Cell surface abnormality in clones of skin fibroblasts from a carrier of Duchenne muscular dystrophy

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SUMMARY We have previously reported that skin fibroblasts from patients with Duchenne muscular dystrophy (DMD) have a lower intercellular adhesiveness than control cells, and that cells from carriers of DMD have normal adhesiveness instead of the expected intermediate value. We have now cloned skin fibroblasts from a carrier of DMD (subject AS) who is also heterozygous for G6PD B/G6PD Mediterranean and determined the intercellular adhesiveness and G6PD phenotypes of the clones. G6PD activity was determined using the 2d-G6P/G6P ratio method. Normal cells had a percentage utilisation of 7.31% and uncloned cells from AS a value of 25.16%. Of 16 clones, 15 had normal values (mean 8.72%) while one clone was G6PD Med with a value of 57.5%. Mean intercellular adhesiveness of normal and uncloned cells from AS were 2.95 and 2.90 respectively. Of 11 clones tested, nine had normal values of adhesiveness (mean 3.1) and all these clones were G6PD B. The single G6PD Med clone had a value of 0.88, compared with 1.39 for DMD cells. We have no explanation at present for the single clone that was G6PD B but DMD-like on aggregation.

Duchenne muscular dystrophy is a severe, degenerative disorder of muscle¹ that may result from some abnormality of the plasma membrane.² Measurements of aggregation kinetics using Couette viscometry³ have shown that skin fibroblasts from patients with Duchenne muscular dystrophy (DMD) have intercellular adhesiveness approximately 40% that of normal cells.^{4 5}

As a result of X chromosome inactivation,⁶ cultures of skin fibroblasts from carriers of DMD should be composed of cells of low (DMD) and high (normal) adhesiveness. These cultures should give intermediate values of adhesiveness⁷ but, as we have shown previously, carrier cells have normal values.⁸ We suggested that this result could be explained (a) if the DMD locus escaped inactivation so that a normal gene was active in all cells, or (b) if there was cooperation between normal and DMD cells in the cultures, or (c) if the cultures contained an excess of normal cells as a result of selection *in vivo* or *in vitro*

against cells expressing the X chromosome bearing the DMD gene.

We have now tested these possible explanations by determining the intercellular adhesiveness of clones of skin fibroblasts from a carrier of DMD who is also heterozygous for another X linked gene, glucose-6-phosphate dehydrogenase Mediterranean variant (G6PD Med).

Methods

Cells from carrier AS were obtained through the kindness of Dr T Webb and Professors A Cao and J H Edwards. Cells from AS were plated at densities of 25 and 50 cells per 60 mm diameter petri dishes in Ham's F10 medium (Earle's salts) supplemented with 15% fetal calf serum, 50 IU/ml penicillin, and 50 µg/ml streptomycin. Individual clones were isolated using glass cylinders and subsequent passaging was carried out using Eagle's MEM with Hank's salts containing 10% fetal calf serum and 10 mmol/l Hepes buffer. There was considerable variability in clonal cell morphology and growth, and many

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isolated clones failed to grow for more than a maximum of two or three passages.

Cell aggregation kinetics were measured as described previously.⁸ Briefly, confluent monolayers of cells were washed with calcium- and magnesium-free Hank's balanced salt solution and gently agitated at room temperature with 2.5 ml of 0.25% trypsin solution for 20 minutes. Cells were collected, washed once with calcium- and magnesium-free saline, and resuspended at a concentration of 0.5×10^6 cells per ml in Eagle's MEM containing 5% (w/v) Ficol 400 (Pharmacia). Single cells made up more than 90% of the total particle concentration. Aggregations were performed at 37°C using Couette viscometers set to a shear rate of 10s^{-1} . All samples were assayed blind.

