DUCHENNE MUSCULAR DYSTROPHY: STUDIES OF CELL MOTILITY IN VITRO

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SUMMARY

Duchenne muscular dystrophy (DMD) is a severe degenerative disorder of skeletal muscle. It has been suggested that an abnormality of the plasma membrane may be responsible for the pathogenesis of DMD, and a number of cell surface changes have been described in DMD muscle fibres and other cell types. Alterations in cell-to-cell and cell-to-substratum adhesiveness have been reported for DMD cells and we have determined whether these alterations in cell adhesiveness affect migration of cells from DMD muscle explants. DMD cells move more rapidly and spend less time at rest than do normal or DMD carrier cells, although the differences were statistically significant only for the latter cells. An inverse relationship between cell speed and contact with surrounding cells was not observed. All cells tended to persist in their direction of movement, and there were no differences between the types of cells studied. Our results support the view that there may be a cell surface defect in DMD.

INTRODUCTION

Much research on the pathogenesis of Duchenne muscular dystrophy (DMD) is based on the hypothesis that there may be a defect in the sarcolemma of the dystrophic muscle fibre and that this in turn may allow an influx of calcium ions that cause hypercontraction and overload mitochondria (Duncan, 1978). The most striking evidence for a membrane defect comes from morphological and dye-penetration studies of the dystrophic muscle fibre that indicate that there may be holes in the dystrophic sarcolemma (Mokri & Engel 1975; Bradley & Fulthorpe 1978). Freeze-fracture studies of the sarcolemma in human and animal dystrophies have also supported the view that these disorders may result from a membrane defect (Shotton, 1982). In addition, membrane abnormalities have been reported in other cells, particularly erythrocytes and lymphocytes, although many of these results are controversial (Rowland, 1980; Jones & Witkowski, 1983a). We have been examining the membrane hypothesis by studying cell surface-mediated behaviour of DMD cells in culture.

Using couette viscometry, we have recently demonstrated abnormal intercellular adhesion of skin fibroblasts from boys with DMD (Jones & Witkowski, 1979, 1981) and from carriers of DMD (Jones & Witkowski, 1983b). Couette viscometry gives precise, reproducible estimates of intercellular adhesiveness, but one requirement is

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that the particles being aggregated should be of similar size and shape. This precludes its use in studies of muscle cell cultures that contain cells of widely differing size.

An alternative method for studying cell surface-mediated behaviour that could be used with muscle cells in vitro is to observe their behaviour in monolayer cultures. For example, we have recently shown that the spreading of monensin-treated DMD skin fibroblasts on a glass substratum is abnormal (Pizzey, Witkowski & Jones, 1984), and Thompson and colleagues have reported that DMD cells prepared by enzymic dissociation of muscle biopsies came together in monolayer cultures to form 'clusters' of cells, several cells deep (Thompson et al. 1977; Yasin et al. 1979; Thompson, Cavanagh & Yasin, 1981; Thompson, Yasin, Lake & Cavanagh, 1983; but see Ecob-Johnston & Brown, 1981). We have not observed 'clustering' of cells in cultures derived from explants of DMD muscle (Witkowski, Durbidge & Dubowitz, 1976), and it may be that enzymic dissociation of DMD muscle biopsies brings about some long-term change in the DMD cell membrane. (However, Delaporte, Dehaupas & Fardeau (1984) reported finding clusters in subcultures prepared from explant cultures.)

Yasin et al. (1979) used time-lapse cinematography to analyse the phenomenon of clustering, but it was not a wholly satisfactory analysis (see Discussion). We have now used the methods employed by Abercrombie & Heaysman (1953, 1966) in their classic analyses of the movements of cells from explants to determine if changes in the cell surface of DMD cells are reflected in changes of cell motility.

