Identification of carriers of Duchenne muscular dystrophy by muscle protein synthesis

Victor Ionasescu, M.D., Hans Zellweger, M.D., Paul Shirk, B.S. and Thomas W. Conway, Ph.D.

■ A previous paper based on a survey of 10 suspected carriers of Duchenne muscular dystrophy (MD) showed that analysis of in vitro amino acid incorporation in fractionated extracts of biopsied muscle was useful for detecting females who carry the defective gene responsible for this X-linked disease.[1] Since then, we have routinely included an evaluation of protein synthesis with the more commonly used serum creatine phosphokinase (CPK) determination and muscle histologic examination. Protein synthesis in this larger group of suspected carriers has been compared with the two conventional methods not only for its value in carrier detection but also in respect to the known increased protein synthesis observed in muscle extracts from patients with the disease[2,3] and, in particular, with elevated in vitro synthesis of collagen.[3]

Materials and methods

Sixty-three female relatives of patients with Duchenne MD and 20 age-matched normal females were examined. The suspected carriers were grouped according to the classification of Dreyfus and associates[4]: known carriers (11), mothers of isolated cases (10), and sisters of patients (42). The *known* carriers have more than one male patient in the family. This group includes both definite and probable carriers according to the classification of Milhorat and Goldstone.[5] Mothers of isolated cases and sisters of patients with Duchenne MD correspond to those designated as possible carriers by the Milhorat and Goldstone classification. The higher risk of being carriers for the mothers of isolated cases than for the sisters of patients accounts for their separate classification.

Muscle specimens of the left vastus lateralis were obtained from both suspected carriers and controls. All subsequent procedures for the extraction of muscle polyribosomes and for in vitro amino acid incorporation were done as previously reported.[3,6] The assay for collagen synthesis represented the difference in the trichloroacetic acid precipitable radioactivity in the absence and presence of purified *Clostridium histolyticum* collagenase (Worthington Biochemical Corp.).[3,6]

Determination of the noncollagen protein in the muscle homogenate was done by the method of Lowry, Rosebrough and Farr,[7] with bovine serum albumin as standard. The method of Hughes,[8] as modified by Bray and Ferren-

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delli,[9] was used for the determination of serum CPK. The activity was expressed in micromoles of creatine liberated per milliliter of serum in one hour at 37° C. The specimens for light microscopy were fixed in Susa's fixative, and longitudinal and transverse sections were obtained. The sections were stained with hematoxylin and eosin, Mallory's trichrome, phosphotungstic acid-hematoxylin, and periodic acid-Schiff reagent.

Results

Clinical findings. Physical and neurologic evaluations were within normal limits for 61 suspected carriers. Two, however, showed large pseudohypertrophic calves, with harder muscle consistency; both were scored as carriers due to increases in ribosomal protein synthesis and also high levels of serum CPK.

Ribosome content. As seen in table 1, the ribosome content in all the classes studied had normal values for the carriers regardless of their protein synthesis levels. The noncollagen protein content of the muscle was slightly but nonsignificantly decreased in the group of carriers with high protein synthesis.

Amino acid incorporation of polyribosomes and monomeric ribosomes. Protein synthesis was measured by means of the major ribosome fraction (table 1). This fraction was further separated on sucrose density gradients into subunits, monomeric ribosomes, polyribosomes, and sedimented polyribosomes, which are found pelleted at the bottom of the tube. Each ribosome fraction was complemented with the concentrated soluble enzymes contained in the supernatant of the initial high-speed centrifugation used to obtain the major ribosome fraction. Details concerning the incorporation of a mixture of carbon 14-labeled amino acids have been previously reported.[3,6] All data presented in the tables are given as the specific activity for incorporating radioactive amino acids in respect to the ribosomes used (counts per minute per microgram of ribosomes). Reactions were always limiting in respect to the ribosomes (1 to 20 mcg), not the supernatant enzymes.

