Intracellular calcium and pathogenesis and antenatal diagnosis of Duchenne muscular dystrophy

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Summary and conclusions

One of the earliest and most important abnormalities of fetal muscle in Duchenne muscular dystrophy is an increase in eosinophilic fibres (those that stain darkly with eosin). A study of normal and at-risk male fetuses after abortion was carried out, which showed that these eosinophilic fibres contain increased intracellular calcium, which suggests that this is an early biochemical change in the disorder.

Since increased intracellular calcium would account for various biochemical and clinical features of the disease, it may be related to the primary defect. Thus an increase in muscle fibres containing increased intracellular calcium in at-risk fetuses may provide an additional means of assessing the validity of any future presumptive antenatal test for Duchenne muscular dystrophy.

Introduction

Duchenne muscular dystrophy is a serious X-linked disorder associated with progressive muscle wasting and weakness. The cause is unknown, but evidence is accumulating of a widespread membrane defect demonstrable not only in muscle tissue but also in lymphocytes and erythrocytes.

We have reported¹ that in muscle from male fetuses at risk for Duchenne muscular dystrophy the proportion of fibres that stain darkly with eosin and are therefore referred to as eosinophilic fibres is appreciably increased. Since eosinophilic fibres were present in the absence of gross histological changes (for example, necrosis and phagocytosis), we concluded that the increase in these fibres must be one of the earliest structural changes in the disorder. Here we report that these same muscle fibres contain increased intracellular calcium, which therefore suggests that this is an early biochemical change in the disorder. Since increased intracellular calcium would account for many of the membrane abnormalities, and even some of the clinical features associated with this disease, this may be related to the primary defect.

Methods

Material from normal male fetuses was obtained at the time of therapeutic abortion in cases with no history of any neuromuscular disorder, and from at-risk male fetuses after therapeutic abortion in mothers at high risk of having an affected son. The gestational ages ranged from 14 to 21 weeks in both groups. Pregnancies were terminated by either hysterotomy or prostaglandin, and, at least in controls, the method of termination did not appear to affect the proportions of eosinophilic fibres and muscle fibres containing increased intracellular calcium, which were also unrelated to gestational age.

Specimens of quadriceps muscle were obtained as soon as possible after termination and fetal death. Transverse cryostat sections were stained with haematoxylin and eosin. Serial 10 µm sections were also stained for calcium (by using alizarin red S² and a fluorescence method

with pentahydroxyflavone (Morin)³ and for ferric iron by using Perl's method.⁴ Methods for determining muscle-fibre size and the proportion of eosinophilic fibres have been reported.⁵ Only the central areas of each section were studied to avoid artefacts, which are commoner toward the periphery of sections.

FIG 1—Serial sections of muscle from patient presenting as early case of Duchenne muscular dystrophy, stained with (a) haematoxylin and eosin (note three centrally placed dark-staining eosinophilic fibres); (b) alizarin red S; and (c) fluorescent Morin. (Original magnification $\times 430$.)

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FIG 2—Serial sections of muscle from at-risk fetus (B118) stained with (a) haematoxylin and eosin (note dark-staining eosinophilic fibres); (b) alizarin red S; and (c) fluorescent Morin. (Original magnification $\times 740$.)

Results

Muscle tissue (adductor longus) was examined from a 7-year-old patient presenting as an early case of Duchenne muscular dystrophy, which showed an appreciable increase in the proportion of those fibres defined as large, dark-staining eosinophilic fibres. Staining of serial sections showed that these fibres contained increased intracellular calcium (fig 1). They did not, however, appear to contain increased amounts of iron.

In two at-risk fetuses (B118, B132) previously considered to be abnormal with increased proportions of eosinophilic fibres,¹ serial sections showed that most of these eosinophilic fibres also contained increased intracellular calcium (fig 2). Occasional muscle fibres that appeared histologically normal, in both at-risk and normal fetuses, also sometimes contained increased calcium. The proportions of

fibres containing increased intracellular calcium, however, were greater in these two at-risk fetuses than in the controls (table). There was no apparent increase in the proportions of these fibres in six other at-risk fetuses in which the proportions of eosinophilic fibres and other variables (mean and variance in fibre size) were normal (table). As in the patient with Duchenne muscular dystrophy, the eosinophilic fibres in at-risk fetuses did not contain increased amounts of iron.