**MATERIALS AND METHODS**

**Tissue culture**

Samples of muscle were obtained by needle biopsy or in the course of orthopaedic operations; details of the subjects are listed in Table 1. Diagnoses were confirmed on clinical, histochemical and electromyographic criteria (Dubowitz, 1978). Fat and connective tissue were trimmed from the muscle and the sample was cut into pieces of approximately 1 mm³. These were transferred to gelatin-coated Petri dishes and covered with a 22 mm × 22 mm glass coverslip to keep them in position; 1.5 ml of medium was added and the medium was changed weekly. The medium used was BME or MEM with 10% foetal calf serum, 50 i.u. of penicillin, 50 µg of streptomycin and buffered with 10 mM-HEPES. All media were purchased from Gibco-Biocult (Irvine, Scotland) except for the antibiotics, which were obtained from the hospital pharmacy.

**Time-lapse cinematography**

A problem encountered in attempting to film cells moving from explants in the primary culture was that the number of cells emerging was initially low and spaced over several days. For this reason, explants in the primary culture were left until cells had begun to migrate away from them, and then they were transferred to another dish and coverslips were again placed over them. The plastic dishes in all these experiments were 35 mm dishes from Lux. The surfaces of the dishes were not treated before use. Figs 1 and 2 show that such explants contained many cells and these migrated synchronously, providing good preparations for filming.

Before filming, the coverslips were removed from the Petri dishes, fresh medium was added and paraffin oil gently layered onto the medium to reduce evaporation during filming. Filming was carried out using a Wild M40 inverted microscope with phase-contrast optics and a hot stage maintained at 36 °C with a Technic circulator. The hot stage was enclosed in a polystyrene shield to minimize fluctuations in temperature. The TLC system used was a 16 mm Bolex H16 camera
<table>
<thead>
<tr>
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<th>No. of cells</th>
<th>Distance moved (μm)</th>
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* Values given are medians with 95% confidence limits.  
† 'Overall' time is the total length of time a cell was filtered; 'Moving' is the time a cell was moving; i.e. excluding periods when the cell moved less than 0.5 μm.  
‡ 'Overall' speed is given by the distance moved by a cell and the total time for which it was observed. 'Moving' is the average speed of a cell excluding those periods in which it did not move.  
§ 'Path ratio' is the ratio of the length of the path followed by a cell, to the length of the direct distance between the initial and final positions of the cell.
controlled by Paillard control and timer units, with Kodak Plus-X reversal film (50 ASA). One frame was exposed for 0.2 s every minute and cultures were filmed for about 8 h. An electromagnetic shutter interrupted the light source except during an exposure so that the cells were illuminated for only 12 s in each hour.

Film analysis

In the initial phases of this study, cells were filmed in the immediate area of the explant from which they were migrating, and for this reason, the analysis developed by Abercrombie & Heaysman (1953, 1966) was used rather than the random-walk model of Gail & Boone (1970).

Films were projected using an analytical film projector in such a way that 1 mm on the screen represented 1 μm. The position of each cell was marked on the screen every 15 frames (corresponding to 15 min 3 s) by using the centre of the nucleus as the reference point. Each cell was recorded when its nucleus became clearly visible as the cell moved into view from under the explant until it moved out of the field of view or went into mitosis. A complete tracing of a film showed the paths of each cell that could be followed as a series of positions at intervals of 15 min 3 s (Fig. 3). The following measurements were then made directly on these tracings.

Step length. This is the linear distance between two successive cell positions measured to an accuracy of 0.5 mm on the tracing.

Direct distance. This is the linear distance between the first marked position of a cell and the last position at which it was marked.

Direction of movement. The field was divided into six equal segments centred on the explant. These segments were called forward (F), backward (B), forward lateral (FL) and backward lateral (BL). The direction in which a cell moved in the 15-min interval was noted.

Zero steps. The minimum movement that could be detected by measuring the tracings was 0.5 μm. If a cell did not move this distance in a 15-min interval this was recorded as a zero step.

Contact number. At each position the number of cells contacted by the cell being traced was noted, and the contact number for a 15-min interval was the mean of this value for the cell positions at the beginning and end of this interval.

From these measurements the following parameters were calculated.

1) Total path length: the sum of the individual step lengths for each cell.

2) Overall speed (μm/h). Calculated from the total path length and total filming time of each cell.

3) Moving speed (μm/h). Calculated from the total path length and the time for which the cell was moving.

