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ORIGINAL ARTICLE

Abnormal expression of myosin heavy chains in early postnatal stages of spinal muscular atrophy type I at single fibre level

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Objective. We investigated myosin heavy chain (MyHC) isoform expression at early postnatal stages of clinically and genetically confirmed spinal muscular atrophy type 1 (SMA1) patients, in order to study the muscle fibre differentiation compared to age-matched controls at single fibre level.
Methods. Open skeletal muscle biopsies were performed from the quadriceps muscle in four SMA1 patients and three age-matched controls. Standard techniques were used for immunohistochemistry of embryonic and foetal MyHCs. Type I, IIa and IIx MyHCs were assessed by applying quadruple immunofluorescence. Western blot was performed to analyse the amount of survival motor neuron (SMN) protein in the muscle samples.
Results. There were profound and early alterations in MyHC expression from 7 days of life compared to age-matched controls. The expression of type IIx MyHC was completely lost in SMA1 and instead developmental isoforms remained highly expressed. Foetal MyHC was still, at 3.5 months of age, expressed in the majority of muscle fibres in SMA1 patients, whereas it was completely downregulated in age-matched controls. The level of SMN protein was reduced in all SMN1 patients.
Conclusions. The abnormal pattern of MyHC expression in postnatal stages of SMA1 was observed early in the newborn period, which may have implications for the effects of gene therapy, since there are clear clinical benefits from early treatment. Whether such aberrant and delayed expression of MyHCs can be completely restored by postnatal gene therapy remains to be studied and may also have implications for new phenotypes that will evolve with new therapies.

Key words: spinal muscular atrophy, SMA, myosin, MyHC isoforms, single fibre

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Introduction

Investigating the myosin heavy chain (MyHC) isoform expression in order to study the muscle fibre type composition has become increasingly important in the pathological assessment of muscle biopsies [1,2]. In addition to the three major adult isoforms, i.e. slow/beta cardiac or type I, type IIa and type IIx MyHC, there is also expression of two developmentally regulated MyHC isoforms i.e. embryonic (developmental) foetal (perinatal/neonatal) MyHC [3].

In extrafusal limb muscle fibres, embryonic MyHC is completely downregulated around birth, whereas foetal MyHC is completely downregulated at 4-6 months of age [4]. In neuromuscular diseases there is often an abnormal pattern and prolonged expression of developmentally regulated MyHCs. Further investigation of these may help to elucidate disease mechanisms and allow development of diagnostic biomarkers.

In the current era, with the development of new therapies for neuromuscular diseases,

such as spinal muscular atrophy (SMA), new phenotypes are developing making it important to understand the natural course of the findings seen in a muscle biopsy. This study describes the expression of adult MyHC isoforms and developmentally regulated MyHCs in muscle biopsy specimens of four patients with clinically and genetically defined spinal muscular atrophy type I (SMA1) aged between 7 days and 3.5 months and three age-matched controls between 5 days and 4 months of age to investigate the expression and co-expression of MyHC isoforms at single fibre level.

Materials and methods

Patients

Muscle samples from four patients with genetically and clinically defined SMA1 were selected for investigation (Tab. I) [5]. SMN1 and SMN2 copy numbers were determined by multiplex ligation-dependent probe amplification (MLPA) for patient 1-4 (SALSA MLPA kit P021-B1 SMA, MRC Holland). Genome sequencing was performed to search for small SNVs in P1.

Skeletal muscle controls included individuals with normal muscle biopsies who had been investigated for a possible mitochondrial disease, but in whom the clinical, biochemical and pathological investigations excluded muscle disease.

Western blot analyses of survival motor neuron (SMN) protein

Western blot analysis of SMN protein was performed on proteins extracted from muscle tissue. Protein extracts were loaded and separated by SDS-PAGE, transferred to a PVDF membrane and probed with anti-SMN (610647, BD Transduction Laboratories, 1:2500). Horseradish peroxidase-conjugated secondary antibody and Super-Signal West Femto substrate (Thermo Scientific) were used for visualization and detection by chemiluminescence using Fujifilm LAS-4000 system.