In Duchenne MD patients, the specific activity of the polyribosomes for protein synthesis was found to be about five times higher than in controls.[3] Similarly, our preliminary report[1] indicated that protein synthesis was elevated for eight of 10 suspected carriers. The increases seen were less than those in patients with the disease, so we were somewhat uncertain as to what constituted a range

TABLE 1
MUSCLE RIBOSOME CONTENT

Suspected carriers	*Noncollagen protein (mg/gm of muscle ± S.D.)*	*Ribosome content (mcg/mg of protein ± S.D.)**			
		Total ribosomes	*Major ribosome fraction*	*Free ribosomes*	*Reextracted ribosomes*
Positive, high protein synthesis (42)	44.5 ± 16.7	10.3 ± 7.0	5.9 ± 3.2	1.3 ± 0.8	2.5 ± 2.2
Controls (20)	49.0 ± 9.8	11.5 ± 3.1	6.8 ± 2.3	1.9 ± 1.2	2.4 ± 1.4
t value	−1.1	−0.7	−1.1	−1.8	0.2
p value	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
Negative, normal protein synthesis (21)	46.7 ± 19.0	10.0 ± 4.0	6.8 ± 3.0	1.4 ± 1.0	1.9 ± 1.2
Controls (20)	49.0 ± 9.8	11.5 ± 3.1	6.8 ± 2.3	1.9 ± 1.2	2.4 ± 1.4
t value	−0.4	−1.1	−0.06	−1.3	−1.0
p value	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05

*The major ribosome fraction was obtained by extraction of an initial 122,000 X g pellet with detergent. The free ribosomes were obtained by recentrifugation of the initial high-speed supernatant fraction at 150,000 X g for two hours. Further extractions of the 122,000 X g pellet with a doubled concentration of detergent produced the reextracted ribosome fraction.

of activity characteristic of the carrier state. The present group of 63 suspected carriers and their 20 normal controls have allowed us to set these limits. Table 2 gives the range of specific activities for the 20 controls. All are tightly distributed between about 30 and 70 cpm per microgram of total polyribosomes. This range does not seem to be related to age; therefore, all the normal values can be considered together, as done in table 2. The specific activities of the suspected carriers covered a much broader range, from normal to about 500 cpm per microgram of polyribosomes, a value close to the upper level seen in patients with Duchenne MD. On careful analysis of these results together with the genealogical evaluation, serum CPK measurements, muscle histologic study, and the extent of collagen synthesis as well as ribosome distribution (see below), it was decided that a specific activity greater than 70 cpm per microgram of ribosomes was consistent with the carrier state. On this basis, 42 of the 63 were scored as carriers. The average specific activity of the total polyribosomes for this group was 171.9 ± 101.6 cpm per microgram of ribosomes. Previously we had found that in patients with Duchenne MD the activity of the monomeric ribosomes was decreased while that of the polyribosomes was increased; consequently, the ratio of the specific activities of the polyribosomes to the monomeric ribosomes was highly significant. The activity of the

TABLE 2
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Suspected carriers	*A. Monomeric ribosomes*	*B. Total polyribosomes, fractionated and sedimented*	*C. Ratio B/A*
Positive, high protein synthesis (42)	23.6 ± 7.6	171.9 ± 101.6	6.1 ± 4.2
Controls (20)	20.8 ± 2.4	50.1 ± 9.7	2.4 ± 0.6
t value	1.5	5.3	3.9
p value	> 0.05	< 0.01	< 0.01
Negative, normal protein synthesis (21)	22.1 ± 9.7	45.3 ± 14.1	2.3 ± 0.8
Controls (20)	20.8 ± 2.4	50.1 ± 9.7	2.4 ± 0.6
t value	0.6	−1.2	−0.5
p value	> 0.05	> 0.05	> 0.05

*Measured as counts per minute per microgram of ribosomes ± S.D.

TABLE 3
SERUM CPK, MUSCLE HISTOLOGIC FINDINGS, AND MUSCLE PROTEIN SYNTHESIS IN CARRIERS OF DUCHENNE MUSCULAR DYSTROPHY

	Known carriers		*Mothers of isolated cases*		*Sisters of patients*	
	+	−	+	−	+	−
Serum CPK	6	5	6	4	12	30
Muscle histologic findings	3	8	4	6	1	41
Muscle protein synthesis	11	0	8	2	23	19
	(100 percent)		(80 percent)		(54 percent)	

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monomeric ribosomes for all the suspected carriers fell within normal limits (table 2, column A), and the ratio of polyribosomes to monomeric ribosomes (table 2, column C) was actually less significant than the difference between the specific activities of the total polyribosomes (table 2, column B).

