POLY A RNA STATUS AND EXPRESSION OF milli (m) AND micro (μ) CALPAINS IN SKELETAL MUSCLE OF DUCHENNE MUSCULAR DYSTROPHY PATIENTS

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ABSTRACT

Poly A RNA status and its *in vitro* translation in a rabbit reticulolysate cell free system, were assessed in the skeletal muscle of young boys with Duchenne muscular dystrophy. Compared to normals there is a significant 48% increase in poly A RNA content of dystrophic muscle and its translatability was increased by 56% based on ^{35}S methionine incorporation into total protein synthesised. Immunoprecipitation of the translated products with monospecific antibodies showed that there is a 2.6 fold and a 2 fold increase in m and μ calpains respectively. This underlines the importance of both synthetic and degenerative activities in the early pathology in DMD muscle.

KEY WORDS

Calpain; DMD, *in vitro* translation; poly A RNA; Protein synthesis; Skeletal muscle.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked recessive genetic disorder characterized by progressive muscle necrosis (1). Muscle necrosis in DMD is largely attributed to the myofibrillar and cytoskeletal protein degradation. Besides necrosis, a characteristic feature of the early stage of DMD is the regeneration of embryonic myoblasts, an attempt to combat the progressive loss of muscle tissue (2). A drastic increase in intracellular levels of calcium is one of the early biochemical changes observed in DMD and subsequently there is an increase in the activity of calcium dependent protease *viz* calpain (3,4). A number of studies have demonstrated an increase in the activity and concentration of calpain in DMD and mdx mice, suggesting its role in muscle necrosis (5,6). Calpain, an ubiquitous intracellular cytosolic protease found in eukaryotes is classified as m- and μ -calpain, based on the requirement of mM and μM concentration of Ca^{2+} for activity respectively (7).

DMD pathogenesis is considered to be the result of imbalance between protein synthesis and degradation, i.e. the rate of muscle protein synthesis is insufficient to replace degraded proteins and enhance the protein mass. While a few studies have recorded the protein synthesis to be low in DMD and hamster (8,9,10), the others have found the protein synthesis to be enhanced in DMD muscle compared to control (11,12). However no report has implicated defective protein synthetic machinery. There are reports of enhanced ribosomal dependent collagen synthesis (12). Nonetheless these observations are based on the studies conducted on the DMD patients aged around 12-18 years, who have lost their ambulation completely, indicating severe muscle loss.

The present study directly aimed at assessing the synthetic state of muscle under dystrophic condition, where poly A RNA is extracted and protein synthesis is monitored using ^{35}S methionine incorporation in *in vitro* translation in rabbit reticulocyte cell free system. Moreover, the muscle tissue used in this study was collected from DMD boys aged 5-12 years, who have retained their ambulatory capacity, despite proximal muscle weakness. Muscle histopathology in four of these shows presence of regenerative fibres. The *in vitro* translated product was immunoprecipitated with

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m/ μ antisera in order to assess the relative amounts of the respective calpains in disease condition.

MATERIALS AND METHODS

Purification of m-and μ -Calpains

m- and μ -calpains were purified from human placenta as described earlier (13). The constituents of m- and μ -calpains were separated on 7% SDS-PAGE & the large subunits (80 K) of respective calpains were electro eluted and used for raising the antisera. Antisera against the antigen (large subunits of m-and μ -calpains) was raised in albino rabbits.

Preparation of rabbit reticulocyte lysate system

Albino rabbit was injected with 2.5% phenyl hydrazine (pH7) (Sigma, USA) with 0.25ml/kg body weight to induce anemia. The dose was repeated for six consecutive days. On the 8th day, 60ml of blood was collected in an equal volume of chilled normal saline containing 0.001% heparin. After 3-5 washes with normal saline, the cells were lysed with equal volume of cold distilled water and clear reticulocyte lysate was obtained by centrifugation at 20,000 g for 20 minutes at 40°C. The lysate was treated with micrococcal nuclease, hemin and creatine phosphokinase type I (Sigma, USA).

