Cytokines and growth factors in Duchene muscular dystrophy patients

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ABSTRACT

Introduction: Dystrophin deficiency associated with Duchene muscular dystrophy (DMD) results in chronic inflammation and severe skeletal muscle degeneration, where the extent of muscle fibrosis contributes to disease severity. The microenvironment of dystrophic muscles is associated with variation in levels of cytokine and growth factors. Most of the current researches test for such cytokines and growth factors in tissue biopsies, which is an invasive technique.

The Aim: Of the present study is to investigate whether cytokines and growth factors, as indicators of inflammatory response, can be detected in blood of DMD patients as non-invasive technique.

Patient and Methods: Accordingly the cytokine tumor necrosis factor alfa (TNF TNF-α), as well as the growth factors basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) were measured in blood of 24 boys with DMD diagnosed clinically and at the molecular level versus 20 age matching healthy boys.

Results: Showed a significant increases in TNF-α (30.2 ± 9.5 vs. 3.6 ± 0.9 pg/ml) and bFGF (21.7 ± 10.3 vs. 4.75 ± 2.2 pg/ml.), while VEGF was significantly decreased (190 ± 115 vs. 210 ± 142 pg/ml) in blood of DMD patients compared to controls.

Conclusion: Results provide further proof that inflammatory response is associated with DMD pathogenesis and favours the use of biomarkers in blood of such patients as a non invasive technique.

Key Words: (basic fibroblast growth factor (bFGF), duchene muscular dystrophy, necrosis factor alfa (TNF TNF-α), vascular endothelial growth factor (VEGF)).

INTRODUCTION

DMD is an X-linked recessive disorder, primarily characterized by progressive muscle weakness and waisting. Mutations in dystrophin gene are the prime
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cause for muscle degeneration associated with DMD. Normally dystrophin interacts with several members of the dystrophin glycoprotein complex, which forms a mechanical as well as signaling link from the extracellular matrix to the cytoskeleton. Mutations in dystrophin result in membrane damage, allowing massive infiltration of immune cells, chronic inflammation, necrosis, and severe muscle degeneration. Normally, muscle cells possess the capacity to regenerate in response to injury signals, however, this ability is lost in DMD, presumably due to an exhaustion of satellite cells during ongoing degeneration and regeneration cycles. Although dystrophin mutations represent the primary cause of DMD, it is the secondary processes involving persistent inflammation and impaired regeneration that likely exacerbate disease progression. This results in chronic inflammation and severe skeletal muscle degeneration, where the extent of muscle fibrosis contributes to disease severity. Elevated numbers of inflammatory cells are known to be present at the sites of muscle injuries and interact together for cytokine and growth factors signaling.

TNF-α is an early and potent pro-inflammatory cytokine that stimulates the inflammatory response. Even minor trauma to muscle will increase levels of TNF-α by release from mast cells; TNF-α is also produced by neutrophils, macrophages and lymphocytes that accumulate rapidly at the site of injury. TNF-α increases rapidly within damaged myofibers and is expressed by myoblasts and myotubes. TNF-α is greatly elevated in injured normal damaged myofibers and myopathic skeletal muscle; it is chemotactic for myoblasts and is mitogenic for satellite cells in vivo, suggesting a direct role in myogenesis of regenerating muscle.

Muscle tissue repair is a complex biological process that crucially involves activation of stem cells. Skeletal muscle contains two different stem cell types: 1) myogenic stem cells, so-called satellite cells (SCs), that reside beneath the basal lamina of muscle fibers; 2) interstitial multipotent stem cells, which are extra-laminal, exhibit fibroblastic morphology and do not express myogenic markers. SCs represent the pre-eminent muscle stem cell type used for muscle growth, repair and regeneration. They are primarily quiescent in skeletal muscle, can self-renew and upon activation, proliferate and further differentiate to become fusion-competent myoblasts and ensure muscle regeneration. Interstitial “muscle-derived” stem cells give rise to several lineages after transplantation and in this setting, contribute to synchronized reconstitution of blood vessels (pericytes, smooth muscle cells [SMCs], and endothelial cells), peripheral nerve (Schwann cells), and muscle cells (myofibers and SCs). However, participation of multipotent interstitial stem cells in physiological muscle repair appears to be limited.

Satellite cells could excrete growth factors including VEGF that would induce angiogenesis and improve cell survival. The VEGF is the prototypic member of a family of secreted, homodimeric glycoproteins with endothelial cell-specific mitogenic activity and the ability to stimulate angiogenesis in vivo. On the other hand, a number of growth factors such as fibroblast growth factor (FGF) can promote the activation and proliferation of skeletal satellite cells.
Basic FGF contributes to promote proliferation and to inhibit differentiation of skeletal muscles.\textsuperscript{22}

The aim of the present study is to investigate levels of cytokines (TNF-α) and growth factors (VEGF and bFGF) involved in muscle regeneration in blood of DMD patients compared to controls.

\textbf{PATIENT AND METHODS}

Subjects were\textsuperscript{23} DMD boys diagnosed clinically and at the molecular level (mean age (8.1 ± 1.9) years versus 20 age and socioeconomic matching healthy boys (mean of age 8.2 ± 2.2). Patients and controls were chosen to be free from any infection and receiving no therapeutic treatment known to increase the oxidative stress. Blood samples were drawn after their parents’ consent.

Plasma analysis was performed for cytokine (TNF-α) using elisa/PCR (24), growth factor VEGF using the ACCUCYTE Human VEGF immunoassay kit\textsuperscript{24} and bFGF using human bFGF immunoabsorbant assay (ELIZA) Quantitin kit.\textsuperscript{25}

\textbf{Statistical Analysis:}

Each experimental condition was performed and expressed as mean ± SD. Comparisons were made by Student’s t-test (two-tailed for independent samples).

