Spreading behaviour of cultured fibroblasts from carriers of Duchenne muscular dystrophy

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Summary

Cultured skin fibroblasts from patients with Duchenne muscular dystrophy (DMD) are more sensitive than normal cells to prolonged exposure to the ionophore monensin. In a cell spreading assay in which cells were preincubated with monensin and subsequently allowed to adhere to and spread on a glass substratum in serum-free medium for 100 min, the mean transformed cell area of normal and DMD cells was 5.97 ± 0.11 and 5.29 ± 0.03, respectively. Cultured fibroblasts from carriers of DMD yielded a value of 5.59 ± 0.03, which is intermediate between, and significantly different from, the values for both normal and DMD cultures. This result would be predicted on the basis of random X-chromosome inactivation in female carriers of this disorder. However, comparison of DMD carrier cell spreading data with data obtained from pooled and summated measurements taken from separate experiments using either normal or DMD fibroblasts suggest a more complex situation. Examination of the variance of the means of cell area for the true carrier population and the summated normal and DMD population provides evidence suggesting that some form of cellular interaction may occur between the two cell genotypes in culture.

Key words: cell spreading, Duchenne dystrophy, monensin.

Introduction

Duchenne muscular dystrophy (DMD) is an X-chromosome-linked wasting disease of skeletal muscle (Dubowitz & Brooke, 1973) and although the aetiology of the disorder is unknown, several reports have suggested that a membrane abnormality may be involved (for reviews, see Rowland, 1976; Lucy, 1980; Jones & Witkowski, 1983a). Consistent with such reports we have previously shown that freshly trypsinized cultured DMD fibroblasts exhibit reduced intercellular adhesiveness (Jones & Witkowski, 1979, 1981, 1983a; Hillier et al. 1985; Pizzey & Jones, 1985) and Kent (1983) has also identified an increased sensitivity to trypsin in these cells using a cell–substratum detachment assay. We have also previously shown that the monovalent carboxylic ionophore monensin inhibits the restoration of high values of intercellular adhesiveness after recovery from trypsinization, and that this effect is much greater in DMD cells (Jones et al. 1985). Furthermore, monensin also suppresses the spreading of human skin fibroblasts when these cells are seeded onto uncoated substrata in serum-free media (Pizzey et al. 1983). In this respect also, DMD fibroblasts are more sensitive than normal cells (Pizzey et al. 1984).

Since DMD fibroblasts are more sensitive to the challenge of monensin than normal cells, it may be predicted that given the same monensin treatment, DMD carrier fibroblast populations would spread to an extent that is intermediate between normal and DMD values. This prediction is based upon the premise that DMD carriers are mosaics of approximately equal proportions of cells of normal and DMD genotypes, and that selection for one type does not occur. However, it is known from several reports that such interactions can occur both in vivo and in vitro (see Discussion). Indeed, we have also shown (Jones...
& Witkowski, 1983b) that, by using an aggregation assay that measures cell–cell adhesion, cellular cooperation may be occurring in DMD carrier cultures. The purpose of the present study was to test the prediction given above by comparing monensin-treated DMD carrier cells with both monensin-treated normal and DMD cells, and with the pooled and summed data from separate experiments using normal and DMD cells. Thus this latter case represents a modelled 'pseudocarrier' population of exactly equal numbers of non-interacting fibroblasts of both normal and DMD genotypes.

Materials and methods

Cell culture

Cultured human skin fibroblasts were established from skin biopsies of normal and DMD subjects, and from obligate DMD carriers. All cultures were maintained as described previously (Jones & Witkowski, 1983b) and used between passages 8 and 20. Cultures were grown to near-confluency in minimal essential medium (MEM) supplemented with 10% foetal calf serum, washed in Ca²⁺/Mg²⁺-free Hanks' basal salt solution (HBSS) and incubated for 20 min at room temperature in 0·25% trypsin in HBSS. Cells were then centrifuged at 150 g for 5 min at 4°C, and resuspended in MEM at a concentration of 2×10⁵ cells ml⁻¹. Cell suspensions were then divided into two aliquots, containing either 5×10⁻⁷ M-ethanolic monensin (Calbiochem) or 0·1% ethanol. Cell spreading assays were then conducted as described below.