G6PD phenotype was determined according to the 2d-G6P/G6P ratio method of Ferraris *et al.*⁹ Washed and trypsinised pellets of cells were lysed and sonicated in 10 mmol/l Tris-HCl buffer, pH 7.6, containing 1 mmol/l EDTA, 0.5% NP-40, 10 $\mu\text{mol/l}$ NADP, and 1 mmol/l E-amino-n-caproic acid. Aliquots for protein determination were precipitated with acetone and the protein precipitate dissolved in 1 mol/l NaOH containing 0.4% SDS. G6PD activity was measured according to the standardised method of the World Health Organization¹⁰ and the relative utilisation of analogue/substrate was calculated as a percentage. All samples were assayed blind.

Results

Results of the G6PD assays are presented in the figure and the table. Uncloned cultures from AS had a percentage utilisation of 2d-G6P of $25.16\% \pm 4.96$ SEM ($n=7$) compared with a value of $7.31\% \pm 0.49$ ($n=26$) for 15 normal subjects. (This group includes both control and DMD cells.) The clones isolated from cultures of AS could be divided into two groups on the basis of the 2d-G6P % utilisation. Of 16 clones, 15 had a mean value of $8.72\% \pm 0.44$, not different from normal values but significantly less than uncloned cells from AS. One clone had a mean utilisation of $57.75\% \pm 5.14$ ($n=7$), significantly greater than uncloned cells and the normal clones.

Results of determinations of intercellular adhesiveness are also given in the figure and the table. The mean intercellular adhesiveness of uncloned cells from AS was 2.90 ± 0.23 SEM ($n=8$), not significantly different from the mean value for normal cells, 2.95 ± 0.72 ($n=18$). Clones from AS could again be separated into two groups: nine of 11 clones had a mean value of 3.10 ± 0.130 , not significantly different from the value for normal cells or for uncloned cells from AS. The remaining two

FIGURE 2d-G6P/G6P % utilisation of cells plotted against intercellular adhesiveness (α). High 2d-G6P/G6P values indicate G6PD Med phenotype. High values of α indicate high (normal) adhesiveness. Normal skin fibroblasts (▲), skin fibroblasts from DMD patients (■), uncloned cultures of AS (●), clones of AS cells (○). Clones 7 and 33 are marked.

TABLE G6PD phenotype and intercellular adhesiveness of clones.

Cells	2d-G6P/G6P ratio (%)	Collision efficiency
Normal	7.31 $\pm$ 0.49(26)	2.95 $\pm$ 0.72(18)
DMD	*	1.39 $\pm$ 0.11(27)
AS (uncloned)	25.16 $\pm$ 4.96(7)	2.90 $\pm$ 0.23(8)
Clone 2	8.28 $\pm$ 1.06(4)	3.16 $\pm$ 0.08(4)
Clone 7	57.75 $\pm$ 5.14(7)	0.88 $\pm$ 0.10(4)
8	8.80 $\pm$ 0.93(6)	2.64
9	7.16 $\pm$ 1.02(4)	NT
12	9.25 $\pm$ 1.20(4)	NT
14	8.00 $\pm$ 0.98(6)	3.13
15	5.98 $\pm$ 0.39(4)	3.18
18	11.38 $\pm$ 2.98(4)	2.76
19	9.80 (2)	NT
20	12.30 $\pm$ 2.21(4)	NT
26	7.19 $\pm$ 1.84(6)	2.95
33	7.50 $\pm$ 0.82(4)	1.11 $\pm$ 0.07(4)
52	8.45 $\pm$ 0.60(3)	NT
54	6.50 (2)	3.39
56	8.62 $\pm$ 1.19(3)	3.93 (2)
57	8.49 (2)	2.80 (2)

*For the G6PD assay, DMD cells were considered normal and included in the normal group. Values are means; the SEM is given except where fewer than three determinations were made. Number of samples is given in parentheses. NT=not tested.

clones had values of 0.88 ± 0.10 ($n=4$) and 1.11 ± 0.07 ($n=4$), not significantly different from the mean value of 1.39 ± 0.11 ($n=27$) for DMD cells.

