Activation of NF-κΒ pathway in Duchenne muscular dystrophy: relation to age

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Muscle degeneration in Duchenne muscular dystrophy (DMD) is exacerbated by increased oxidative stress and the endogenous inflammatory response, with a key role played by nuclear factor kappa-B (NF-κB) and other related factors such as tumor necrosis factor (TNF-α) and interleukin (IL)-6. However the time course of expression of these molecules and the relation with the amount of necrosis and regeneration have never been investigated.

The expression of NF-κB, the cytokines TNF-α and IL-6 and the antioxidant enzyme glutathione peroxidase (GPx) was studied in muscle samples from 14 patients with DMD aged between 2 and 9 years. Moreover a quantitative morphological evaluation was performed to evaluate necrotic and regenerative areas.

The highest percentage of necrosis was revealed within 4 years of age, with a significant negative correlation with age (p < 0.003), which paralleled to a significant decrement of regenerating area (p < 0.0004). We reported the novel observation that the number of NF-κB positive fibers and the NF-κB DNA-binding activity, revealed by EMSA, are high at two years of life and significantly decline with age (p < 0.0005 and p < 0.0001). The expression of TNF-α, IL-6 and GPx was upregulated in DMD muscles compared to controls and significantly increased with age on real-time PCR analysis (p < 0.0002; p < 0.0005; p < 0.03 respectively) and ELISA (p < 0.002; p < 0.02; p < 0.0001 respectively).

Since anti-inflammatory and anti-oxidant drugs are nowadays being translated to clinical studies in DMD, the reported insights on these therapeutic targets appear relevant. Further studies on the interactions among these pathways in different DMD phases and on the response of these cascades to treatments currently under investigation are needed to better elucidate their relevance as therapeutic targets.

Key words: DMD, NF-κB, TNF-α

Introduction

Nuclear Factor Kappa-B (NF-κB) is a major transcription factor expressed in a wide variety of cells and modulating the cellular immune, inflammatory and proliferative responses (1). In unstimulated cells, NF-κB is inactive via interaction with its inhibitor protein (I-κB). NF-κB activation is regulated by the I-κB kinase (IKK) complex composed of catalytic subunits, IKKα and IKKβ, and a regulatory subunit IKKγ/NEMO (2). Classical stimulatory signals such as proinflammatory cytokines (e.g. tumor necrosis factor [TNF]-α, interleukin [IL]-1β, IL-6) result in IKKβ-mediated site-specific phosphorylation and subsequent degradation of I-κB. Loss of I-κB allows nuclear NF-κB entry and subsequent transcription of a diverse set of genes encoding growth factors, cytokines, chemokines, antiapoptotic proteins and cell adhesion molecules (3). Moreover, NF-κB is an important mediator of redox-responsive gene expression and actively involved in the upregulation of antioxidant enzymes, such as glutathione peroxidase and catalase, in response to oxidative stress (4).

Duchenne muscular dystrophy (DMD) is a progressive muscle wasting disorder leading to loss of ambulation by thirteen years of age (5). The disease is caused by the absence of the protein dystrophin, which results in the loss of muscle membrane integrity. Consequently, this initial damage progresses to myofiber necrosis, phagocytosis, infiltration of inflammatory cells and loss of muscle fibers with subsequent fibrosis and fat replacement leading to impaired muscle function (6).

The mechanisms responsible for the progression from initial membrane damage to pathological hallmarks of the dystrophic process have not been fully identified. There are many evidences that the initial myofiber damage is exacerbated by the endogenous inflammatory response (7-10) and increased oxidative stress (11). Inflammatory cells and cytokines may also further damage the sarcolemma resulting in myofiber necrosis rather than in the repair of minor membrane lesions (8).
Our group demonstrated in DMD muscle immunoreactivity for the activated form of NF-κB in all regenerating fibers and in 20-40% of necrotic fibers. Western blot analysis of nuclear extracts and especially electrophoretic mobility shift assay (EMSA) analysis confirmed activation of NF-κB (12). In the murine model of DMD, the mdx mouse, a skeletal muscle-specific activation of NF-κB has been demonstrated even before the onset of dystrophic damage (13). We have also reported that oxidative stress/lipid peroxidation and NF-κB activation occur in mdx mice and that their inhibition significantly ameliorate functional, morphological and biochemical parameters (14-16).

Nevertheless the NF-κB contribution to dystrophic damage in humans has been poorly investigated (10, 12, 17, 18) and the time-course of its activation remains unstudied. Therefore the aim of this study is to define the NF-κB activation and the NF-κB-related genes expression profiling in different phases of DMD course. This study might also help to choose the most effective time-frame to administer pharmacological modulators of NF-κB activity in future clinical trials.