In addition to this short-term exposure to monensin, the effect of prolonged monensin treatment on DMD carrier cell spreading was also studied by incubating cell cultures with 5×10⁻⁷ M-monensin for 20 h prior to dissociation. For these monensin-preincubated cells, monensin was not reintroduced into the cell suspensions at any later stage. This was because we have previously shown that although monensin-preincubated DMD cells seeded in monensin-supplemented media spread significantly less than normal cells given the same treatment, the difference in spreading between normal and DMD fibroblasts is greater if such preincubated cells are subsequently seeded in monensin-free media (Pizzey et al. 1984).

Cell spreading assay

From each cell suspension, 50-μl samples were dispensed into the wells of acid-washed glass Multitest slides (Flow Laboratories), and cells were allowed to settle and spread for 100 min at 37°C. After this period, cells were briefly rinsed in 0·1 M-sodium cacodylate buffer (pH 7·2) and fixed for 30 min in 2% glutaraldehyde/0·1 M-sodium cacodylate buffer at room temperature. Random diameters were drawn across the slides and from each sample 50 cells falling on these diameters were photographed using differential interference contrast microscopy. Cell areas were then measured as described previously (Pizzey et al. 1984). For normal and DMD cell suspensions, six cultures (established from six different individuals in both cases) were used. Thus in both cases, a total of 300 cells were measured. For DMD carrier cultures, 100 cells were measured from each of six cultures (derived from five different individuals). Data for the 'pseudocarrier' culture represent the pooled and summated measurements derived from the separate experiments using normal and DMD cells.

Distributions of the area measurements of spreading cells have been compared either by Gaussian analyses of transformed data by Pizzey et al. (1984) or by non-parametric tests of significance (Cassiman et al. 1982; Pizzey et al. 1983). In this study we have used both the two-sample Kolmogorov-Smirnov two-tailed test (Sokal & Rohlf, 1981) and, for the analysis of the transformed data, Student's t-test modified for inequality of variances. For these latter analyses, significance tests were conducted on the means of ln(area), which were calculated for each culture examined (Pizzey et al. 1984). Therefore in this case, n = 6 for the normal, DMD and DMD carrier populations. In addition, the variances of the DMD carrier and pseudocarrier populations were calculated by measuring the spread of the pooled, transformed values for all the cells in the cultures used. Thus, n = 600 in both these cases.

Results

Trypsinized DMD carrier cells that were allowed to spread at 37°C for 100 min onto an uncoated glass substratum exhibit a well-defined radially spread cytoplasmic 'skirt' (Fig. 1A), which is much reduced if monensin is added to the seeding medium (Fig. 1B). No significant differences are observed between the spreading of normal, DMD and carrier fibroblasts and all are equally inhibited by short-term exposure to monensin. When cultures were preincubated with monensin for 20 h prior to trypsinization and subsequently seeded in monensin-free medium, the measured mean area of carrier cells is intermediate between the values for both normal and DMD fibroblasts, and significantly different from both (P < 0·01). This applies both to comparison of the area frequency distributions (Fig. 2A) and to the ln-transformed area data; the mean ln-transformed values for the normal and DMD cultures are 5·97 ± 0·11 and 5·29 ± 0·03, respectively (Fig. 3).

We have previously shown that, after equivalent treatment, DMD cells fail to spread significantly (Pizzey et al. 1984) and since many carrier fibroblasts also exhibit minimal spreading (Fig. 1C), it is possible that these latter cells are of DMD genotype. If this is so, the appearance of normal and DMD genotypes within the carrier cell cultures may be reflected by the presence of bimodality in the area distributions. However, inspection of these distributions does not reveal two discrete populations. This result may arise because the extent of cell spreading, under the conditions of the assay, is too small to allow for any bimodality to be detected (see below); the mean (back-transformed)
Fig. 1. Representative skin fibroblasts from DMD carriers after being seeded onto uncoated glass slides in serum-free medium and allowed to spread for 100 min as described (Pizzey et al. 1983). Untreated cells (A) characteristically demonstrate well-defined radial cytoplasmic spreading. Spreading is much reduced in B, where cells were seeded in the presence of $5 \times 10^{-7}$ M-monensin. After 20 h preincubation with monensin, and subsequent seeding in monensin-free medium (C), cell spreading is yet further reduced and some cells have failed to spread at all. For all experiments, cell viability was determined by Trypan Blue exclusion and found to be $>95\%$. Bars, 20 μm.

Cell area for monensin-preincubated carrier cells is only 266 μm$^2$, compared to a mean value of 936 μm$^2$ for untreated carrier fibroblasts (see also Fig. 1). Another explanation for the apparent absence of two populations in the area frequency distributions is that a form of cooperation may occur between cells expressing either the normal or the DMD gene and this affects the characteristics of the distribution. Alternatively, a combination of both possibilities may apply.

