Expression of the gene for large subunit of m-calpain is elevated in skeletal muscle from Duchenne muscular dystrophy patients

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Abstract

Calpain is an intracellular nonlysosomal protease involved in essential regulatory or processing functions of the cell, mediated by physiological concentrations of Ca^{2+}. However, in an environment of abnormal intracellular calcium, such as that seen in Duchenne muscular dystrophy (DMD), calpain is suggested to cause degeneration of muscle owing to enhanced activity. To test whether the reported increase in calpain activity in DMD results from *de novo* synthesis of the protease, we have assessed the quantitative changes in mRNA specific for m-calpain. mRNA isolated from DMD and control muscle was analysed by dot blot hybridization using a cDNA probe for the large subunit of m-calpain. Compared to control a four-fold increase in specific mRNA was observed in dystrophic muscle. This enhanced expression of the m-calpain gene in dystrophic condition suggests that the reported increase in m-calpain activity results from *de novo* synthesis of protease and underlines the important role of m-calpain in DMD.

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Introduction

Calpain is a calcium-dependent nonlysosomal thiol protease widely distributed in cytosol of eukaryotes (Suzuki and Ohno 1990). The enzyme is identified as μ-calpain or m-calpain depending on the requirement of concentration of Ca^{2+}, in μM or mM respectively, for activation (Suzuki *et al.* 1987). Although the precise function of calpain *in vivo* has not yet been clearly identified, the ubiquitous expression of mammalian μ-calpain and m-calpain strongly suggests that they are involved in essential and regulatory processing functions such as proteolytic activation of protein kinase C (Cressman *et al.* 1995), regulation of cell division (Malcov *et al.* 1997), and modulation of brain function (Neumar *et al.* 1996).

On the other hand, calpain, particularly the m-calpain, has been implicated in the pathogenesis of various degenerative disorders, including Duchenne muscular dystrophy (DMD). The gross increase in the intracellular calcium of dystrophic muscle provides an environment for widespread activation of m-calpain (Morandi *et al.* 1990; Dunn and Radda 1991). There are a number of reports on increased m-calpain activity as well as concentration in DMD and mdx mice (Ashmore *et al.* 1986; Kumamoto *et al.* 1995; Spencer *et al.* 1995). Increase in m-calpain activity reported in lymphocytes from Alzheimer's disease is found to be responsible for the proteolysis of amyloid precursor, the abnormal feature in this disease (Karlsson *et al.* 1995). m-Calpain, responsible for cortical cataract formation by proteolysis of crystallin, vimentin and actin, may promote degeneration of optic nerve in optic neurites (Ma *et al.* 1997). m-Calpain has also been implicated in neuronal cytoskeletal protein degeneration in brain traumatic injury (Mansoor *et al.* 1996).

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In addition to the observed increase of m-calpain activity in various disorders, studies employing the quantitative reverse transcriptase polymerase chain reaction demonstrated enhanced expression of the gene for m-calpain in certain pathological conditions such as neurological degenerative disorder and cataract formation (Li *et al.* 1996). The present study has been focussed to evaluate the status of m-calpain gene expression under dystrophic condition in DMD muscle by measuring the quantitative changes in mRNA of the large subunit of m-calpain in DMD muscle using dot blot hydridization.

Materials and methods

Diagnosis of DMD: Diagnosis of DMD in young boys was carried out by clinical examination, electromyogram (EMG), muscle histology and estimation of serum creatine kinase. Histological details of muscle tissue from studied individuals are presented in table 1.

Collection of clinical materials: Part (about 500 mg) of the muscle tissue (vastus lateralis or gastrocnemius muscle) biopsied from DMD patients who were referred for histopathological examination was made available for the study with consent from family members. Similarly skeletal muscle (about 650 mg) was collected, with consent of the respective families, from healthy boys of age five to eight years, who were referred for lumbar amputation or other abdominal surgeries. The present study was approved by the ethical committee constituted by the Nizam's Institute of Medical Sciences.

Isolation of total RNA from DMD and control muscle tissue: All glassware and autoclavable plasticware were treated with a solution of 0.1% diethylpyrocarbonate (DEPC) for 12–14 h at room temperature to denature any RNAases. To remove traces of DEPC, the glassware and plasticware were autoclaved for 20 min at 120°C. Similarly all solutions required were prepared with DEPC-treated autoclaved distilled water. Total RNA from DMD and control muscle tissue was isolated by acid guanidinium phenol chloroform (AGPC) method as described by Chomczynski and Sacchi (1997).