Materials and methods

We studied vastus lateralis muscle samples from 14 patients with DMD aged between 2 and 9 years. The diagnosis was based on clinical features, muscle biopsy with dystrophin analysis by immunocytochemistry and study of the dystrophin gene. Fourteen muscle samples taken from age-matched normal subjects (2-9 years), undergoing orthopedic surgery, were tested as controls. All individuals or their parents had given informed consent for the scientific use of the muscle biopsy. The Medical School Ethical Committee, University of Messina, authorized the study.

Histological studies

All specimens were frozen in isopentane cooled in liquid nitrogen and stored at –70°C. Transverse cryostat sections (10 μm) were stained with hematoxylin-eosin, and then examined by a blinded observer, using the Axioscope 2.05 image analysis system equipped with Axiocam camera scanner (Zeiss, Munchen, Germany). The following two areas were recognized with intermingled distribution on three different sections: (i) necrotic fibers, identified by pale cytoplasm and phagocytosis; (ii) regenerating fibers, identified by small size, basophilic cytoplasm and central nuclei. The results were expressed as the ratio of the area occupied by necrotic or regenerating fibers divided by the total surface area as a percentage.

Immunocytochemistry

Seven-μm-thick transverse cryostat sections from vastus lateralis muscles were incubated for 120 minutes at 37°C in rabbit polyclonal antibody against phospho-NF-κB p65 subunit (Ser276) (1:50; Cell Signaling Technology, Beverly, MA). It selectively binds to the NF-κB p65 only when phosphorylated at serine 276, ie, it is activated and can then undergo nuclear translocation.

Non-specific binding of immunoglobulin was blocked with 5% normal horse serum. Immunodetection was performed using a biotin-avidin system (Dako, Milan, Italy) followed by horseradish peroxidase staining with 3,3′-diaminobenzidine tetrahydrochloride.

NF-κB DNA-binding activity by electrophoresis mobility shift assay (EMSA)

Isolation of nuclear proteins in approximately 50 mg of frozen muscle was performed according to elsewhere detailed methods (12). Twenty micrograms of nuclear extract were incubated for 30 min at room temperature with 50 fmol of biotin-end-labeled 45-mer double-strand NF-κB oligonucleotide from the HIV long terminal repeat, 5′-TTGGTACAGGAGCCTGCCGCTGGAGCTTCAGGAGCGTG-3′ containing 2 (underlined) NF-κB binding sites. Both complimentary oligos were end-labeled separately and then annealed prior to use. Binding reaction mixtures were prepared in a final volume of 20 μL HEPES buffer containing 1 mg double-strand poly dI/dC, 10% glycerol, 100 mM MgCl2 and 1% Nonidet P-40. The shift was performed by LightShift™ Chemiluminescent EMSA Kit (Pierce, Milan, Italy), according to the manufacturer’s instructions. Competitive assays were also performed by addition of 50-fold excess of unlabeled probe to nuclear extract at room temperature for 10 min before the addition of the labeled probe. Bound complexes were separated on 7.5% nondenaturating polyacrylamide gels, blotted onto nylon membrane and visualized on Kodak X-ray film (Kodak, Milan, Italy) by autoradiography. The results are expressed as relative integrated intensity compared with normal controls and internal positive controls, considering exposure time, background levels, and known protein concentration of an Epstein–Barr virus nuclear antigen-1 extract, with its consensus sequence provided with the Light-Shift Chemiluminescent kit (Pierce), which was used as EMSA control.

RNA isolation and real-time PCR

Total RNA was extracted from each muscle biopsy specimen using TRIzol reagent, followed by DNase I treatment (Invitrogen, Carlsbad, CA). The RNA quality and quantity were checked respectively on agarose gel
and by spectrophotometry. 3 µg of total RNA from each sample was reverse-transcribed by Archive kit (Applied Biosystems, Milan, Italy). Generated cDNA was used as template for real-time quantitative PCR analysis using gene expression products according to the manufacturer’s recommendations. For each real-time PCR reaction, we used 2.5µl of cDNA in a total volume of 50 µl. We performed reactions with a 7300 Sequence Detection System apparatus (Applied Biosystems) to quantitatively compare the mRNA levels. IL-6, glutathione peroxidase (GPx), TNF-α and β-actin (as an endogenous control) assays were obtained from Applied Biosystems. Real time PCR was performed in duplicate with 2X TaqMan Universal PCR Master Mix. The thermal cycling conditions consisted of one cycle each for 2 min at 50°C and 10 min at 95°C, and 40 cycles for 15s at 95°C and 1 min at 60°C. All gene expression levels were normalized to β-actin mRNA level, which was determined simultaneously in the same tube. The comparative cycle threshold (Ct) method was used to analyse the data by generating relative values of the amount of target cDNA. Relative quantification (RQ) for these genes was expressed as fold variation over control, and was calculated by the ΔΔCt method, using control samples as calibrators.

