Short Communication

X-Linked Vacuolated Myopathy

TNF- α and IFN- γ Expression in Muscle Fibers with MHC Class I on Sarcolemma

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The presence and the distribution of tumor necrosis factor- α , interferon- γ , and p65 subunit of nuclear factor- κ B, molecules known to induce synergistically and to mediate major histocompatibility complex (MHC) class I expression, were determined in muscle sections from control and X-linked vacuolated myopathy patients. MHC class I colocalized with tumor necrosis factor- α and interferon- γ , as well as with p65, in most of the membrane attack complex- and/or calcium-positive muscle fibers in X-linked vacuolated myopathy. These results suggest that the expression of MHC class I in X-linked vacuolated myopathy could be induced by tumor necrosis factor- α and interferon- γ and partly mediated by nuclear factor- κ B. (*Am J Pathol* 2001, 158:355–359)

X-linked vacuolated myopathy (XLVM) was first described by Kalimo et al and is characterized by predominant proximal muscle involvement, juvenile onset, and slow progression, without cardiac or intellectual impairment.¹ We have described a French family presenting with similar clinical and histopathological features. The role of complement in the pathogenesis of this muscular disease was emphasized by the deposition of the complement C5b-9 membrane attack complex (MAC) over all histologically abnormal muscle fibers,² and by the elevation of the complement components C5 and C9 in the sera of the patients.³ An accumulation of calcium was also demonstrated on the sarcolemma of muscle fibers in XLVM.⁴

We have demonstrated MHC I expression in XLVM,² but the mechanism underlying this expression was not clearly understood. It is known that interferon (IFN)- γ especially, but also tumor necrosis factor (TNF)- α , induce upregulation of major histocompatibility complex (MHC) I and II molecules in cultured muscle cells, whereas TNF- β , interleukin (IL)-2, IL-1 α , and IL-1 β displayed no such induction.^{5,6} However, IFN- γ and TNF- α are absent most of the time in the biopsy samples of patients with inflammatory myopathies, for example.

MHC class I gene expression is synergistically induced by the cytokines TNF- α and IFN- γ . A portion of this cooperativity is mediated by synergism between two distinct transcription factors, nuclear factor (NF)- κ B and signal transducer and activator of transcription-1 (STAT-1).⁷ TNF- α induces binding of NF- κ B subunits p50 and p65 to the NF- κ B-like element of the MHC class I promoter, whereas IFN- γ induces the expression of STAT-1.⁷ Furthermore, IFN- γ , which typically does not activate NF- κ B, synergistically can enhance TNF- α -induced NF- κ B nuclear translocation via a mechanism that involves the induced degradation of I κ B- α .⁸ Thus, NF- κ B, involved in the inducible regulation of a large number of genes, appears to participate in the control of MHC class I genes' basal expression as well as in their transcriptional upregulation after treatment by TNF- α and IFN- γ .⁹

The goals of the present work were (i) to characterize the putative expression of TNF- α and IFN- γ in muscle fibers of patients with XLVM and the possible colocalization of these cytokines in muscle fibers exhibiting MHC class I, and (ii) to study the putative immunolocalization of

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NF- κ B and its relationship with TNF- α , IFN- γ , and MHC class I in XLVM.

Materials and Methods

Muscle Biopsy

Skeletal muscle biopsy samples were from deltoid muscles of patients presenting with XLVM ($n = 5$) originating from three French families and from deltoid muscles from nonweak control subjects ($n = 5$) obtained during surgical intervention. The patients with XLVM presented with the symptomatology previously reported.² All of these patients fulfilled the pathological criteria for the diagnosis of XLVM (vacuoles in muscle fibers, deposition of MAC in the sarcolemma, and deposition of calcium on the membrane of muscle fibers). We also used biopsy samples of patients diagnosed with acid maltase deficiency, a myopathy with vacuoles in the muscle fibers, as a control. The biopsy specimens of patients with XLVM and acid maltase deficiency were considered as "pre-existing pathological specimens" obtained for diagnostic purposes and did not require informed consent. Informed consent was obtained from control subjects, and the study was approved by the Ethical Committee of the institution.