To investigate these alternatives, two series of experiments were conducted. First, we modelled a pseudocarrier culture by pooling data of area measurements from equal numbers of monensin-preincubated normal and DMD fibroblasts. These data were obtained from separate experiments on normal and DMD cell preparations, rather than by using mixed normal/DMD cell combinations. This ensured that (1) exactly equal numbers of normal and DMD cells were measured, and (2) any possible cooperation (or competition) between normal and DMD fibroblasts during seeding and subsequent spreading were obviated. Naturally, the modelling of such a pseudocarrier loses information about the distribution of the individual values used to construct the population. It is also possible that if one or more cultures was significantly different from the others (of the same genotype) then the pseudocarrier value could be misleading. However, this was not found to be the case and the spread of mean area values for both normal and DMD cultures was small; standard errors were <2% of the mean in both cases (from values given above). Second, having recently used the ester, 6-carboxyfluorescein diacetate (CFDA) to identify living cells of normal and DMD genotypes in an analysis of the composition of mixed-cell aggregates (Pizzey & Jones, 1985), we used the same label here in a series of experiments to identify normal and DMD cells following preincubation with monensin. In a mixed population consisting of equal numbers of normal and DMD fibroblasts (in which the normal cells had been labelled with CFDA and mixed with unlabelled DMD cells immediately prior to the spreading assay), the two genotypes could be readily identified. It was found, consistent with our previous observations, that following preincubation with monensin DMD fibroblasts spread significantly less than normal cells given equivalent treatment (Pizzey et al. 1984). However, when the area distributions for the labelled and unlabelled cells were combined, no bimodality (representing the two genotypes) could be detected. Indeed, the area distributions of the mixed-cell preparations (data not shown) were indistinguishable from that of the pseudocarrier population (see below). This

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Fig. 2. Area frequency distributions of cells preincubated for 20 h with $5 \times 10^{-7}$ M-monensin and then allowed to spread for 100 min in monensin-free media. In A, values are shown for normal (O-O), DMD (●-●) and DMD carrier (O-——O) cells. For normal and DMD distributions, 50 cells were randomly identified and measured (Pizzey et al. 1984) from each of six cultures (established from six different individuals) for both genotypes. Thus in both cases $n = 300$. For each data point, the s.e.m. was $<5\%$ of the mean. For the DMD carrier cultures, 100 cells were measured from each of six cultures. The DMD carrier distribution is also shown in B together with the pseudocarrier culture distribution (●-——●), pooled and summated from the normal and DMD data (see the text). Thus in B, $n = 600$ for both distributions.

indicates that, in terms of the differential spreading response of the two genotypes, any possible metabolic cooperation or cross-feeding via the extracellular medium, does not occur in the mixed-cell preparations during the short period (100 min) of our spreading assay.

Analysis of the pseudocarrier population, consisting of 300 normal and 300 DMD fibroblasts, shows that following preincubation with monensin, the mean ln-transformed cell area is within the range of the true carrier cell populations (Fig. 3). Similarly, a Kolmogorov-Smirnov analysis (Sokal & Rohlf, 1981) of the area frequency distributions failed to show a significant difference ($P > 0.05$) between the distributions of pseudocarrier cells and true carrier fibroblasts (Fig. 2B). Although there is no significant difference between the mean ln-transformed values for the cell areas from these two populations*, the scatter of values for the former is much greater; when all 600 transformed cell areas are pooled from the DMD carrier cultures, the mean value is 5.558, compared to the value for the pseudocarrier, which is 5.631 ($P > 0.05$). However, the respective variances are $0.239$ and $0.424$, which yield an F-statistic that is significant at $P < 0.001$.