Morphological analysis

Open skeletal muscle biopsies were performed, and specimens were snap-frozen in liquid propane chilled by liquid nitrogen for cryostat sectioning and histochemistry. Standard techniques were used for enzyme histochemistry [6]. As previously described [7], type I, IIa and IIx MyHCs were assessed by applying quadruple immunofluorescence and scanning in a Hamamatsu S60 digital scanner with fluorescence equipment, including a DAPI/FITC/TRITC/Cy5 quad-band filter set (Semrock, New York). The tissue sections were processed in a Dako Autostainer. Primary antibodies were for type I, slow/beta cardiac MyHC: BA-D5 (mouse IgG2b, DSHB, 1:50), for MyHC IIa: SC-71(mouse IgG1, DSHB, 1:50), for MyHC IIx: 6H1 (mouse IgM, DSHB, 1:10) and for perlecan (basement membranes): Anti-Heparan Sulfate Proteoglycan, MAB1948P (rat IgG2a, cloneA7L6, Merck, 1:200). Secondary antibodies were BV421 (goat-anti-mouse IgG2b, Jackson Immunoresearch Laboratories, 1:200), Alexa Fluor488 (goat anti-mouse IGg1, Invitrogen, 1:200), Alexa Fluor 647 (goat anti-mouse IgM, Invitrogen, 1:200) and Alexa 568 (goat anti-rat IgG, Invitrogen, 1:200). For other immunostainings brightfield microscopy was applied and tissue sections were processed in a Dako Autostainer using the EnVision FLEX DAB+ Substrate Chromogen System kit and incubated with the following primary antibodies for one hour: anti-embryonic MyHC, F1.652 (DSHB, 1:20) and anti-foetal MyHC, MHn (Leica,1:20).

Results

Western blot of SMN protein

To investigate the amount of SMN protein we performed western blot on protein extracted from muscle tissue from the four SMA1 patients together with two SMA type III (SMA3) patients and three control samples. The result demonstrated that all SMA1 patients showed profound reduction of SMN protein compared to control samples (Fig. 1).