4) % Time moving: the percentage of the total time a cell was filmed that it was moving.

5) Mean velocity for movement in a particular quadrant.

6) Velocity for each step as a function of the contact number for that step.

7) The ratio of the total path length followed by a cell to the direct distance between its initial and final positions. For cells moving in other than a straight line (including those that reverse direction by 180°) this value will exceed unity. This should be regarded as only an approximate indication of the tendency of cells to maintain their direction of movement, depending both on the total time of observation and the intervals chosen for measurement.

The distributions of the data are such that the median rather than the mean was used, and the 95% confidence limits on the median were calculated (Snedecor & Cochran, 1980). Statistical comparisons were performed using the non-parametric Mann-Whitney test (Siegel, 1956).

RESULTS

Details of the subjects used in this study and of the numbers of cells measured are given in Table 1.

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Fig. 1. Section of muscle explant at time of 're-explantation'. Many cells are present on the surface of the muscle fibres. Toluidine-stained, 1 μm section. ×450.

Fig. 2. Detail of the interior of a muscle explant, showing the presence of multinucleate myotubes within the explant. ×3000.
There were considerable variations in morphology between cells in the same culture and between different cultures from the same individual. There were no consistent features of cell form that could be used to distinguish the sources of the cells. Human skin fibroblasts were rarely clearly polarized as are chick heart fibroblasts, with a single well-defined leading edge with ruffled membrane. Generally, they had a very variable shape with many long extensions that contacted other cells at considerable distances from them. The nucleus was clearly visible and could be followed easily as the reference mark for the position of the cell.

Fig. 3 illustrates a typical tracing from a film, although some cells have been omitted for clarity. It is clear that the general movement of the cells is radially away from the explant in the forward or forward lateral directions. One cell in this film reversed its direction of travel, and in other films some cells that were already away from the edge of the outgrowth when filming began moved 'backwards' throughout the period of filming, and other cells 'oscillated', moving backwards and forwards along approximately the same path. The maximum and minimum distances moved by any cell were 249 \( \mu m \) and 20 \( \mu m \), and the maximum and minimum periods over which cells were analysed were 9·75 h and 1·75 h. Details of the analysis are given in Table 1.
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'Overall' speed is the mean speed of the cells over the distance travelled, including intervals when the cells were not moving. Although the DMD cells moved more quickly than either normal or carrier cells, the difference was statistically significant only for DMD and carrier cells \((P=0.007)\), the difference between normal and DMD cells just failing to reach significance at the 5% level \((P=0.051)\). 'Moving' speed is the mean rate of cell movement excluding intervals when the cells were stationary. Again the only statistically significant difference was between DMD and carrier cells \((P=0.008)\); the difference between normal and DMD cells is significant at \(P=0.072\).

All cells moved predominantly away from the explants. The speed of movement and the directions in which chick cells move from an explant are strongly influenced by the contacts a cell has with neighbouring cells (Abercrombie & Heaysman, 1953; Abercrombie & Gitlin, 1965). In the present case, plots of contact number against \(\log (\text{speed} + 1)\) for normal, DMD and carrier cells failed to show the inverse relationship between contact number and speed demonstrated by Abercrombie & Heaysman (1953). This might be due to the relatively small number of values, but even when all values were pooled there was still no evidence of an inverse relationship.

Human cells move non-randomly when migrating from an explant, tending to persist in the direction in which they are moving. There were no statistically significant differences in the persistence of movement between the three types of cell studied.

**DISCUSSION**

These experiments were undertaken to determine if the changes in cell surface-mediated behaviour of DMD cells found using a variety of methods (Jones & Witkowski, 1983a) could be detected by measurements of cell motility. Is there any evidence from other systems to suggest that differences in intercellular adhesiveness might be reflected in altered cell movement on inert substrata \textit{in vitro}? Steinberg & Garrod (1975) have shown that the differences in cell adhesiveness that lead to cell sorting in mixed cell aggregates (Steinberg, 1963) also operate in mixtures of cells in monolayer culture. Furthermore, Ede & Flint (1975a), using couette viscometry, have shown that limb-bud mesenchyme cells of the \textit{talpid} \(^3\) chick mutant are more adhesive than normal cells. This increased adhesiveness of \textit{talpid} \(^3\) cells was also reflected in reduced cell motility, with the \textit{talpid} \(^3\) cells spending more time at rest (Ede & Flint, 1975b).