*The mean transformed value for the pooled ($n = 600$) DMD carrier cells (5.631) is not identical to the grand mean value based on the means of the individual carrier cultures ($n = 6$). This latter value is 5.583 (given above as anti-ln 266) and the difference represents a rounding-up error of $0.45\%$ between the two means.
The mechanism(s) whereby monensin suppresses the spreading of DMD fibroblasts to a greater extent than normal fibroblasts is unclear. Monensin is known to affect the surface-directed translocation and post-translational modification of Golgi-mediated glycoproteins such as collagen and fibronectin (Uchida et al. 1980; Ledger et al. 1983) and the inhibitory effects of monensin on cell spreading and stress-fibre formation are known to be overcome if fibroblasts are allowed to spread in serum-supplemented media (Pizzey et al. 1983) or on fibronectin-coated substrata (Lehto & Virtanen, 1985), respectively. Furthermore, collagen and fibronectin are components of the extracellular matrix (Yamada, 1983), and there are indications that the synthesis, secretion or interaction of matrix and cell surface glycoproteins may be associated with the aetiology or pathogenesis of DMD (Ionesescu et al. 1977; Duance et al. 1980; Jones & Witkowski, 1983a; Dunn et al. 1984; Rodemann & Bayreuther, 1984). Indeed, recent evidence from studies exploiting recombinant DNA techniques are consistent with suggestions that a membrane-associated abnormality may be implicated with the disease (Francke et al. 1985). However, monensin affects other cellular systems in addition to those mentioned above (Basu et al. 1981; Tartakoff, 1983) and the reduced spreading of fibroblasts of DMD genotype (compared to normal cells) following prolonged exposure to the drug may not necessarily be directly related to the amount or form (Ledger et al. 1983) of the adhesive glycoproteins secreted by skin fibroblasts. It is of interest to note that a recent detailed study has shown that protein degradation is much increased in cultured DMD fibroblasts (Rodemann & Bayreuther, 1986) and although monensin does not appear to affect protein synthesis in normal cells (Uchida et al. 1980), it is possible that a combination of the monensin-induced reduction of glycoprotein secretion and an intrinsic higher rate of protein degradation could be responsible for the behavioural abnormality of DMD cells noted in our assay.

The results and discussion presented here are based on the assumption that the initial skin biopsy samples from the DMD carriers contain approximately equal numbers of cells in which either the maternal or paternal X chromosome was inactivated. This assumption is justified by the known intermixing of skin fibroblast clonal progeny during embryonic and foetal development, which results in a fine mosaic of very small patch size; this has been demonstrated and exploited in another X-chromosome-linked disorder (Gartler et al. 1969, 1971). However, there is evidence that in vivo selection for the normal genotype can occur in certain tissues in some inherited disorders. For example, there is selection for normal erythrocytes in G6PDMed (Rinaldi et al. 1976) and for normal peripheral blood cells in Wiskott-Aldrich syndrome (Prchal et al. 1980). In the absence of such in vivo selection, i.e. assuming that the initial carrier biopsy sample contained an approximately equal number of fibroblasts of normal and DMD genotypes, the increased variance of the pseudocarrier population may offer an argument against a straightforward explanation of our results in terms of the Lyon hypothesis (Lyon, 1961), and may suggest that some form of cooperation is occurring between the two genotypes present in the true carrier cultures. This effect has been reported for cultures derived from carriers of Hunter syndrome, in which mutant cells may acquire sufficient wild-type gene product to appear phenotypically normal (Migeon et al. 1977), and from carriers of adrenoleukodystrophy, where analysis of skin fibroblast clones suggests that there may be selection against the normal cells (Migeon et al. 1981). Nevertheless, if such cooperation is occurring, it clearly does not fully confer normal (or DMD) phenotype, in terms of spreading behaviour, to all monensin-treated cultured carrier cells (Figs 2, 3), since the mean value for the degree of carrier cell spreading in our assay is clearly intermediate between those of normal and DMD values.

Fig. 3. Distribution of mean cell areas (μm²) after natural-logarithmic transformation of the individual measurements. Values are shown for normal (N), DMD carrier (C), pseudocarrier (PC) and DMD (D) cultures. In all cases (except PC), 50 cells were measured from each of six cultures of each genotype and these values were then transformed. The mean transformed value was then calculated and the figure shows the distribution of these six means ± s.e.m. For PC, the grand mean of the six normal and six DMD means is shown.
We are further studying the usefulness of this assay as an aid in DMD carrier detection; in parallel with this work we are also investigating cells of embryonic derivation. If embryonic fibroblasts respond to the grants from the Medical Research Council and the Muscular

similar manner to that of postnatal fibroblasts, the challenge of prolonged incubation with monensin in a as an aid in DMD carrier detection; in parallel with

of DMD.

excellent technical assistance. This study was supported by valuable comments on this work, and to J. Carter for other methods available for the antenatal diagnosis of DMD.

We are indebted to Professor Sir Andrew Huxley for his valuable comments on this work, and to J. Carter for

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(Received 3 July 1986 – Accepted, in revised form, 3 October 1986)