Distribution of ribosomes. Muscle extracts from 10 of the 63 suspected carriers showed an abnormal distribution of monomeric ribosomes on sucrose density gradient analysis (figures 1 and 2). The abnormality took the form of an increase in the size of the 60S ribosomal peak. One carrier in this group of 10 showed a 60S peak larger than the 80S peak (figure 1, solid line). Two others displayed roughly equal amounts of these ribosomes, while seven showed a prominent 60S peak but no 80S peak at all (figure 2, solid line). All 10 women with abnormally distributed ribosomes had increased protein synthesis and were scored as carriers. The family histories showed that seven of the 10 were known carriers, while three were sisters of patients with Duchenne MD.

In vitro synthesis of collagen. Synthesis of both collagen and noncollagen was increased in 13 of 42 subjects (31 percent). In 12 (28.5 percent), the increase involved only collagen. Thus, the increase in protein synthesis in more than one-half of our carriers was due to increased collagen formation. In the other 17 (40.5 percent) scored as carriers, high noncollagen synthesis accounted for the overall increase in amino acid incorporation. The relation between collagen synthesis and age of the carriers showed that 20 of 28 carriers (71.4 percent) under 30 years of age had polyribosomes with higher collagen synthesis, while only 5 of 14 carriers (35.7 percent) above 30 years of age revealed the same disorder.

Comments

Our findings indicate an increase in protein synthesis of muscle polyribosomes from carriers of Duchenne MD. A similar increase has been found in polyribosomes from patients with early stages of the disease.[2,3] Our previous studies[3] showed that protein synthesis is also qualitatively abnormal in Duchenne MD, because more collagen than noncollagen protein is synthesized in vitro by dystrophic polyribosomes. All these data suggest a disorder of muscle regeneration. Histochemical and electron-microscopic studies by Mastaglia and associates[10,11] also indicate abnormal regeneration of muscle from Duchenne patients.

Some of our present findings are consistent with a defective protein synthesis in the carriers as well. The abnormal separation of monomeric ribosomes on sucrose density gradients with a decrease or absence of the 80S peak and enlargement of 60S ribosomal subunits in a

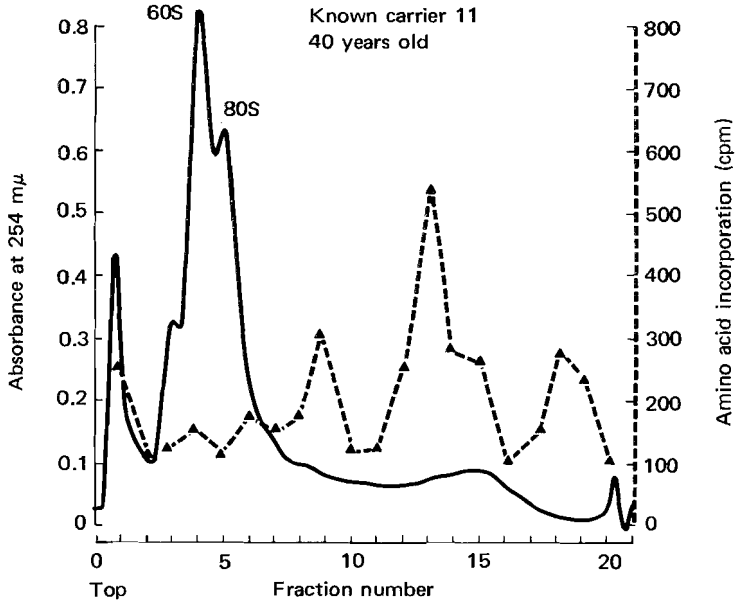

Figure 1. Sucrose density gradient analysis of ribosomes from muscle vastus lateralis of a 40 year old known carrier (carrier 11). The gradient was layered with 180 mcg of the major ribosome fraction. The amount of the soluble enzyme used for protein synthesis was 150 mcg per tube.