Dot-blotting of RNA onto nitrocellulose membrane: Total RNA isolated from DMD patients and controls was serially diluted (300–2.4 ng) in sterile distilled water and mixed with formamide, formaldehyde and SSC (50 mM NaCl, 15 mM sodium citrate) to give a final concentration of 50%, 7% and 1% respectively. The samples were incubated at 68°C for 15 min and chilled on ice. Two volumes of $20 \times$ SSC were added, and the samples were dot-blotted onto nitrocellulose (NC) membrane using dot-blotting apparatus. The NC membrane was then vacuum baked at 80°C for 2 h.

Preparation of cDNA probe for the m-calpain gene: Competent calcium chloride treated cells of DH5α strain of *E. coli* were transformed with 25 ng of plasmid DNA (pUC8) containing the cDNA insert (kindly gifted by Prof. K. Suzuki, University of Tokyo, Japan). Large-scale isolation of plasmid DNA from transformed cells selected on LB agar containing 50 µg/ml of ampicillin was carried out essentially by the method described by Sambrook *et al.* (1989). Plasmid DNA was purified by Sephadex G-25 column chromatography.

Restriction enzyme digestion of plasmid DNA: Plasmid DNA (5 µg) was incubated with *Eco*RI (25 units) in a 50 µl reaction buffer (90 mM Tris HCl pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$) at 37°C for 4 h. Digested plasmid DNA was electrophoresed on 0.8% low-melting agarose and a 1.2-kb cDNA fragment was purified from the gel.

Random primer labelling of cDNA probe: Twentyfive ng of cDNA probe was denatured by heating at 95–100°C for 2 min followed by cooling on ice. Random primer labelling reaction was carried out in a volume of 50 µl containing 20 µM each of unlabelled dNTPs (dCTP, dGTP, dATP), 25 ng of cDNA probe, 20 µg of nuclease-free BSA, 50 µCi of [$\alpha-^{32}$P]dATP, 5 units of Klenow enzyme, 50 mM Tris HCl, 5 mM $MgCl_2$, 2 mM DTT and 0.2 mM HEPES. The contents were gently mixed and incubated at 37°C for 2 h. Reaction was terminated by heating at 95–100°C for 2 min and subsequent chilling on ice. Unincorporated label was removed by Sephadex G-50 column chromatography.

Prehybridization and hybridization reactions: Vacuum-baked nitrocellulose membrane containing the immobilized RNA was prehybridized for 2 h at 68°C in prehybridization

Table 1. Histopathological details of muscle tissue from three DMD patients.

	Fibre condition	Hyalinized fibres	Regenerating fibres	Contral migration	Interstitial adipose tissue infiltration	Inflammatory infiltration (macrophages)
1.	RF, V, SAF	++	+++	++	++	++
2.	V, RF, LHF	++	−	+	+	+
3.	V, SAF	++	++	++	+++	++

V, Variation in fibre size; SAF, small atrophied fibres; LHF, large atrophied fibres; RF, rounded fibres; +, mild ($< 8\%$); ++, few ($< 15\%$); +++, many (30%).

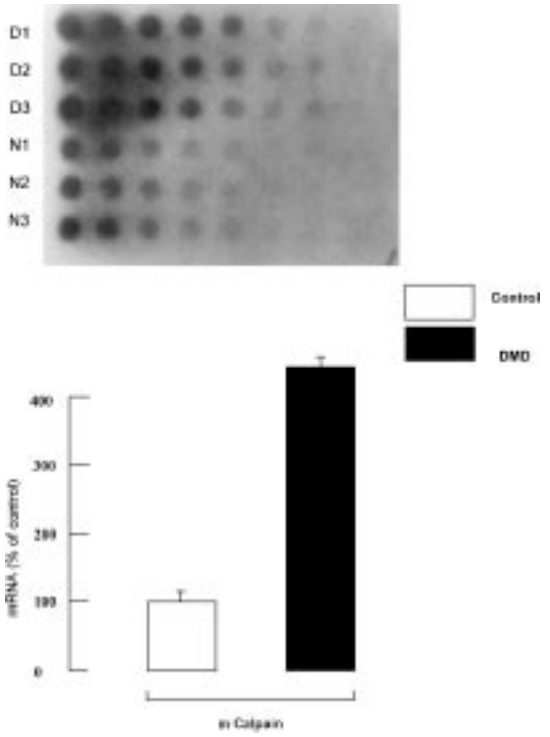

Figure 1. Dot-blot hybridization analysis of m-calpain mRNA in skeletal muscle of DMD patients and controls. Serial dilutions of total RNA (300–2.4 ng) were spotted onto nitrocellulose filter. The lower panel shows results of quantification of mRNA by scanning densitometry, expressed as percentage of controls. The error bars are mean $\pm$ SD for $n = 3$.

