhibition (Reporter & Norris, 1973). The effect of phospholipase C upon myoblasts appears to be solely upon phosphatidylcholine (Kent, 1979).

Lipid composition can be altered by the addition of exogenous lipids (see Lucy, 1982). This approach has been applied to the study of myoblast fusion. Prives & Shinitzky (1977) added fatty acids to the culture medium and found that oleic and linoleic acids facilitated fusion, whilst stearic and elaidic acids delayed fusion (also shown by Horwitz *et al.*, 1978). Nakornchai *et al.* (1981) surveyed a variety of fatty acids, both unsaturated (fusogenic) and saturated (non-fusogenic) which affect other fusion systems. Addition of myristic acid, oleic acid, linolenic acid, arachidonic acid and glycerol mono-oleate inhibited fusion, linoleic acid moderately enhanced fusion and arachidic acid was without effect. Lysophosphatidylcholine (Reporter & Norris, 1973) and dimethyl sulphoxide (Blau & Epstein, 1979; Miranda *et al.*, 1983), which fuse erythrocytes, also reversibly inhibit fusion. The results obtained in the various studies on the addition of lipids to the culture medium are not in total agreement. It is possible that this is due to the known variations in lipid content in different batches of horse or foetal calf serum. It is clear, however, that addition of lipids which can act as fusogens in the other systems either has no effect or inhibits the fusion of myoblasts. This is probably due to intrinsic properties of myoblast fusion. It is likely to be a tightly regulated, programmed event involving several biochemical or biophysical changes. Experiments with poly(ethylene glycol) serve to illustrate this proposal. Poly(ethylene glycol) does not stimulate normal myoblast fusion (M. J. O. Wakelam, unpublished work): in fact, it inhibits normal myoblast fusion (G. Fiorini, D. Fisher & J. A. Lucy, personal communication). Poly(ethylene glycol) does, how-

ever, promote the fusion of myoblasts with other cells or with myoblasts of other species (e.g. Quinn *et al.*, 1981; Blau *et al.*, 1983a).

Changes in the phospholipid metabolism of myoblasts upon fusion have been examined by using the system in which Ca^{2+} is added after 50 h to Ca^{2+} -depleted cultures (see above). The incorporation of [^{32}P]P_i into phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and sphingomyelin was thus studied in 50 h cultured chick embryonic myoblasts that were stimulated to fuse by raising the medium Ca^{2+} concentration from 0.1 μM to 1.4 mM (Wakelam & Pette, 1982). Only phosphatidylinositol exhibited an increased [^{32}P]P_i incorporation. This was probably secondary to an increased breakdown. Moreover, the polyphosphoinositides were found to be rapidly broken down (Wakelam, 1983). When fusion was inhibited, and when 24 h cultured myoblasts (fusion-incompetent) were stimulated with Ca^{2+} , no lipid breakdown was observed. Concomitant with the breakdown of inositol phospholipids, an apparent synthesis of 1,2-diacylglycerol and phosphatidic acid was observed (Wakelam, 1983). As these two lipids are known to be fusogens in other systems (see Cullis *et al.*, 1983), their production could be stimulating myoblast fusion.

Plasma membrane changes during myoblast fusion

Upon fusion, Fulton *et al.* (1981) observed extensive reorganization of the cytoskeletal framework of detergent-extracted myoblasts. Numerous lacunae were observed to develop, which they suggest are regions of lipid bilayer devoid of glycoproteins. Following fusion, a stable, extensively crosslinked internal structure characteristic of myotube morphology is reformed. Bar-Sagi & Prives (1983) have shown that calmodulin-antagonists inhibit fusion and they suggest that the antagonists act by inhibiting cytoskeletal reorganization. Consistent with this, cytochalasin B (Holtzer *et al.*, 1975) and taxol (Antin *et al.*, 1981), two inhibitors of microtubule disassembly, also inhibit fusion. However, it should be noted that calmodulin antagonists may be inhibiting the activity of many enzymes and not simply one event.

