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Light-microscopic study of phosphoprotein B-50 in myopathies

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Abstract The growth-associated protein B-50, also termed GAP-43, is a membrane-bound phosphoprotein that is expressed in neurons. It is particularly abundant during periods of axonal outgrowth in development and regeneration of the central and peripheral nervous system. In this paper we study the expression of B-50 in inflammatory and dystrophic myopathies. To investigate the state of regeneration, N-CAM and vimentin serial sections were performed, because N-CAM and cytoskeletal protein vimentin are excellent markers for regenerating muscle. Light-microscopic evaluation showed that muscle fiber regeneration in myopathies corresponds closely to B-50 immunoreactivity in satellite cells, myoblasts, myotubes and small regenerating myocytes in cytoplasmatic distribution. In normal muscle and in biopsies of neurogenic muscular atrophy, however, no light-microscopically demonstrable B-50 staining was found. B-50 in muscles apparently plays a role in the growth morphology of regenerating myocytes, and the phosphoprotein B-50 can no longer be regarded as a neuron-specific molecule.

Key words Phosphoprotein B-50 (growth-associated protein GAP-43) · N-CAM · Vimentin · Immunohistochemistry · Human skeletal muscle

Introduction

Phosphoprotein B-50 is present in synaptosomal plasma membranes and in fetal and neonatal growth cones [1, 8]. B-50 has been shown to be identical with growth-associated protein GAP43 [27] and is highly enriched in the growth cones of axons, where it is associated with the membrane cytoskeleton on the inner face of the plasma membrane [23, 30]. Furthermore, a striking correlation has been found between increased synthesis and transport of GAP43 after axotomy of peripheral nerves and the ability of these neurons to regenerate [29, 32]. These and other observations suggest that B-50 plays a part in the maintenance of growth cone morphology and/or mobility [11] and that is clearly a protein of growing interest in studies of neurite formation during neuronal development [33] and regeneration [19].

With regard to muscle, light- and electron-microscopic evaluation of the reinnervation process showed that the period of fiber reinnervation corresponds closely to the time in which high B-50 immunoreactivity was observed in nerve fibers invading muscle and in the newly formed neuromuscular junctions [34]. Only Stocker et al., in 1992 [31] have suggested the possibility of B-50 expression in non-neuronal cells of the embryonic chicken limb, some of which may be part of the muscle cell lineage. Thus, little is known about the expression of B-50 in myocytes of human skeletal muscle.

Our present study, performed with immunohistochemical procedure that allows to assess changes at light-microscopic level, attempts to demonstrate the existence of phosphoprotein B-50 in regenerating human skeletal muscle cells. To investigate the state of regeneration we examined B-50, N-CAM and vimentin serial sections of diseased and normal human skeletal muscle. Lanier et al. 1988 [21] have shown that Leu19 antigen is similar to or identical to neural cell adhesion molecule (N-CAM), and Leu19 antigenicity has been detected in skeletal muscle. After labelling with a monoclonal antibody, the surface of cultured myoblasts [15] and satellite cells was Leu19 positive in both normal and diseased muscles [28]. The expression of Leu19 antigen in muscle-specific structures related to developing and/or regenerating muscle fibers has led to the conclusion that Leu19 antigen represents a molecular marker of muscle fiber regeneration [16, 28].

The intermediate filament vimentin is a component of the cytoskeleton of striated muscle fibers. Light-microscopic observations of muscle biopsies from patients with neuromuscular disorders and studies of experimental regeneration in rats suggest that regenerating fibers may transiently react with vimentin [5, 17].

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Thus, we regard N-CAM and vimentin as excellent markers for regenerating myocytes, which are thought to express the growth-associated phosphoprotein B-50 (GAP43).

Materials and methods

We obtained muscle biopsy specimens from 91 patients (Table 1). The following conditions were represented: non-weak control subjects (14 cases), polymyositis (12 cases), dermatomyositis (14 cases), interstitial myositis (26 cases), lymphocytic vasculitis (8 cases), Duchenne muscular dystrophy (2 cases), neurogenic muscular atrophy (15 cases).

Control muscles were obtained through diagnostic biopsies of patients complaining of muscle symptoms who were ultimately found to have no muscle disease. None of the controls had muscle weakness, elevated serum CK level, electromyographie abnormality, or histological abnormality of muscle according to ordinary light-microscopic and histochemical criteria or on immunohistochemical procedures for revealing and phenotyping inflammatory cells.

Open muscle biopsy was performed on limb muscles (Table 1) under local anesthesia, and the tissues were processed for light microscopy. Specimens were immediately frozen in isopentane, which was cooled in liquid nitrogen and then cut in a cryostat. For

Table 1 List of clinical data by diagnosis (*m* male, *f* female, *r* right, *l* left); figures show age in years; muscles named are those biopsied