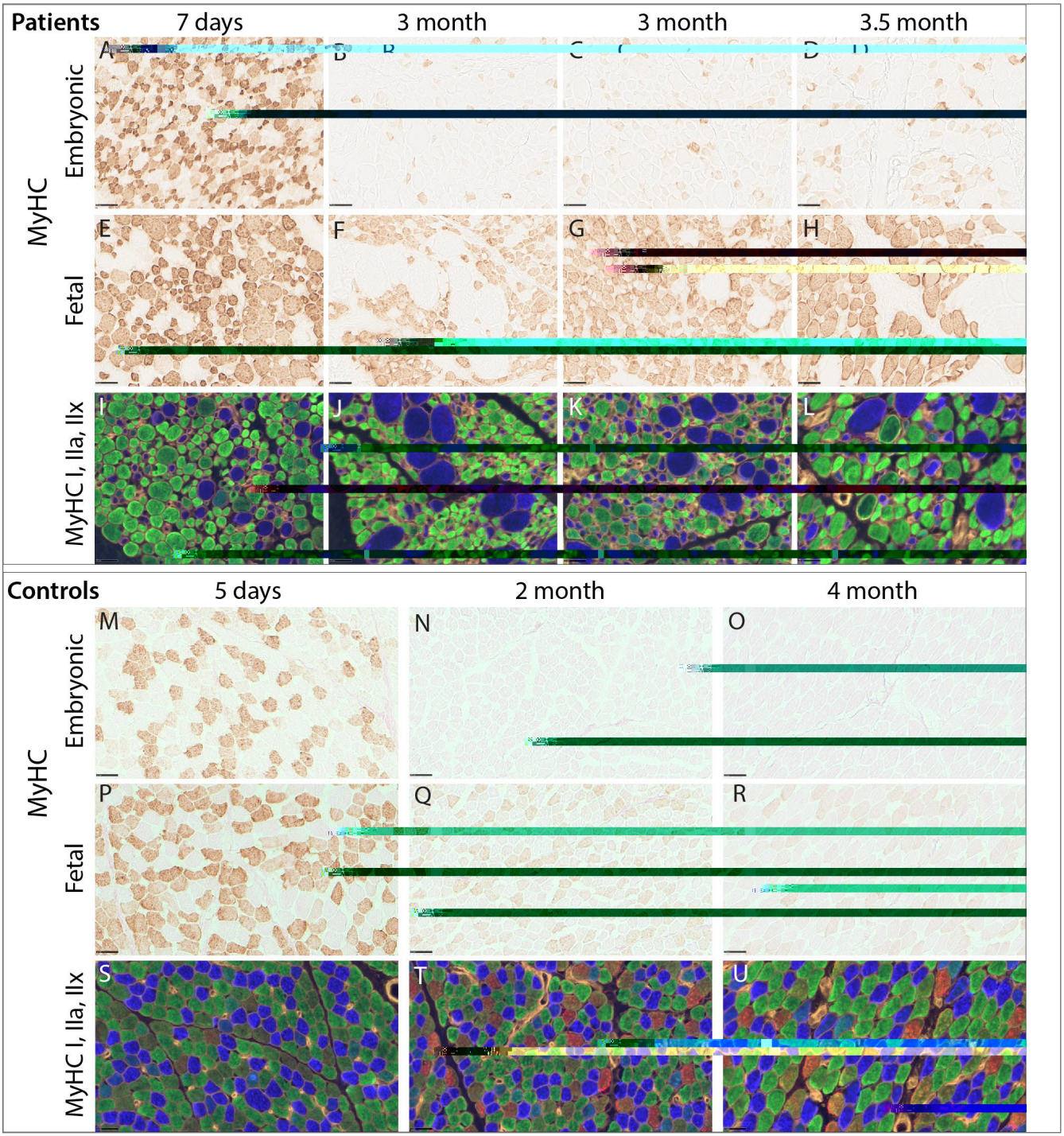

Figure 2. Myosin heavy chain isoforms in SMA1 patients and controls. Embryonic and foetal MyHC are stained with immunoperoxidase histochemistry. MyHC I, IIa and IIx are stained by immunofluorescence. MyHC I: blue, IIa: green, IIx: red. Note: remaining expression of embryonic and foetal MyHC throughout the study period in SMA1 patients (A-H) and complete absence of MyHC IIx in SMA1 patients (I-L). In the controls, MyHC IIx is expressed at all ages, but at age 5 days only as hybrid IIa/IIx fibres (S-U). Scale bar 25 µm.

patients than in controls at all ages (Fig. 2 A-D, Fig. 3C). In the 7-day-old SMA1 patient it was seen in the vast majority of the fibres but not in clearly hypertrophic fibres (Fig. 2A and 3C). In the 5-day-old control, it was present in less than 50% of the fibres (Fig. 2M and 3G). In both SMA1 and controls embryonic MyHC was co-expressed with MyHC IIa and to a lesser degree with foetal MyHC in most fibres (Fig. 3). Embryonic MyHC was not seen in fibres expressing IIx MyHC (Fig. 3F). In some fibres, embryonic and MyHC I were co-expressed in both SMA1 and controls. The expression of embryonic MyHC was completely downregulated in controls at 2 months of age whereas it