Using freeze-fracture electron microscopy, Kalderon & Gilula (1979) observed particle-free regions in myoblast membranes at the fusion sites of myoblasts. They also observed vesicles near the intracellular surface of the plasma membrane, which they suggested might be involved in the fusion process. However, other workers have not observed such vesicles (Lipton & Konigsberg, 1972; Rash & Fambrough, 1972; Fumagalli *et al.*, 1981). Lipton & Konigsberg (1972) suggested that

fusion is initiated at a single site, with a single cytoplasmic bridge forming between the fusing cells. Myoblast membranes need to be in a fluid state for fusion to occur. The fusion rate increases by a factor of 15–20-fold between 28 and 40°C (van der Bosch *et al.*, 1973). The activation energy of fusion changes at about 35°C [$E_a = 302\text{--}318\text{ kJ/mol}$ (72.5–76 kcal/mol) for $t < 35^\circ\text{C}$; $E_a = 74.4\text{--}92\text{ kJ/mol}$ (17.8–22 kcal/mol) for $t > 35^\circ\text{C}$]. Several studies have demonstrated an increase in myoblast membrane fluidity upon the onset of fusion. This has been observed with various methods: microviscosity (Prives & Shinitzky, 1977), fluorescence polarization (Weidekamm *et al.*, 1976), resonance energy transfer (Herman & Fernandez, 1982), fluorescence photobleaching recovery and fluorescent depolarization (Elson & Yguerabide, 1979). The study of Weidekamm *et al.* (1976) utilized myoblasts cultured for 50h in a low Ca^{2+} -containing medium. The rise in fluorescence polarization was observed within 5–10min of adding millimolar Ca^{2+} to the cells; it then fell over a 2–3h period. This time course parallels that of fusion in cells cultured under these conditions. Herman & Fernandez (1978, 1982) also showed that areas of cell contact between fusing cells exhibited higher fluidity than other membrane regions on the same cells, possibly corresponding to the particle-free regions observed by Kalderon & Gilula (1979). Changes in fluidity may also account for the redistribution of antigenic sites during fusion observed by Kaufman & Foster (1984).

Relationship between membrane organization and fusion mechanisms

Biological membranes may exist in both bilayer and non-bilayer configurations. The H_{II} (hexagonal) structure of membrane phospholipids is a non-bilayer inverted micellar structure that is more fluid than a bilayer (Cullis *et al.*, 1983) and has been proposed to be the preferred membrane state for fusion (Verkleij *et al.*, 1984). It is possible that on stimulation of fusion such structures are formed. However, the involvement of these inverted micellar structures in fusion processes remains controversial. The presence of these structures in cellular systems has not been clearly demonstrated and no X-ray diffraction data in support of the hypothesis have been presented. Thayer & Kohler (1981) have shown, in a theoretical study, that spectra typical of the H_{II} structure can be generated for phosphatidylethanolamine in a bilayer by changing the conformation of the head group. For further discussion of this issue see Verkleij *et al.* (1984) and Lucy (1984a,b). An alternative view is that membrane fusion proceeds

by removal of hydration barriers between apposed membranes (see Parsegian *et al.*, 1984). Inhibitors of fusion may therefore be envisioned as preventing either the formation of H_{II} structures or the dehydration. The inhibitors could be lipid or protein or both.

It is attractive to consider the breakdown of specific proteins and lipids as initiating events in fusion. Polyphosphoinositide breakdown is of particular interest in that the head-groups of these lipids are the bulkiest, most negatively charged and thus heavily hydrated of all phospholipids. The head-groups can be envisioned as binding to both proteins and other lipids in the myoblast membrane resulting in rigidity. The time course observed by Weidekamm *et al.* (1976) for the increase in fluorescence polarization in myoblast plasma membranes on the stimulation of fusion by 1.4mM- Ca^{2+} is the same as that observed for polyphosphoinositide breakdown (Wakelam, 1983). It has also been shown in vesicle studies that the polyphosphoinositides, and phosphatidylinositol 4-phosphate in particular, inhibit fusion (Sundler & Wijkander, 1983). Breakdown of these lipids has been shown to have both a Ca^{2+} -independent and an apparently Ca^{2+} -dependent stage (Wakelam & Pette, 1984a,b). It is possible that the Ca^{2+} -independent lipid breakdown is involved in Ca^{2+} entry, as proposed for other systems (Michell *et al.*, 1981). The Ca^{2+} -dependent lipid breakdown and the possibly Ca^{2+} -dependent proteolysis (see above), could result from the Ca^{2+} entry into myoblasts known to occur before fusion (David *et al.*, 1981). Either of these effects, or the two acting in concert, could result in the removal of inhibitors of fusion, which could then proceed through the known fusogens present in the myoblast membrane. The high level of phosphatidylethanolamine present in the outer leaflet (Sessions & Horwitz, 1983), the ganglioside GD_{1a} (Whatley *et al.*, 1976) and the 1,2-diacylglycerol and phosphatidic acid produced (Wakelam & Pette, 1982; Wakelam, 1983) are all capable of giving the H_{II} structure (Cullis *et al.*, 1983). Thus, this structure may be formed upon the stimulation of fusion and be involved in membrane union. The situation with regard to 1,2-diacylglycerol is complicated, however, in that treating myoblasts with phospholipase C (Nammerof *et al.*, 1973; Schudt & Pette, 1976) and with the tumour promoter 12-*O*-tetradecanoylphorbol 13-acetate (Grove & Schimmel, 1981) both inhibits myoblast fusion and increases membrane levels of 1,2-diacylglycerol. This would suggest that the lipid has no role in fusion. However, the source phospholipid has been found to be phosphatidylcholine (Kent, 1979; Grove & Schimmel, 1982). The metabolism of this lipid is unchanged in