We chose to use the methods of Abercrombie & Heaysman (1953, 1966) rather than other models such as random-walk analysis (Gail & Boone, 1970; Noble & Peterson, 1974). The random-walk model failed to detect the differences in adhesiveness between normal and \textit{talpid} \(^3\) cells (Ede & Flint, 1975b). Furthermore, we were interested in cell migration from explants, and the methods of choice for such an analysis are those of Abercrombie & Heaysman. These results indicate that DMD cells move more rapidly than do normal or carrier cells, although only the difference between carrier and DMD cells was significant at the 5% level. (It is interesting to note that Ede & Flint (1975a,b) found that the more adhesive \textit{talpid} \(^3\) cells also moved more slowly...
than the less adhesive normal chick wing bud cells.) These cells from adult human tissue move much more slowly (about 14 μm/h) than do, for example, embryonic chick heart cells (about 80 μm/h; Abercrombie & Heaysman, 1966). Stephenson & Stephenson (1978) also found that adult human skin fibroblasts moved slowly (16 μm/h).

Although others claim to be able to recognize myoblasts in clonal cultures (Blau, Webster & Pavlath, 1983), it was not possible to determine if the mononucleate cells in the present study were fibroblasts or myoblasts. In addition, the irregular outlines of these cells (in contrast to the compact, polarized shape of embryonic cells) made determination of contact number difficult and may have contributed to the failure to observe the dependence of cell speed on contact number found by Abercrombie & Heaysman (1953).

It is difficult to compare the results of the present study with the previous measurements of DMD cells in vitro made by Yasin et al. (1979). Firstly, Yasin et al. evaluated cell–cell interactions by ‘nuclear crossover time’, defined as ‘...the time for the nucleus of a cell within the culture to crossover (or to be crossed) by any visible projection of another cell’ (our emphasis), and it is very difficult to understand how this parameter can be related to those normally used to measure cell motility. Furthermore, although Yasin et al. were interested in examining the properties of clustering cells, only one of the two DMD cultures studied exhibited clustering, and no measurements were made of cells within the cluster. Finally, there seems to be some doubt about the diagnosis of this case; it was suggested that it might be a case of an autosomal recessive disorder, rather than DMD.

We have not observed cluster formation in explant cultures prepared from DMD muscle (Witkowski et al. 1976) and none was found in the present series of experiments. It has been suggested that the failure to observe cluster formation in explant cultures may be due to the retention within the explant of those cells with abnormally high adhesiveness that are believed to give rise to clusters (Yasin et al. 1979). However, Delaporte et al. (1984) have recently reported observing clusters in 'few' dishes of subcultures from muscle explants; in two of these cases, clusters formed in one dish but not in another. It is now clear that cluster formation is a more variable phenomenon than was at first thought.

An attractive explanation for the findings of abnormal cell surface behaviour of DMD cells is that there is some underlying abnormality in the cytoskeleton of these cells. Shay and colleagues have described reduced tubulin content in dystrophic chick cells (Shay & Fuseler, 1979) and more recently have reported similar findings for monocytes from patients with DMD (Shay, Thomas & Fuseler, 1982). These latter cells also spread less well than cells from normal subjects. Unfortunately, other groups have found normal microtubular complexes in DMD skin fibroblasts and muscle cells (Connolly, Kalnins & Barber, 1979; Rungger-Brandle et al. 1980; Walsh, Yasin, Kundu & Thompson, 1980) and at present the consensus must be that cytoskeletal defects are not present in DMD cells.

While the small numbers of individuals used in the present study preclude drawing definitive conclusions about the effects of DMD on cell motility, these results together
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with those from other studies (reviewed by Jones & Witkowski, 1983a) support the hypothesis that an alteration in cell surface properties may underlie the pathogenesis of DMD.

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REFERENCES


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