**ELISA**

GPx, TNF-α and IL-6 gene expressions were evaluated for protein expression using ELISA. Muscle levels of GPx, TNF-α and IL-6 were measured using conventional double sandwich ELISA kits from Invitrogen Inc. (Carlsbad, CA). Assays were performed according to the manufacturer’s instructions and expressed as ng or pg/mg of non collagen protein.

**Statistical analysis**

Mann-Whitney U and ANOVA tests (StatView software, version 5.0.1) were used for group comparisons. The relationship between variables was studied using Pearson’s correlation coefficient and Fisher two-tailed test. A level of significance of p < 0.05 was considered.

**Results**

A pathological consequence of the absence of dystrophin from the sarcolemmal membrane is the altered mechanical and signaling functions which contribute to membrane fragility, necrosis, inflammation, and progressive muscle wasting (19). The time course of DMD pathology during early disease phases and the contribution of inflammatory pathways is an understudied area. To better detail these aspects we performed quantitative morphological evaluation of vastus lateralis muscle in patients of different ages. It revealed the highest percentage of necrosis within 4 years of age being the mean value 5.1 (SD ± 1.1), whereas it was 2.7 (SD ± 0.8) in DMD patients between 5 and 9 years (p < 0.001). The percentage of necrosis had a significant negative correlation with age (r: -0.75; p < 0.003; Fig. 1A).

In DMD, the regeneration process is stimulated by muscle necrosis and fails to keep pace with the repeated cycles of degeneration. Therefore, the imbalance between

![Figure 1](image-url)
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Muscle damage and muscle repair leads to a loss of muscle fibers and an increase in the amount of fibrosis. In keeping with this evidence in our experiment, we showed that the decrease of necrosis paralleled to a significant decrement of the percentage of regenerating area with age (r: -0.82; p < 0.0004; Fig. 1 B).

No immunoreactivity for NF-κB was found in muscle specimens from normal controls (Fig. 2 A). In DMD patients NF-κB immunoreactivity was seen in the nuclei of the majority of regenerating fibers seen on serial hematoxylin-eosin sections and also in the nuclei of few apparently normal fibers. NF-κB immunoreactivity was also observed in a low percentage of fibers with features of necrosis and phagocytosis (Fig. 2 B). Following the trend of the regenerating fibers, we showed a significant decrease in the number of NF-κB positive fibers with age (r:-0.81; p < 0.0005; Fig. 2 C).

NF-κB DNA binding activity revealed by EMSA was absent in normal controls and with wide variability among patients ranging from 70 to 15 (integrated intensity, arbitrary units) in relation to the increase in age (r:-0.87; p < 0.0001; Fig. 2 D,E).

We found that the expression of GPx was upregulated in DMD muscles compared to controls and had a significant positive correlation with age on real-time PCR analysis (r: 0.59, p < 0.03, Fig. 3A) and confirmed by ELISA (r: 0.89; p<0.0001, Fig. 4A). Similarly, TNF-α and IL-6 expression was augmented in dystrophic muscles and significantly increased with age as demonstrated with real-time PCR (r: 0.89; p < 0.002 and r: 0.86, p < 0.0005, respectively; Fig. 3B,C) and confirmed by ELISA (r: 0.76; p < 0.002 and r: 0.62; p < 0.02, respectively Fig. 4B,C). Moreover real-time PCR analysis revealed that GPx, TNF-α and IL-6 mRNA levels were increased respectively 97-, 1.8- and 25.9-fold (mean values) in muscles belonging to DMD patients within 4 years of age, whereas 537-, 27.7- and 148-fold (mean values) in DMD patients between 5 and 9 years, compared to control muscles (p < 0.001).