Table 1. *Biochemical changes before and after myoblast fusion*

Proteins	
(i) Changes in plasma membrane protein synthesis	
Electronection	Gartner & Podleski (1975)
M_r 70000 protein	Cates & Holland (1978)
M_r 200000–250000 protein	Yoshioka & Suroka (1983)
(ii) Changes in protein phosphorylation	Senechal <i>et al.</i> (1982)
(iii) Monoclonal antibody studies	
Stage-specific surface antigens	Walsh <i>et al.</i> (1984); Kaufman & Foster (1984)
Changes in surface distribution of antigens	Kaufman & Foster (1984)
(iv) Iodination of patterns of surface proteins	
Lower amounts of high- M_r proteins	Moss <i>et al.</i> (1978)
Higher amounts of low- M_r proteins	Pauw & David (1979)
(v) Enzymes	
Metalloendoproteinases	Couch & Strittmatter (1983)
Ca^{2+} -activated neutral proteinases	Kaur & Sanwal (1981)
Lipids	
(i) Unique phospholipid asymmetry	Sessions & Horwitz (1983)
(ii) Changes in ganglioside pattern	Whately <i>et al.</i> (1976)
(iii) Inositol phospholipid breakdown	Wakelam & Pette (1982); Wakelam (1983)
Plasma membrane structure	
(i) Reorganization of cytoskeletal framework	Fulton <i>et al.</i> (1981)
(ii) Particle-free domains at fusion sites	Kalderon & Gilula (1979)
(iii) Increase in membrane fluidity	Weidekamm <i>et al.</i> (1976); Prives & Shinitzky (1977); Herman & Fernandez (1978, 1982); Elson & Yguerabide (1979)

normal myoblast fusion (Wakelam & Pette, 1982). Table 1 summarizes the various changes in myoblast membrane structure known to occur at fusion.

Fusion regulation

When myoblasts reach fusion-competence, it seems likely that there is some form of diffusable signal that stimulates the onset of fusion. The earliest attempt to demonstrate such a signal comes from the work of Zalin and her co-workers. Zalin & Montague (1974) observed a 10-fold increase in the content of cyclic AMP in myoblasts 5–6 h before fusion onset. Prostaglandin E_1 was found to promote precocious fusion (Zalin & Leaver, 1975) and indomethacin, an inhibitor of prostaglandin synthesis, inhibited fusion (Zalin, 1979). These results implicate a rise in the concentration of cyclic AMP in myoblasts in response to the initiation of fusion by prostaglandins. This proposal has been supported by David & Higginbotham (1981), but cyclic AMP has been shown in some studies to inhibit fusion (Aiu *et al.*, 1973; Wahrman *et al.*, 1973; Moriyama & Murayama, 1977). Schutzle *et al.* (1984) were unable to detect the rise in cyclic AMP before fusion, finding it instead after fusion. The increased concentration of cyclic AMP was inhibited by indomethacin, but the cyclo-oxygenase inhibitor did not inhibit fusion. These results suggest that prostaglandin synthesis and the increase in cyclic AMP are a consequence