Results obtained in muscle from normal and at-risk fetuses. (Values outside normal range given in *italics*)

Method of termination*	Fibre diameter		Eosinophilic fibres (%)	Fibres containing increased intracellular calcium (%)	
	Mean (μ m)	Variance (σ^2)		Alizarin stain	Morin stain
<i>Ranges in normal fetuses</i>					
H/P	6.9-10.7 (n = 16)	0.8-3.6 (n = 16)	0.5-0 (n = 16)	0-3.9 (n = 9)	0-7.0 (n = 9)
<i>At-risk fetuses</i>					
B118	H	9.6	2.5	8.0	12.0
B132	P	10.0	4.3	6.9	8.4
B119	P	8.5	1.3	1.0	2.0
B125	P	8.5	1.7	4.9	0.0
B135	H	9.4	1.8	0.0	0.0
B141	H	8.3	0.9	0.5	1.0
B142	H	9.0	1.7	0.5	0.0
B143	H	8.7	2.3	1.8	3.8

*H = Hysterotomy. P = Prostaglandin.

Discussion

In their histological and histochemical studies Bodensteiner and Engel⁶ showed an increased proportion of non-necrotic muscle fibres containing increased intracellular calcium in biopsy specimens from patients with Duchenne muscular dystrophy compared with controls and patients with various other neuromuscular disorders. We found similar fibres in an early case of Duchenne muscular dystrophy as well as in muscle from two at-risk fetuses presumed to be affected. Since in the fetal material no gross histological changes (for example, necrosis and phagocytosis) were evident, increased intracellular calcium is apparently a very early biochemical change in the disorder. An increase in the calcium content of muscle nuclei⁷ and mitochondria⁸ in dystrophy has been reported, and mitochondrial calcium overload has been suggested as a possible mechanism for cell necrosis in muscle diseases.⁹

The various membrane changes that have been reported in Duchenne muscular dystrophy might be accounted for by increased intracellular calcium; these include echinocyte formation,^{10,11} increased potassium efflux^{12,13} and reduced deformability in erythrocytes,¹⁴ and reduced capping in lymphocytes.¹⁵ In muscle increased intracellular calcium might account for degeneration and increased protein degradation^{16,17} and, through enhancement of calcium-activated proteases,^{18,19} lead to muscle necrosis and weakness; and the resultant increase in interaction between myofilaments might account for the development of muscle contractures. Finally, even the efflux of creatine kinase, one of the most consistent features of Duchenne muscular dystrophy, has been induced in vitro in mouse²⁰ and human muscle (Anand and Emery, unpublished observations) by increasing the concentration of calcium in the incubating medium.

If increased intracellular calcium is an important factor in the pathogenesis of Duchenne muscular dystrophy several therapeutic approaches are possible.²¹ Though the influx of calcium could be secondary to a generalised membrane defect,²² the primary defect might possibly reside in the active extrusion of intracellular calcium²³ or even in calcium transport per se since uptake of calcium by the sarcoplasmic reticulum is decreased in Duchenne muscular dystrophy.²⁴

Our finding of an increased proportion of muscle fibres containing increased intracellular calcium in at-risk fetuses

provides an additional variable¹ for determining whether a particular fetus may have been affected and therefore for assessing the validity of any future presumptive antenatal test based on the study of amniotic-fluid cells or fetal blood.

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Detection of unrecognised nocturnal hypoglycaemia in insulin-treated diabetics

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Summary and conclusions

Cortisol to creatinine ratios in overnight urine samples, urinary glucose excretion, and plasma glucose concentrations were determined in 43 diabetic inpatients. All initially had normal cortisol to creatinine ratios ($<55 \times 10^{-6}$) and were initially treated by increasing their long-acting insulin component. Nine patients in whom this ratio became raised then had their long-acting insulin component reduced until their fasting plasma glucose concentration was 4-7 mol/l (72-126 mg/100 ml). The 34 patients who had never had a raised ratio were treated by increasing their long-acting insulin component until their fasting plasma glucose concentration was in the range 4-7 mmol/l. All the raised cortisol to creatinine ratios were clearly separate from the other values. A

mean reduction in total insulin dose of 23% and in long-acting insulin dose of 53% was achieved, abolishing presumptive nocturnal hypoglycaemia by reducing the ratio to normal and dramatically improving diabetic control.

Although there was no definite evidence that the patients who had raised cortisol to creatinine ratios had suffered from nocturnal hypoglycaemia, these results strongly support the view that a raised ratio indicates an otherwise unrecognised episode of this condition.

Introduction

In insulin-treated diabetes the occurrence of fasting hyperglycaemia may be due either to insufficient long-acting insulin to last the night or to overtreatment with insulin, which initially produces hypoglycaemia and then rebound hyperglycaemia.¹ It is important to distinguish between these two causes of morning hyperglycaemia so that an appropriate alteration in insulin dose can be made. Nocturnal hypoglycaemia may be recognised by the patient or his doctor, but clinical diagnosis may be difficult.² The definitive method of distinguishing between the two possibilities is frequent blood-glucose monitoring during the night; this, however, is not

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