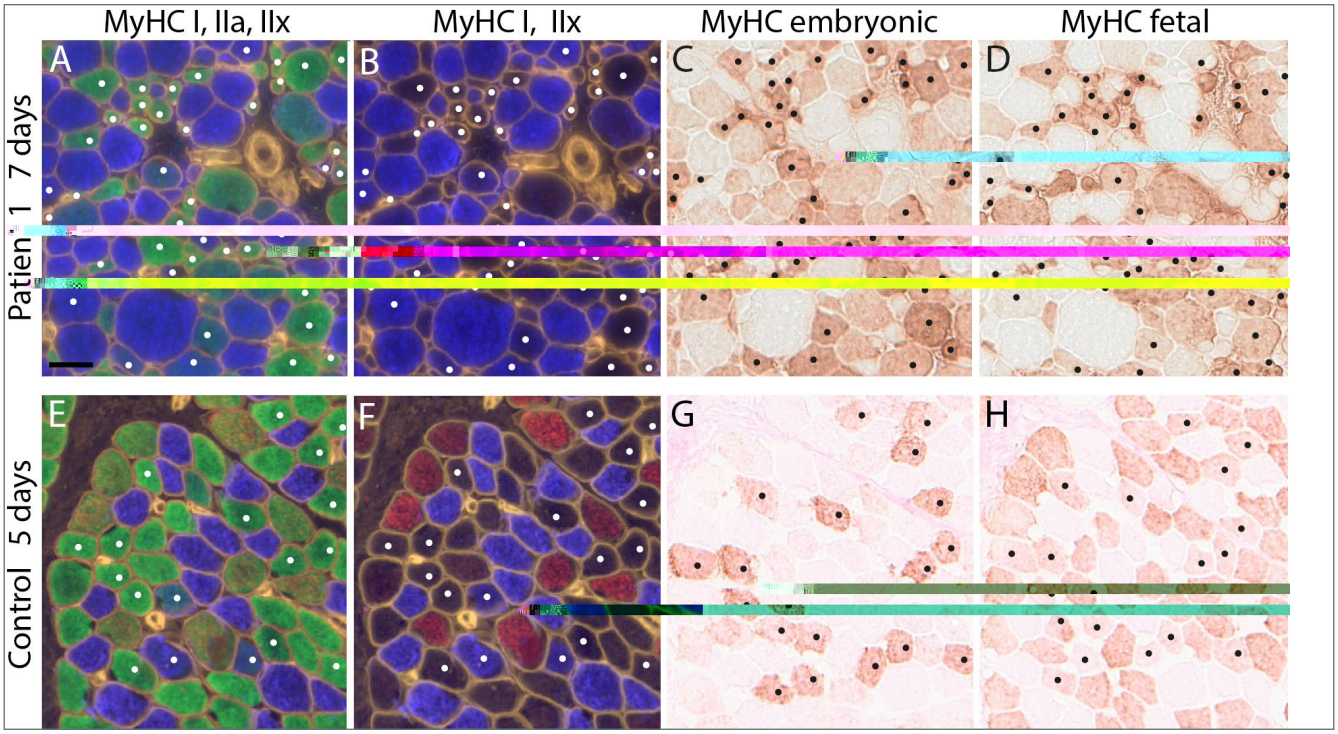

Figure 3. Myosin heavy chain (MyHC) isoforms in a SMA1 patient (A-D, age 7 days) and control (E-H, age 5 days) in serial sections. MyHC I, IIa and IIx are stained by immunofluorescence. MyHC I: blue, IIa: green, IIx: red. Embryonic and foetal MyHC are stained by immunoperoxidase histochemistry. The majority of fibres with high expression of embryonic MyHC are marked in all panels, showing that they are hybrids (IIa/embryonic, majority) or (I/embryonic) in both SMA1 and controls. There is no expression of MyHC IIx in the SMA1 patient (B). No fibres expressing IIx in the control co-express embryonic MyHC (F and G). Scale bar 25 µm.

declined but persisted in some, mainly small, atrophic fibres of SMA1 patients during the study period up to 3.5 months of age (Fig. 2A-D and 2M-O).

Foetal MyHC expression showed an overall high expression in SMA1 from 7 days to 3.5 months (Fig. 2E-H). It was generally co-expressed with MyHC IIa but not with MyHC I (Fig. 3B and D). In controls, foetal MyHC was expressed at 5 days with high co-expression of IIa and in some fibres IIa and IIx MyHC (Fig. 2P, Fig.3F and H). There was in general very little co-expression of fetal and MyHCI. At 2 months of age foetal MyHC was markedly downregulated and at 4 months it was completely downregulated (Fig. Q-R).

Type I (slow/beta cardiac) MyHC was present in some normal, hypertrophic and small (hypotrophic or denervated) muscle fibres in SMA1 at age 7 days (Fig. 2I, Fig. 3A-B). Occasionally, these fibres co-expressed developmental isoforms and/or type IIa MyHC. From 3 months of age, all hypertrophic fibres expressed only type I MyHC (Fig. 2J-L). Many small fibres expressed type I MyHC and these were sometimes hybrids expressing also IIa MyHC and developmental isoforms but the majority of these small type I fibres expressed only type I MyHC. In the controls, around 40% of the fibres expressed type I MyHCI, in a mosaic pattern, the majority being pure MyHCI fibres (Fig. 2S-U, Fig. 3E-F).

Type IIa MyHC was expressed in a majority of the muscle fibres in SMA1 at all ages (Fig. I-L, Fig. 3A-B). At 7 days of age MyHC IIa was present in normal, hypertrophic and small (hypotrophic or denervated) muscle fibres (Fig. 2I, Fig. 3A-B). At later stages of SMA1, MyHC IIa was not observed in any hypertrophic fibres (Fig. 2J-L). The majority of fibres expressing IIa MyHC were co-expressing both developmental MyHC isoforms, and some of them were also expressing MyHC I. In the controls, at all examined ages, type IIa MyHC was expressed either alone or co-expressed with type IIx MyHC (Fig. 2S-U, Fig. 3E-F).

Type IIx MyHC was not expressed in SMA1 muscle at any age (Fig. 2I-L, Fig. 3A-B). In the control aged 5 days, all fibres expressing MyHC IIx also expressed MyHC IIa (Fig. 2S, Fig. E-F). At later ages (2 and 4 months), control muscle included both pure IIx MyHC expressing fibres (2B fibres) and hybrid fibres expressing both MyHC IIx and IIa (Fig. 2T-U). It should be noted that the IIa antibody (SC-71, DSHB) reacts to some extent with IIx MyHC [6,8]. The definition of fibres with pure IIx in this study is based on intense staining of IIx MyHC in combination with weak IIa staining. Although it seems that a majority of fibres expressing IIx are hybrids with IIa MyHC, there is a proportion of fibres with pure MyHC IIx expression in controls.