Diagnosis of DMD

Diagnosis of DMD in 6 patients with age range of 5-12 years was carried out by clinical examination, EMG, muscle histology and by estimation of serum CK. The part of biopsied muscle (580 mg) from DMD patients referred for histopathological examination was made available for this study with prior consent from the parents and with the consent of the ethical committee of Nizams Institute of Medical Sciences, Hyderabad, India. Biopsied muscle was processed for the isolation of total RNA. Total RNA from DMD & control muscle tissue was isolated by the method described by Chomczynski et al (14). Poly A RNA was isolated using Oligo dT-cellulose (Boeringer Mannheim, Germany) chromatography and concentration was measured by spectrophotometer (Shimadzu, Japan) at 260 nm wavelength.

***In vitro* translation in reticulocyte lysate system**

A translation cocktail containing 6.2 mM spermidine, 10mM creatinine phosphate, 0.3 mM each of all amino acids except methionine, 25 mM dithiothreitol & 250 mM HEPES (pH 7.4) (Sigma, USA) was prepared. The reaction mixture (26 μ l) included 2 μ l of translation cocktail, 2 μ l of 1M KCl, 0.05 μ l of 32.5 mM MgCl₂, 10 μ l of 100 μ Ci ³⁵S methionine, 10 μ l of treated rabbit reticulocyte lysate, 0.5 μ l of autoclaved deionised water & 1 μ l of poly A RNA ranging from 0.05 – 2.4 μ g. At 0.6 μ g of poly A RNA dependent incorporation was linear which was used for comparison.

The reaction was allowed to proceed at 37°C for one hour and stopped by the addition of an equal volume of stop-mixture (10% cold TCA containing 10 mM unlabelled methionine) for assessing the total mRNA. For immunoprecipitation, the reaction was terminated by adding an equal volume of stop mixture containing 10% triton X-100, 5mM EDTA and 10mM unlabelled methionine, followed by 5 μ l of anti m or μ calpains antisera, respectively. Both, the TCA & immunoprecipitates were washed 3-5 times with the respective stop mixture. The precipitates obtained by centrifugation were resuspended in 100 μ l of formic acid and radioactivity measured in 3 ml of toluene based scintillation cocktail [0.4% PPO (diphenyloxazole), 0.12% POPOP (1,4-bis-2- (5-phenyloxazolyl) benzene) and 33% triton X-100] using the LKB ultra-beta model 1210 scintillation counter. The translation products were analyzed on SDS-PAGE. For fluorography, the gels were treated with two changes of dimethyl sulphoxide (DMSO) for 30 minutes each, followed by gentle shaking in 20% PPO dissolved in DMSO, for 2 hours. The gel was washed thoroughly under running water, dried on whatman No.1 and subjected to autoradiography.

In vitro synthesis of the large 80 K subunits of m-calpain & that of μ -calpain was assessed by measuring the pico moles incorporation of ³⁵S-methionine in respective immuno precipitates which were expressed as percentage incorporation of ³⁵S-methionine in the immuno precipitate with respect to that incorporated in TCA precipitate. Specificity of the immnoprecipitate was assessed by SDS-PAGE analysis (fig 1). Immunoprecipitates with preimmune serum lacked the specific bands corresponding to 80 and 30 Kd bands. Similar patterns were obtained with milli and micro antisera as the subunit sizes are same. *In vitro* synthesis of total protein in DMD and normal control was

assessed by measuring the pico mole incorporation of ^{35}S -methionine in the TCA precipitate.

RESULTS

Quantitation of poly A RNA

Poly A RNA isolated from the muscle had an $\text{OD}_{260}/\text{OD}_{280}$ ratio of ~ 1.87 and the quantity of RNA was expressed per milligram of cellular DNA to allow an unbiased comparison. Poly A RNA in normal samples amounted to an average of 0.0296 ± 0.0053 mg/mg of muscle DNA and in the dystrophic samples to an average of 0.044 ± 0.073 mg/mg of muscle DNA. This amounts to a significant 48% increase in transcription in DMD muscle.