Antibodies

For immunocytochemistry, we used antibodies anti-developmental myosin heavy chain (d-MHC), anti-slow myosin heavy chain (s-MHC), anti-fast myosin heavy chain (f-MHC) (Novocastra, Newcastle upon Tyne, UK), anti-MAC C5b-C9 (Dako, Carpinteria, CA), anti-p65 subunit of NF- κ B, anti-TNF- α , and anti-IFN- γ (Santa Cruz Biotechnology, Santa Cruz, CA). All antibodies were used according to the recommendations of the manufacturers.

Morphological Analysis, Histochemistry, and Immunocytochemistry

Muscle specimens were frozen in isopentane cooled in liquid nitrogen. Transverse cryostat sections (10 μ m thick, Frigocut 2800; Reichert-Jung, Nussloch, Germany) were stained by hematoxylin and eosin. Calcium staining and immunocytochemistry were performed as previously reported on 10- μ m-thick serial cryostat sections.^{2,4} For immunocytochemistry, negative controls consisted of omission of the primary antibody, preincubation with PBS, and substitution of non-immune isotype-matched control antibody for primary antibody. Mouse IgG1 and IgG2a (Dako), rabbit IgG (Dako), and goat IgG (Dako) were used as controls. The three types of controls mentioned above were performed for each experiment. p65-, cytokines (IFN- γ , TNF- α)-positive fibers were counted in cross-sections of muscle appropriately stained and the number of each was calculated as a percentage of at least 300 fibers randomly chosen from five different re-

gions within a section of the muscle using an image analyzer (Phase Three Imaging Systems, PA).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from muscle samples stored at -80°C using RNA TRI reagent (Gibco BRL) as instructed by the manufacturer. First strand cDNA synthesis for RT-PCR for cytokines was primed from 1 μ g total RNA with 50 ng random primer under the conditions suggested by the manufacturer (Qiagen Inc., Chatsworth, CA). PCR was performed with cytokine-specific primers (Clontech, Palo Alto, CA) according to the manufacturer's instructions. PCR conditions were 35 cycles at 94°C for 30 seconds, 55°C for 1 minute, and 72°C for 1 minute, followed by a final extension at 72°C for 10 minutes. The sequences of the primers for TNF- α were, for the sense primer, cgggaccgtggagctggcgaggag and, for the antisense primer, caccagctggtatctctcagctc. For IFN- γ , the sequences of the primers were for the sense primer, atgaaatatacaagtatatcttgctt and, for the antisense primer, gatggtcttcgacctcgaacagcat. The sequences of the primers for β -actin were for the sense primer, tgacggggtcaccacactgtgccatcta and, for the antisense primer, ctagaagcatttgcggtggacgatggaggg. According to personal unpublished data and previous results¹⁰ showing a strong expression of β -actin gene expression in muscles, only 1/20th of the cDNA that was used for amplifying cytokine cDNAs was used to amplify β -actin cDNA.

Results

NF- κ B Is Overexpressed in the Cytoplasm of Muscle Fibers in XLVM

Figure 1A shows that the immunoreactivity of the p65 subunit of NF- κ B was faint in the muscle of normal subjects or in muscle of patients with acid maltase deficiency (not shown) compared to patients with XLVM. In XLVM, p65-positive fibers could be small or normal-sized fibers, either vacuolated or not. No immunoreactivity for p65 was observed in the vessels and in the conjunctive tissue. Regenerating muscle fibers are rare in XLVM,² and p65-positive fibers were rarely d-MHC-positive fibers ($<1\%$ of the p65-positive fibers), but mostly f-MHC-positive fibers (not shown). The percentage of p65-positive fibers in XLVM is figured in Table 1. No staining was seen after substitution of primary antibody by non-immune isotype-matched control antibody, or after omission of the primary antibody.