Discussion

Only one clone out of 16 was found to have a G6PD Med phenotype. It is possible that the G6PD assay was performed on a selected population not representative of the uncloned cultures. Because large numbers of cells were required for the adhesion assay, the G6PD assay was performed on only those clones that grew well, and one or other of the two cell types present may have been better able to grow at clonal densities. Furthermore, the disproportion of Med and normal cells in the uncloned cultures may not be as extreme as indicated by the 1/16 proportions of the clones. Because of the small sample size, this result could have been obtained by chance from a population in which the ratio of the two cell types was in fact 1/4. Nevertheless, wide deviations from the expected 1:1 ratio have also been reported in studies of the Lesch-Nyhan¹¹ and testicular feminisation¹² syndromes.

The normal intercellular adhesiveness data obtained with uncloned carrier cells⁸ could be explained if the DMD locus were not inactivated. However, although XgA¹³ and STS¹⁴ escape inactivation, they are a considerable distance from the DMD locus believed to be at Xp21.¹⁵ Furthermore, inactivation of the DMD gene is the most satisfactory explanation for the variable expression of the disorder in carriers¹⁶ and in a pair of identical twins who are carriers of DMD.¹⁷ The data in the table and the figure show that, with one exception, there is concordance between expression of the G6PD phenotype and intercellular adhesiveness (α) for clones from subject AS: G6PD positive clones have normal values of α while the G6PD Med clone has a low value of α .

Two other reports have been published recently that claim to demonstrate the occurrence of X chromosome inactivation in skin fibroblasts and muscle cells from carriers of DMD. Ionasescu *et al*¹⁸ have reported data describing the occurrence of two types of skin fibroblast clone, one with high (DMD-like) and one with normal incorporation of hydroxyproline. Unfortunately they did not give the proportions of the normal and mutant clones found. Thompson *et al*¹⁹ have examined cultures of muscle cells from DMD heterozygotes for the phenomenon of 'clustering', described by them in DMD patient muscle cultures (but see reference 20 for contradictory data). However, they did not use clones of cells from carriers, but found that cultures of seven out of

14 carrier biopsies showed clustering. Their conclusion that they have demonstrated X chromosome inactivation in DMD carriers depends on the assumption that the patch sizes of normal and DMD cells in these carriers is large relative to the biopsy size.

Clone 33 is exceptional in that it is unequivocally G6PD positive but has unequivocally low intercellular adhesiveness characteristic of DMD. Preliminary karyological analysis (Dr Daker, Paediatric Research Unit, Guy's Hospital) indicates that there is no major chromosomal structural abnormality to account for this. Recent studies of the expression of recessive alleles in retinoblastoma have found evidence for what may be an example of in vivo mitotic recombination,²¹ and we may have detected a similar phenomenon in a somatic cell in vitro. Alternatively, reactivation of the G6PD B locus on the inactivated X chromosome carrying the (normal-G6PD B) loci would result in cells of the clone 33 phenotype. However, the G6PD phenotype of clone 33 cells appeared completely normal, although Migeon *et al*²² found that the derepressed locus produced only half the enzyme subunits produced by the normally active locus.

For this carrier, the high proportion of G6PD+ve/normal adhesiveness clones would account for the normal aggregation of her uncloned cells. It is unfortunate that we are unable to continue cloning experiments with this carrier as her cells have been difficult to grow at clonal densities. Our current data do not exclude cooperation between the normal and DMD cells in an uncloned carrier culture as the explanation for the normal aggregation of cells from other carriers.⁸

The results obtained in the present study are particularly important because we can now use measurements of intercellular adhesiveness in obligatory heterozygotes for DMD who do not carry a marker for a second X linked heterozygosity. Polymorphic differences between these clones will be restricted to X linked loci, that is, to about 6% of the length of the genome.²³ Differing autosomal loci may be active in the two sets of clones as a result of the expression of the DMD locus in one set of clones, but the total differences between clones will be considerably reduced compared with those between genetically unrelated normal and DMD subjects. We are now searching for protein differences between such clones using two dimensional polyacrylamide gel electrophoresis.²⁴

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