^{35}S methionine incorporation into total protein synthesized

The protein synthetic activity of the isolated poly A RNA was tested using a range of poly A RNA concentration from 0.05-2.4 μg per assay. Incorporation of ^{35}S methionine in total protein synthesized was maximum at the RNA concentration of 0.8 μg . However at 0.6 μg at which concentration the incorporation was linear, 610 pico moles of ^{35}S methionine was incorporated in the DMD sample, while for the same amount of poly A RNA of normal muscle gave 390 pico moles of ^{35}S methionine incorporation. There is 56% increase in total protein synthesis in DMD.

m and μ calpain synthesis

Specific activity of calpain mRNA in dystrophic and normal muscle for m80K and μ 80K was monitored in 6 dystrophic and 6 normal samples. The ^{35}S -methionine incorporated in both the μ 80K and m80K immunoprecipitates was expressed as percentage incorporation with respect to the radioactivity incorporated in the TCA precipitate (total protein synthesized). Fig 2, a scatter plot, gives this data for m and μ 80K for normal and dystrophic samples. The mean values are marked by the arrow. The ratio of the mean between DMD and normal indicates a 2.7 and 2-fold increase for m80K and μ 80K mRNA expression respectively and the difference is statistically significant.

DISCUSSION

The net effect of DMD is explained and understood in terms of insufficient rate of protein synthesis to compensate protein degradation. Increased rate of

protein synthesis was observed in hamsters and mice with DMD-like muscular dystrophy (10,15) and increased ribosomal protein synthesis is reported in DMD patients (12). Report of drastic decrease in the rate of total protein is observed based on infusion studies performed on boys with DMD aged 12-18 years (8,9), who were wheel chair bound hence with hardly any muscle to conclude on protein turnover, much less on protein synthesis. The DMD patients in this study however are ambulatory and the histopathology of muscle revealed presence of regenerative fibres. Hence the $\sim 48\%$ increase in poly A RNA content of the dystrophic muscle and the observed 56% increase in protein synthesis assume significance, considering the dilemma regarding the status of muscle protein synthesis in DMD. This may well reflect the adaptation of muscle tissue to the adverse condition, viz the progressive loss of muscle protein.

This study was primarily carried out to investigate the expression of calpain gene in a pathologic condition, such as DMD, which is associated with increased intracellular calcium. There are a number of reports demonstrating an increase in calpain activity in DMD implicating the same in muscle necrosis (5,6,16). The present data clearly indicates a new synthesis of both m and μ calpains in DMD, albeit m calpain expressed much more under adverse condition (17).

As in case of total poly A RNA, the possibility of regenerating fibres, adipose tissue and macrophages contributing to calpain mRNA cannot be overlooked. Calpain mRNA contributed by adipose tissue and macrophages would be insignificant considering the low activity of calpain recorded in these non-muscle cells (18). Contribution of calpain mRNA by regenerating fibres however is of relevance as these myoblasts represent a cell system in a state of responsiveness with respect to gene expression during cell differentiation and growth.

Considering the pathogenesis of DMD muscle tissue, the requirement for an enhanced synthesis of calpain arises from the fact that it is implicated in gene expression via the protein kinase C phosphorylating pathway (19) and cell division (20), alternately the enhanced synthesis of calpain may be needed to mobilize muscle protein reserves in order to compensate the progressive loss of muscle protein (21).

In conclusion, present study demonstrates that the mRNA dependent total protein synthesis is elevated in DMD, which is also reflected by a 48% increase in mRNA content in dystrophic muscle and the 56% increase in poly A RNA dependent protein synthesis. Nevertheless the magnitude of these events is incapable of combating necrosis and the effect diminishes as the disease advances.

Furthermore, the study also demonstrates a *de novo* synthesis of m- and μ -calpains in DMD, which underlines an enhanced expression of respective genes in disease condition. This is apparently to combat the adversity of progressive degeneration by activating the synthetic processes, via gene expression.

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Fig 1. Product analysis on 10% SDS-PAGE. Immunoprecipitation was performed with anti-m calpain antisera (lane b, control sample; lane d, dystrophic sample) and with pre-immune sera (lane a, control sample; lane c, dystrophic sample). Markers are (from top) phosphorylase b (97.4Kd), bovine serum albumin (66Kd), egg ovalbumin (45Kd) carbonic anhydrase (29Kd). Arrows indicate the position of 80Kd and 30Kd subunits, respectively.

Fig 2. Percentage incorporation of ³⁵S-methionine in the immunoprecipitates of m (i) and m (ii) calpain in DMD in comparison to normal control.