Discussion

We analysed, at single fibre level, the expression of the five major MyHC isoforms in limb muscles in four patients at early stages of clinically and genetically defined SMA1 and compared them with age-matched controls. Unlike the controls, there was a persistent expression of developmentally regulated MyHC isoforms, mainly foetal MyHC.

Embryonic MyHC predominated in the early stage (age 7 days) and was expressed in both atrophic, normal and hypertrophic muscle fibres and co-expressed with either MyHC I or IIa. Foetal MyHC was mainly co-expressed with MyHC IIa. After this early stage there was a rapid decline in the expression of embryonic MyHC, while a high expression of foetal MyHC remained throughout the study period, but was restricted to fibres expressing MyHC IIa.

Overall, there seems to be a major difference between embryonic and foetal MyHC expression pattern in SMA1. The very high expression of embryonic MyHC in the early stages of SMA1, involving both type 1 and type 2 fibres, may be a sign of delayed maturation due to the SMA disease with denervation and lack of mobility and perhaps also the reduced levels of SMN protein in the muscle as shown by western blot analysis.

The persisting very high expression of foetal MyHC in MyHCIIa fibres throughout the study period indicates that the expression is associated with denervation of MyHCIIa fibres. As seen in control muscle, the foetal MyHC, still present around birth, is mainly expressed in fibres expressing IIa/IIx MyHC and not in type 1 fibres. Foetal MyHC, unlike embryonic MyHC, is re-expressed in denervated muscle fibres in various neurogenic muscle diseases, which may indicate that denervation itself triggers foetal MyHC expression [4].

The expression of embryonic MyHC in scattered, mainly small fibres may be a sign of attempts to regenerate from satellite cells, since embryonic MyHC seems to be a good marker for muscle fibre regeneration. Another explanation would be that there is not a complete downregulation of embryonic MyHC in all denervated fibres.

There was no expression of MyHC IIx at any age in the SMA1 patients, whereas the controls showed differentiation of first hybrid IIa/IIx fibres at 5 days of age and later fibres expressing only MyHCIIx or co-expression of MyHCIIa and IIx. The absence of embryonic MyHC in all fibres expressing IIx myosin in controls may indicate that the fibres expressing IIx MyHC are at a later stage of development, and that MyHC IIx is developmentally regulated. This normal development of different type 2 fibres may be compromised in SMA1 by denervation and/or deficiency of SMN protein in the muscle leading to absence of MyHCIIx expressing fibres in SMA1. It may be anticipated that the changes we observed in thigh muscles are also present in respiratory muscles since they are also denervated in SMA1, but this was not investigated.

Our findings, to some extent support results from earlier investigations on MyHC expression in SMA of different types [4,9] and also studies on mouse models [10]. However, our study involved patients and controls at an earlier stage and younger age, with clinically and genetically defined SMA1 and verified deficiency of SMN protein in muscle. In addition, we could take advantage of the immunofluorescence technique and new antibodies to characterize the expression of both IIa and IIx MyHC, which had not been possible in the earlier studies [9]. Therefore, we were able to analyse the expression of the five MyHC isoforms at single fibre level.

We demonstrated reduced SMN protein levels in the muscle samples from our patients. This reduced SMN expression may, in addition to denervation, influence muscle maturation as described in muscle cell lines [11].

Conclusions

We studied the myosin heavy chain expression in early postnatal stages of SMA1, and found profound alterations compared to age matched controls. The expression of fast type IIx MyHC was completely lost in SMA1, instead developmental isoforms remained highly expressed. Foetal MyHC was still, at 3.5 months of age, expressed in the majority of muscle fibres. This developmental delay was observed already in the newborn period, which may have implications for the effects of gene therapy, since early treatment has clear clinical benefits [12,13]. Whether such delayed development can be completely restored by gene therapy remains to be studied and may also have implications for new phenotypes that will evolve with new therapies.

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Conflict of interest statement

The authors declare no conflicts of interest.

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Authors contributions

CH-O and AO designed the study and experiments. CH-O, EJ, KV and AO performed experiments. CH-O, EJ, KV, and AO interpreted data. CH-O and AO wrote the manuscript. All authors commented and approved of the manuscript.

Ethical consideration

The study was approved by the Swedish Ethical Review Authority (No 2022-00026-01) and conducted according to the Declaration of Helsinki of 1975.

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