solution containing $6 \times$ SSC, $5 \times$ Denhardt's reagent (1% Ficoll, 1% polyvinylpyrrolidone and 1% BSA) and 100 µg/ml denatured, fragmented salmon sperm DNA. Double-stranded cDNA probe was denatured by heating for 2 min at 100°C followed by chilling on ice before adding to the prehybridization solution. Hybridization was carried out for 16 h at 68°C in a water bath. X-ray film was exposed to the blot for 72 h at −7°C and the developed autoradiogram was scanned on a densitometer (Molecular Dynamics, USA) for dosage analysis.

Results

The difference in m-calpain mRNA content in DMD and normal controls was obtained by scanning the autoradiogram of the dot blot. After establishing that the absorbance and RNA concentration were linearly related, the absorbance of blots from DMD was expressed as a percentage of the absorbance of blots from normal controls (figure 1). Proportionate relationship between absorbance and RNA concentration was observed with RNA concentration ranging from 300 to 37.5 ng, which is the range optimal for cDNA hybridization. A four-fold increase in the specific mRNA content was observed in dystrophic muscle compared to normal control (figure 1).

Discussion

Abnormally high concentration of intracellular Ca^{2+} is the characteristic feature of muscle from DMD and mdx mice (Dunn and Radda 1991; Turner *et al.* 1993). This abnormal increase in the concentration of Ca^{2+} has been attributed to the absence of dystrophin (the protein product of the dystrophin gene) causing decreased membrane stability and thus contributing to the increased influx of calcium (Franco and Lansman 1990). Alternatively, if dystrophin directly regulates the Ca^{2+} influx into the cell, in its absence there is enhanced calcium (Moens *et al.* 1993). The relationship between the increase in Ca^{2+} and pathology of dystrophin-deficient muscle suggests that the increased level of Ca^{2+} provides an environment for uncontrolled activation of calpains, particularly the high-calcium-requiring m-calpain (Morandi *et al.* 1990; Dunn and Radda 1991). In addition to increased m-calpain activity in DMD, a number of reports have also demonstrated a quantitative increase in m-calpain (Ashmore *et al.* 1986; Kumamoto *et al.* 1995). Such an increase in calpain activity is also evident from the observation of increased processing function of calpain seen from the calpain-dependent increase in the activities of protein kinase C and Ca^{2+}-ATPase in muscle and nonmuscle cells of DMD (Jagadeesh *et al.* 1990; Kishimoto 1990).

In the present study, the observation of a four-fold increase in muscle m-calpain mRNA levels in DMD, compared to control, clearly shows enhanced expression of the m-calpain gene in dystrophic condition, explaining the enhanced activity of the enzyme as due to its *de novo* synthesis, and underlining its importance in DMD pathology.

Knowledge of the mechanism underlying the regulation of genes for ubiquitously expressed (housekeeping) intracellular proteins is quite limited compared to knowledge of those that are expressed in a tissue-specific manner (Wang *et al.* 1990). Regulation of enzymes at the transcriptional level is usually seen only for enzymes that are needed in special circumstances or at a particular stage of development (Samis *et al.* 1991).

The ubiquitous distribution of the calpains in animal cells lends support to the notion that calpains are housekeeping enzymes (Mathews and Van Holde 1990). On the other hand, a distinct unevenness in the absolute and relative amounts of m-calpain and µ-calpain and large variations in levels of the respective mRNAs among various tissues could be interpreted as an indication of the relative importance of the calpain system in different cells (Emori *et al.* 1986; Murachi 1989).

Treatment of HeLa cells with the tumour-promoting phorbol ester, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), resulted in an enhanced expression of the gene for the large subunit of human m-calpain, suggesting its specific function in response to cellular stimuli (Hata *et al.* 1992).

Study of atrial natriuretic factor mRNA has revealed that the regulation of transcription and translation is mediated by Ca^{2+} ions (LaPointe *et al.* 1990). Similarly m-calpain

expression is markedly enhanced in affected muscle fibres in acute quadriplegic myopathy resulting from an altered calcium homeostasis (Showalter and Engel 1997). These experimental evidences indicated the involvement of Ca^{2+} as a cellular stimulus responsible for enhanced expression of the respective genes. Since enhanced cellular calcium has been the pathologic feature of dystrophic muscle, such a Ca^{2+}-dependent stimulus may possibly be involved in the induction of the m-calpain gene in DMD, thereby contributing to the *de novo* synthesis of m-calpain in DMD.

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