**Figure 2.** Representative images of NF-κB-immunolocalization in control (A) and in DMD muscle (B) scale bar 50 μm, graph showing the correlation between the number of NF-κB-positive fibers expressed as percentage and patients’ age (C). Electrophoretic mobility shift assay of muscular NF-κB binding activity: representative autoradiograms (D) and graph showing the correlation between the NF-κB binding activity expressed as integrated intensity (arbitrary units) and patients’ age (E).
Membrane defects and mechanical injury are important factors promoting dystrophic disease pathology, but they do not fully explain DMD disease onset and progression (20, 21). Aberrant intracellular signaling cascades that regulate both inflammatory and immune processes contribute substantially to the degenerative process (10, 13, 22, 23). Our group has extensively investigated the mechanisms underlying the dystrophic process in mdx mice, demonstrating that a cross-talk between oxidative stress/lipid peroxidation and NF-κB activation occurs, in turn triggering inflammatory cascades involving TNF-α, IL-1β, mitogen-activated protein kinases (MAPKs) and cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) and contributing to muscle damage. Finally modulation of these cascades, obtained through NF-κB inhibitors, led to a significant decrease of muscle necrosis and increase of regeneration, improving mdx mice functional performances (14-16). Several other studies supported our results showing that NF-κB inhibition, through IKKβ depletion (24) or ablation of 1 allele of the p65 NF-κB subunit (17) improved muscle force, decreased necrosis and facilitated muscle regeneration in mdx mice and after muscle damage. Furthermore, specific pharmacological inhibition of IKK also resulted in improved muscle function and pathology in mdx mice (17).

NF-κB signalling pathway has been shown to be aberrantly activated in DMD muscle during early disease stages (17, 25). However the time course of NF-κB expression over time and the relation with the amount of necrosis and regeneration have never been investigated. In our study we reported the novel observation that in DMD patients the number of NF-κB positive fibers and the NF-κB

**Figure 3.** Real-time PCR: graphs showing the correlation between GPx (A), TNF-α (B) and IL-6 (C) expression, expressed as fold change compared to controls, and patients’ age.
kappa B DNA-binding activity are high at two years of life and significantly decline with age, being very low by the 9th year of age. The decrement in NF-kappa B DNA-binding activity in muscle was very pronounced with a reduction of nearly 80% between patients of 2 and 9 years of age.

The same trend was monitored in the percentage of necrotic fibers that was around 6% at 2 years of age and significantly declined up to 2-3% at 9 years. This is in keeping with previously reported morphological data showing a typical dystrophic pattern in DMD patients since few months of age and a subsequent age-related decline (26).

Moreover, it has been reported that the most evident presence of infiltrating mononuclear cells occurs between the age of 2-8 years (27, 28) and the peak of mast cell infiltration, implicated in the initiation and progression of muscle lesions, takes place by the age of 3 years (29).

As expected the decrement of necrosis was mirrored by a decrease of regeneration over time. This could be partially explained by the diminished inflammatory and necrotic stimuli, but also by the exhaustion of the regenerative spurt leading to connective and adipose tissue replacement.

It has been pointed out that numerous components of chronic inflammatory response, such as TNF-alpha, IL-1beta, IL-6, modulated by NF-kappa B, are overexpressed in dystrophic muscle (7, 16, 23, 30) and their increased expression precedes the onset of dystrophic changes (13). TNF-alpha levels have been shown to be approximately 1,000 times higher in serum of DMD patients than controls (31). Several lines of evidence suggested TNF-alpha role in promoting muscle wasting, and its inhibition has been demonstrated to have beneficial effects in mdx mice (7, 14-16, 30). In our study, we confirmed previous findings of a marked enhancement of TNF-alpha and IL-6 expression in DMD muscle compared to controls and showed their increase with age. Although oxidative stress, TNF-alpha and IL-6 are
known to be potent NF-κB inducers, in our study the expression of NF-κB tended to diminish over time. Whereas downregulation of NF-κB signaling could be explained by the decrease of regenerating and necrotic fibers with age that are replaced by adipose and connective tissue, the increase of TNF-α and IL-6 in older DMD muscles could account for the amount of cytochines produced by adipocytes (32, 33).

Although the role of oxidative stress in contributing to dystrophic damage has been extensively investigated, controversy still remains concerning whether oxidative stress is a necessary precondition for death of dystrophic muscle or is a consequence of pathology that does not advance the disease (34). Several studies focused on the levels of oxidative stress markers in dystrophic muscle (34), but a time-course of these parameters in DMD also in relation to muscle pathology has never been defined. In this study we confirmed the increase of oxidative stress in dystrophic muscles compared to controls as proved by the augmented expression of GPx, most likely in a compensatory attempt to counterbalance it, and demonstrated for the first time its increase with age despite the decrement of muscle necrosis.

Since anti-inflammatory and anti-oxidant drugs are nowadays being translated to clinical studies in DMD, the definition of the time-course expression of these therapeutic targets is crucial.

Our group has recently started a pilot trial with flavocoxid, a NF-κB inhibitor shown to be effective in mdx mice (16), in young ambulant DMD patients and the data obtained in the present study have been of help to define in 4-6 years range the more effective time frame for treatment administration.

Further studies on the interactions among these pathways in different DMD phases and on the response of these cascades to treatments currently under investigation are needed to better elucidate their relevance as therapeutic targets.

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