TNF- α and IFN- γ Are Expressed in Muscle Fibers of Patients with XLVM

The percentages of TNF- α - and IFN- γ -positive fibers are figured in Table 1. Figure 1B shows that TNF- α and IFN- γ were immunolocalized in muscle fibers of patients with

Figure 1. A: Transverse serial cryostat sections (10 μ m thick) of a deltoid muscle from control (**left column**) and from a patient with XLVM (**right column**) stained by H&E (**upper row**) and immunostained by antibody anti-p65 (**lower row**). Muscle fibers from control patient exhibited faint immunoreactivity for p65 whereas a cytoplasmic staining for p65 was observed in XLVM muscle fibers. No immunostaining of connective tissue or vessels was seen in XLVM. **B:** Transverse serial cryostat sections (10 μ m thick) of a deltoid muscle from a XLVM patient stained by H&E and immunostained by antibodies anti-MAC, TNF- α , IFN- γ , p65. **Left upper, middle, and lower rows** show MAC-positive fibers which were TNF- α -, IFN- γ -, and p65-positive. **Right lower row** shows MAC-positive fibers that were p65-positive. **C:** Results of RT-PCR for TNF- α , IFN- γ , and β -actin mRNA expressions in muscle samples from control patient (**lane 1**) and patients with XLVM (**lanes 2 and 3**). TNF- α mRNA and IFN- γ mRNA expressions were observed in muscle samples from patients with XLVM but not in muscle sample from a control patient.

Table 1. Muscle Biopsy Results for XLVM Patients

Patient	p65-positive fibers (%)	TNF- α -positive fibers (%)	IFN- γ -positive fibers (%)
1	54	55	54
2	49	52	51
3	35	37	40
4	45	47	44
5	48	50	49

XLVM, and that these cytokines were most of the time colocalized in muscle fibers exhibiting a deposition of MAC on the sarcolemma. About 89% of TNF- α -positive fibers were IFN- γ -positive. p65 was commonly coimmunocolocalized with IFN- γ and TNF- α in MAC-positive muscle fibers. Respectively, 92% of TNF- α -positive fibers and 88% of IFN- γ -positive-fibers were p65-positive. No expression of TNF- α and IFN- γ was observed in the muscle biopsies of control subjects or in the muscle biopsies of patients with acid maltase deficiency. Substitution of non-immune isotype-matched control antibody for primary antibody resulted in no staining, as well as omission of primary antibody. Figure 1C shows the expression of TNF- α mRNA and IFN- γ mRNA by RT-PCR in patients with XLVM, whereas no expression of the mRNA of these cytokines was observed in normal muscles.

MHC I in Muscle Fibers of XLVM Patients Is Coimmunocolocalized with TNF- α and/or IFN- γ

We have previously shown that the MAC-positive fibers exhibited a staining of the cell surface membrane with the antibody against the MHC class I antigen.² Figure 2 shows that MHC I immunostaining colocalized with MAC and calcium deposition in XLVM. About 84% of MHC class I-positive fibers were p65-positive fibers, whereas not all p65-positive fibers were MHC class I-positive. Most of the MHC class I-positive fibers exhibited immunoreactivity for TNF- α or IFN- γ (91 and 88%, respectively).

Discussion

MHC Class I Expression in XLVM

The class I MHC is not found in normal muscle fibers.¹¹ Several studies described expression of MHC I and II molecules in inflammatory myopathies and muscular dystrophies.^{12–16} However, the relationship between MHC class I expression and cytokines known to induce it has never been clearly demonstrated. In fact, if various cytokines and chemokines have been found in muscle biopsies of inflammatory myopathies and Duchenne muscular dystrophy (DMD) by a variety of techniques,¹⁰ the expression of cytokines by muscle fibers themselves was rarely demonstrated *in situ* in muscle disorders.¹² Because there are very few inflammatory cells in XLVM,² it could be suggested that TNF- α mRNA and IFN- γ mRNA expressions observed on RT-PCR are due to the expression of the mRNA of these cytokines by the muscle fibers