of, as opposed to the cause of, myoblast fusion. The rise in cyclic AMP following fusion may be related to the increase in protein synthesis following fusion (Schutzle *et al.*, 1984; Zalin & Entwistle, 1984). It is also proposed that an eicosanoid is involved in stimulating Ca^{2+} entry into myoblasts in a cyclic AMP-independent manner (Entwistle *et al.*, 1983; Zalin & Entwistle, 1984). These authors propose that the eicosanoid involved is eicosatrienoic acid, but this fatty acid is not found in myoblasts (Kent *et al.*, 1974; Boland *et al.*, 1977). Linoleic acid, which is found in the myoblast, could however act as a precursor, though this remains to be demonstrated. Entwistle *et al.* (1983) also propose that the entry of Ca^{2+} is controlled by a voltage-dependent mechanism, being maximally stimulated by 16 mM- K^+ . However, high K^+ concentrations have been shown to inhibit fusion (Schudt *et al.*, 1973), but the ionic strength of the culture medium was not maintained in this study and this could explain the contrary results. In addition, Kolb & Wakelam (1983) showed that ATP-activated cation channels occur in fusion-competent myoblasts (for Na^+ and K^+), but ATP was not found capable of stimulating myoblast fusion (M. J. O. Wakelam, unpublished work).

It is probable that myoblast fusion is stimulated by receptor activation. Embryo extract has been shown to be essential for primary myoblasts to fuse (see Wakelam & Pette, 1983), and it is probable that the fusion-promoting component is neuronal in origin (Wakelam & Pette, 1983). Two neuropep-

tides, vasopressin (Wakelam & Pette, 1983) and angiotensin II (Wakelam & Pette, 1984a,b), are capable of stimulating fusion in a serum-free medium and also of stimulating myoblast inositol phospholipid breakdown. The breakdown of inositol phospholipids observed on the stimulation of fusion by Ca^{2+} appears to be stimulated, initially, by embryo extract in a Ca^{2+} -independent manner, although Ca^{2+} seems to induce further breakdown (Wakelam & Pette 1984b). Thus embryo extract may contain a receptor-stimulant that initiates fusion. Kent (1982), Wakelam & Pette (1984a) and Zalin & Entwistle (1984) have shown that lysosomotropic amines inhibit myoblast fusion. Kent (1982) has proposed that this is due to effects upon cell-surface receptors, but many non-specific effects are probably also taking place.

Three research groups have proposed that transferrin-like molecules of neuronal origin stimulate muscle differentiation (Ii *et al.*, 1982; Oh & Markelonis, 1982; Beach *et al.*, 1983). However, the involvement of this factor in fusion as opposed to differentiation has not been clearly demonstrated; indeed, it may simply be a myoblast mitogen.

Myoblast fusion and disease

The possible involvement of defects in myoblast fusion and differentiation in various myodegenerative diseases is an attractive proposition to explain the biochemical lesions. In a study of myogenesis in hamster hereditary polymyopathy (a model for human muscular dystrophy), Tautu & Jasmin (1980) observed that myoblasts fused, but formed round multinucleated cells as opposed to the normal structure of myotubes. Blau *et al.* (1983b) observed defective myoblasts in cultured muscle explants from patients with Duchenne muscular dystrophy. These cells had a defective growth potential, but could, however, fuse, producing cells which did not demonstrate the typical morphology of myotubes. The tumour-promoting phorbol esters inhibit myoblast fusion. Grove & Schimmel (1981) have shown that this inhibition is accompanied by an 2-fold increase in 1,2-diacylglycerol content, within 15 min of phorbol ester treatment, which is the result of a stimulated breakdown of phosphatidylcholine (Grove & Schimmel, 1982). Phosphatidylcholine metabolism is unaffected in normal myoblast fusion (Wakelam & Pette, 1982) and it is thus possible that inhibition of myoblast fusion by the tumour-promoter has the same mechanism as inhibition by phospholipase C (see above). Viral transformation also inhibits myoblast fusion (Cohen *et al.*, 1977) whereas interferon accelerates it (Fisher *et al.*, 1983); in both cases the mechanism is unclear.

Conclusions and future prospects

Myoblast fusion is a tightly regulated and programmed event during the differentiation of skeletal muscle. It involves modifications of both lipids and proteins in the cells, though the roles and contributions of these remain unclear. Whilst it is unlikely that an understanding of the fusion of myoblasts will assist in the understanding of diseases such as dystrophy, it may be important in clarifying the mechanisms of recovery from muscular injury in which satellite cells fuse in a manner analogous to that in the embryonic development of muscle. The fusion of myoblasts with other cells to form stable heterokaryons is potentially also of great use in studying the regulation of gene expression during differentiation (see, for example, Blau *et al.*, 1983a), and, in addition, the study of muscle cell fusion may aid the understanding of other membrane fusion processes.

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