\textbf{RESULTS}

TNF and bFGF were significantly higher in Blood of DMD patients compared to controls (Table 1, Figures 1 & 2) TNF was (30.2 ± 9.5 vs. 3.6 ± 0.9 pg/ml) and bFGF was (21.7 ± 10.3 vs. 4.75 ± 2.2).

In contrast, VEGF significantly lower in blood of DMD patients compared to controls. (190 ± 115 vs. 210 ± 142).

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<th>TNF pg/ml</th>
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<th>VEGF pg/ml</th>
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<tr>
<td>DMD Patients</td>
<td>30.2 ± 9.5</td>
<td>21.7 ± 10.3</td>
<td>190 ± 115</td>
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<tr>
<td>Controls</td>
<td>3.6 ± 0.9</td>
<td>4.75 ± 2.2</td>
<td>210 ± 142</td>
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Fig. 1: TNF, and bFGF in Blood of DMD patients compared to controls.

Fig. 2: VEGF in Blood of DMD Patients Compared to Controls.
DISCUSSION

In normal skeletal muscles, damage due to contractile force is followed by an inflammatory response involving multiple cell types that subsides after several days. This transient inflammatory response is a normal homeostatic reaction to muscle damage that induces muscle repair. However in DMD patients a persistent inflammatory response in their skeletal muscles leads to an altered extracellular environment, including an increased presence of inflammatory cells (e.g., macrophages) and elevated levels of various inflammatory cytokines and growth factors. Unfortunately, the signals that lead to successful muscle repair in healthy muscle may promote muscle waisting and fibrosis in dystrophic muscles\textsuperscript{26}. Our results point to increase in inflammatory cytokines TNF-\(\alpha\) and growth factor bFGF in blood of DMD patients, while VEGF exhibited a significant decrease compared to controls.

TNF-\(\alpha\) is an important mediator of inflammatory and autoimmune diseases. It was reported that the mean serum TNF-\(\alpha\)concentration in Duchenne muscular dystrophy patients was approximately 1,000 times higher than that in healthy subjects\textsuperscript{10} and that TNF-\(\alpha\) levels are upregulated in dystrophic muscles from animal models and DMD patients\textsuperscript{4,6}. Among its pleiotropic effects, TNF-\(\alpha\) acts as a potent inducer of the inflammatory response transcription factor NF-\(\kappa\)B\textsuperscript{27,28}. Although dystrophin mutations represent the primary cause of DMD, it is the secondary processes involving persistent inflammation and impaired regeneration that likely exacerbate disease progression. The microenvironment of dystrophic muscles consists of elevated numbers of inflammatory cells that act as a complex interface for cytokine signaling\textsuperscript{4,6}. It is emphasized that TNF contribute to myofibre necrosis, where antibody depletion of host neutrophils resulted in a delayed and significantly reduced amount of skeletal muscle breakdown in young dystrophic mdx mice\textsuperscript{29}.

Results of the present study showed increased levels of bFGF compared to controls. Growth factors, represent essential elements in the modulation of muscle cell regeneration and differentiation\textsuperscript{4}. Interestingly, many growth factors, including basic fibroblast growth factor (bFGF), has been shown to be upregulated in mdx mice\textsuperscript{30} and serum levels has been shown to increase in DMD patients compared to controls\textsuperscript{31}. However, bFGF role in DMD pathogenesis is still unclear. Moreover, disruption of muscle membrane repair machinery results in progressive muscular dystrophy in the presence of a functional dystrophin glycogen\textsuperscript{32}. It is suggested that DMD lack dystrophin and as a result their skeletal muscles show extensive muscle fiber damage and fibrosis, and regeneration\textsuperscript{33}. This is also supported by data obtained from experiments carried out on mdx mice, which are larger than normal mice. Mdx have been noted to show larger limb muscles than normal mice; however, the larger muscles of mdx mice are actually weaker than those of normal mice due to extensive fibrosis and the lower potential for force generation by newly regenerated fibers\textsuperscript{34,35}. Mdx mice have a large number of degenerating and regenerative muscle fibers in the first 4 months of life, after which the number of degenerative and necrotic fibers declines\textsuperscript{35}.

In the present study VEGF was significantly lower in DMD patients compared
Exercise is known to increase muscle VEGF mRNA and DMD patients usually have limited physical activity, which can explain the lower level of VEGF in our DMD patients. Data obtained from comparative studies on young and ageing muscle and on exercised and sedentary muscles, indicated that in aged compared with young men, muscle capillary contacts and capillary-to-fiber perimeter exchange index were lower and that VEGF muscle protein decreases with ageing. Replicative aging of myogenic cells (satellite cells), owing to enhanced myofiber turnover, is an accepted common explanation of the progression of DMD pathogenesis. Studies supporting our finding can be obtained from a previous study that showed that intramuscular delivery of VEGF using recombinant adeno-associated virus (rAAV) vectors in mdx mice induced an increased forelimb strength and strength normalized to weight. Another example supporting replicative ageing theory is that mdx mice transplant injected with muscle precursor cells into tibialis exhibited Graft success. This was evaluated as the percentage of improvement of hybrid fibers by 1.9-fold after swimming 3 times per week during 4 weeks. It was suggested that exercise induced fiber breaks, which improved muscle precursor cells recruitment and fusion and increased long-term graft success and also transverse and longitudinal distribution of hybrid fibers.

CONCLUSION

Growth factors and cytokines are associated with DMD pathogenesis, where TNF, bFGF and VEGF can give a reflection of the severity of DMD pathology. Detecting such growth factors and cytokines biomarkers in blood of DMD patients represents for the first time a non invasive technique compared to the invasive technique of muscle biopsy previously used.

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