Figure 2. Transverse serial cryostat sections (10 μm thick) of a deltoid muscle from XLVM patient stained by Alizarin Red S (RA; **rows 1 and 3, right**), immunostained by antibody anti-MAC (**rows 1 and 3, left**), double-immunostained by antibodies anti-MHC class I, anti-p65, anti-IFN-γ. **A:** MHC class I-positive fibers were mostly p65-positive fibers. Not all of the p65-positive fibers were MHC class I-positive. MHC class I-positive fibers were mostly MAC- and/or calcium-positive fibers. **B:** MHC class I-positive fibers were most of the time IFN-γ-positive fibers.

themselves and not by inflammatory cells, in contrast with inflammatory myopathies, for example.¹⁰

We previously showed an expression of MHC class I in XLVM.² In the present paper, we showed that MHC I expression on the sarcolemma colocalized with TNF-α and IFN-γ expression in the cytoplasm of MAC- and/or calcium-positive fibers. To our knowledge, it is the first time that such colocalization has been demonstrated in muscle fibers. The colocalization of IFN-γ and TNF-α in the same muscle fibers expressing MHC class I could suggest that they act synergistically to induce MHC class I expression.

Expression of p65 in XLVM

NF-κB regulates the expression of many genes involved in immune and inflammatory responses, as well as in cell

proliferation and growth: MHC class I genes, immunoglobulins, κ light chains, IL-2 and its receptor, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), iNOS, interferon-β, T cell receptor β chain.⁹

The role of NF-κB in muscle biology is not clear yet. During muscle development, NF-κB is required for membrane fusion of chicken embryonic myoblasts, and down-regulation of transcription factors activator protein-1, Sp-1, and NF-κB precedes myocyte differentiation.¹⁷ It has also been shown that cytokines such as TNF-α proved to be potent inducers of transient NF-κB activation in myoblasts.¹⁸ Abnormalities of IκB/NFκB pathway have rarely been reported in muscle disorders.¹⁹

Illa et al demonstrated high expression of the transcription factor STAT-1 in many perifascicular atrophic muscle fibers from dermatomyositis patients.²⁰ As we observed it with p65 in XLVM, the localization was cytoplasmic and not nuclear. STAT-1 is involved in cell response to IFN-γ. *In vitro*, STAT-1 was stimulated in human myotubes by IFN-γ.²⁰

Comparing XLVM and DMD, we observed that the main differences between them were the lower percentage of TNF-α-positive muscle fibers in DMD (<5%) compared to XLVM (~50%) and the expression of p65 in regenerating (dMHC-positive) and in some fast-twitch (f-MHC-positive) muscle fibers in DMD, whereas this expression was seen only in fast-twitch muscle fibers in XLVM (manuscript in preparation).

Role of MAC Deposition

Sublytic concentrations of MAC have been shown to induce endothelial IL-8 and MCP-1 through NF-κB activation and, in this way, it has been proposed that MAC could be considered as an important mediator of proinflammatory genes expression via NF-κB activation.²¹

If the present data could suggest that MAC deposition on the muscle sarcolemma could activate NF-κB, the relationship between MAC deposition and expression of IFN-γ, for example, is not clear. It is known that a loop exists between NF-κB and TNF-α, but nothing similar has been reported between NF-κB and IFN-γ.

In XLVM, we demonstrated a colocalization of p65, IFN-γ, and TNF-α in MAC-positive muscle fibers and a colocalization of MHC I, IFN-γ, and TNF-α in MAC- and/or calcium-positive muscle fibers. These results could suggest that in XLVM, IFN-γ, and TNF-α induce MHC I expression, partly via NF-κB activation. However, the role of other transcription factors in the expression of MHC class I in XLVM is currently under investigation.

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