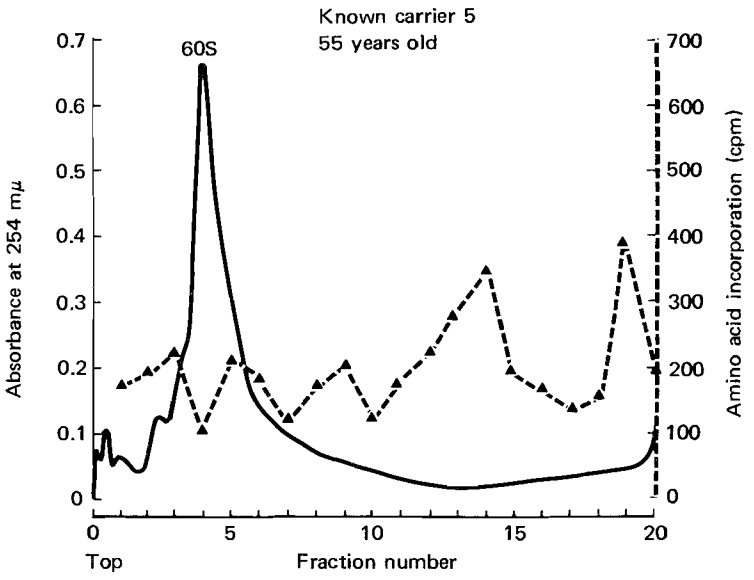

Figure 2. Sucrose density gradient analysis of ribosomes from muscle vastus lateralis of a 55 year old known carrier (carrier 5). The gradient was layered with 102 mcg of the major ribosome fraction. The amount of the soluble enzyme used for protein synthesis was 120 mcg per tube.

prominent peak might indicate a disorder of initiation of protein synthesis or a defect in termination of protein synthesis, which would leave peptide chains attached to the 60S subunit.[12]

High amino acid incorporation of polyribosomes was present in 42 of the 63 suspected carriers studied. The increase in protein synthesis in 25 of these carriers also involved high collagen formation. The relation between collagen synthesis and the carriers' age was striking. A large proportion (71.4 percent) of those under 30 years of age showed high collagen synthesis, while only 35.7 percent of those over 30 had the same disorder. The polyribosomes of younger carriers, like those of children with Duchenne MD, synthesize large amounts of collagen. In older carriers, the ability to synthesize collagen is decreased, and the polyribosomes synthesize mostly noncollagen protein. It is difficult to explain these changes and their correlations with the primary mechanism of the disease. However, the abnormal ratio of collagen to noncollagen in the young carriers may be considered a sign of pathologic change.

The usefulness of ribosomal protein synthesis in carrier identification is illustrated in table 3. None of the known carriers fell within the range of normal test values. Genealogically all should be carriers and 100 percent were so scored. Eighty percent of the mothers of isolated cases were classed as carriers, suggesting that many sporadic cases may be inherited. Fifty-four percent of the sisters of patients with Duchenne MD were scored as carriers, which is close to the theoretical ratio of 50 percent.

Mention must be made that 23 of 24 suspected carriers with abnormal serum CPK levels and all with dystrophic histologic findings were scored as carriers by the protein synthesis test. The one exception was a 12 year old sister of a patient with Duchenne MD. Her increased CPK was only slightly above normal (8 μM per milliliter per hour; normal, 3.5 ± 1.4). Protein synthesis analysis showed her to be normal. Gardner-Medwin, Pennington and Walton[13] have suggested that evidence of slightly elevated serum CPK in young girls is not sufficient to class them as carriers until the tests are repeated after a few years.

Interestingly, five of our known carriers had normal serum CPK levels, and eight had normal muscle histologic findings. Yet all were judged to be carriers by our procedure. So far, serum CPK has been considered the best index for detection of the carrier state by Gardner-Medwin, Pennington and Walton[13]; Moser, Mummenthaler and Wiesmann[14]; and Hausmanowa-Petrusewicz and associates.[15] However, all agree that only 66 to 80 percent of the known carriers can be scored by this method. Our test, when coupled with the CPK evaluation, probably identifies more than 90 percent of Duchenne carriers.

Ribosomal protein synthesis, thus, should not replace serum CPK determination but rather should be used to complement it. A high serum CPK level is good evidence of the carrier state; therefore, it is not necessary to do the protein synthesis analysis unless for research purposes. The same comment is valid for known carriers based on genealogical studies. We feel that ribosomal protein synthesis is indicated only in mothers of isolated cases or sisters of Duchenne patients who have three serum CPK determinations within normal limits. In our experience, the test has been very helpful with genetic counseling in these cases.

Summary

The in vitro amino acid incorporation by polyribosomes extracted from biopsied muscle (vastus lateralis) was studied in 63 suspected carriers (21 mothers and 42 sisters) of Duchenne muscular dystrophy and in 20 normal females. A significant increase in specific activity of total polyribosomes was found in 42 subjects, while the serum creatine phosphokinase levels were high in 24 and dystrophic histologic changes were present in eight. Higher collagen synthesis was found in 20 of 28 carriers below 30 years of age, while only five of 14 carriers over 30 showed abnormal collagen synthesis.

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