Normal muscle		
m	15	r deltoid
m	21	r tibialis anterior
f	32	r quadriceps
f	33	r deltoid
f	19	r biceps brachii
m	34	r quadriceps
f	43	r gastrocnemius
f	28	l deltoid
f	57	l biceps brachii
f	48	r tibialis anterior
f	29	l quadriceps
m	26	l gastrocnemius
m	30	l biceps brachii
f	16	r deltoid
Idiopathic polymyositis		
f	59	r biceps brachii
m	81	l biceps brachii
f	65	l quadriceps
m	29	r quadriceps
m	50	l quadriceps
f	45	r gastrocnemius
f	67	r quadriceps
m	59	l gastrocnemius
f	67	r quadriceps
f	71	l quadriceps
f	57	l deltoid
m	71	l quadriceps
Dermatomyosits		
f	20	l quadriceps
f	38	r deltoid
m	15	l deltoid
f	35	r deltoid
f	76	l quadriceps
f	58	l quadriceps
f	79	r deltoid
f	53	l gastrocnemius
f	34	l deltoid
f	52	l deltoid
m	63	r biceps brachii
m	58	l deltoid
f	64	r deltoid
f	83	r deltoid
Interstitial myositis		
f	64	l deltoid
m	39	l biceps brachii
f	60	l biceps brachii
f	23	r biceps brachii
f	70	r quadriceps
f	47	r quadriceps
Interstitial myositis		
f	38	r deltoid
f	67	l tibialis anterior
m	62	r gastrocnemius
m	70	l quadriceps
f	48	r deltoid
f	42	l quadriceps
f	49	r quadriceps
m	31	l gastrocnemius
m	25	l deltoid
f	68	r biceps brachii
f	54	r gastrocnemius
m	21	r deltoid
f	68	l quadriceps
f	27	r gastrocnemius
m	58	l gastrocnemius
f	77	r gastrocnemius
f	35	r quadriceps
f	27	r quadriceps
f	13	l quadriceps
m	37	r deltoid
Lymphocytic vasculitis		
f	62	r tibialis anterior
f	51	l gastrocnemius
f	54	r gastrocnemius
f	62	r gastrocnemius
m	53	l gastrocnemius
f	65	l quadriceps
m	44	r tibialis anterior
f	58	l deltoid
Duchenne dystrophy		
m	8	l deltoid
m	5	r quadriceps
Neurogenic atrophy		
f	60	l gastrocnemius
f	60	l tibialis anterior
f	60	l quadriceps
m	63	r gastrocnemius
f	60	l quadriceps
f	60	r quadriceps
f	28	l tibialis anterior
f	74	r quadriceps
m	49	l gastrocnemius
m	33	r deltoid
m	50	l deltoid
m	78	r quadriceps
f	42	l quadriceps
m	46	r deltoid
m	45	r quadriceps

routine histological examination the following methods were used to stain 8-μm-thick sections: hematoxylin-eosin, Gomori's trichrome, periodic acid-Schiff, oil red, NADH tetrazolium reductase and metachromatic dye ATPase at pH 9.4 and after incubation at pH 4.3 and 4.5 [9]. To demonstrate acetylcholinesterase activity, frozen sections were stained essentially following the method described by Koelle and Friedenwald in 1949 [20] and modified by Lewis in 1961 [22] and again by Henderson in 1967 [12].

The following antibodies diluted in 0.05 molar TRIS buffer were used for immunohistochemical studies: monoclonal mouse anti-GAP43 (clone 5E7), 1:5 (Oncogene Science); monoclonal mouse anti-CD56 (Leu19=muscle specific isoform of N-CAM) (clone MY31), 1:40 (Becton Dickinson); monoclonal mouse anti-vimentin (clone VIM 3B4), undiluted (Camon); monoclonal mouse anti-CD8 (Leu2a) (clone SK1), 1:40 (Becton Dickinson); monoclonal mouse anti-CD4 (gp32), 1:20 (Biotest); monoclonal mouse anti-CD16 (GRM1), 1:100 (Biotest); monoclonal mouse anti-CD22 (gp130), 1:10 (Dakopatts); monoclonal mouse anti-CD68 (KP1), 1:100 (Dakopatts), monoclonal mouse anti-MHC-class-I (W6/32), 1:800 (Serotec), monoclonal mouse anti-MHC-class-II (Tü34), 1:10 (Biotest), monoclonal mouse anti-MAC (membranolytic attack complex C5b-9) (aE11), 1:25 (Dakopatts). The bound primary antibodies were visualized using the APAAP (alkaline phosphatase anti-alkaline phosphatase) method. Controls for the staining specificity of all primary antibodies were performed with non-immune immunoglobulin instead of the first antibody. Light hemalum counterstaining was used to reveal cellular structures.

Table 2 Numbers of cases with B-50 immunoreactive myocytes

Diagnosis	No. of cases	Expression of B-50
Normal muscle	14	No cases
Polymyositis	12	7 cases
Dermatomyositis	14	5 cases
Interstitial myositis	26	5 cases
Lymphocytic vasculitis	8	6 cases
Duchenne dystrophy	2	2 cases
Neurogenic muscular atrophy	15	No case

Results

The diagnoses reached in the 60 cases of inflammatory myopathies, 2 cases of Duchenne muscular dystrophy and 15 cases of neurogenic muscular atrophy were based on conventional criteria taking account of clinical and myopathological data, including immunohistochemistry for detection of the CD4, CD8, CD16, CD22, CD68, MHC class I, MHC class-II and MAC antigens [2–4, 10, 18].

In muscle biopsies from non-weak control subjects, immunostaining for B-50 and N-CAM was consistently negative, except for some satellite cells that were N-CAM positive. Furthermore, N-CAM, but not phosphoprotein B-50, immunoreactivity was restricted to a few tiny structures close to the outer side of the plasmalemma. These were associated with sole plate nuclei, and thus might have been nerve endings. This is corroborated by the fact that in serial sections at same locations acetylcholinesterase staining was positive. Fibroblasts, capillaries and other blood vessels showed vimentin immunoreactivity. We found some interstitial CD68-positive cells.

In some, but not in all, cases (Table 2) of polymyositis, dermatomyositis, lymphocytic vasculitis and, less often, in interstitial myositis, satellite cells (Fig. 1), myotube-like fibers (Fig. 2) and small myocytes (Fig. 3), sometimes with large vesicular nuclei (Figs. 4A, 6A) showed mainly cytoplasmatic B-50 immunostaining. A few muscle cells exhibited B-50 reactivity in peripheral

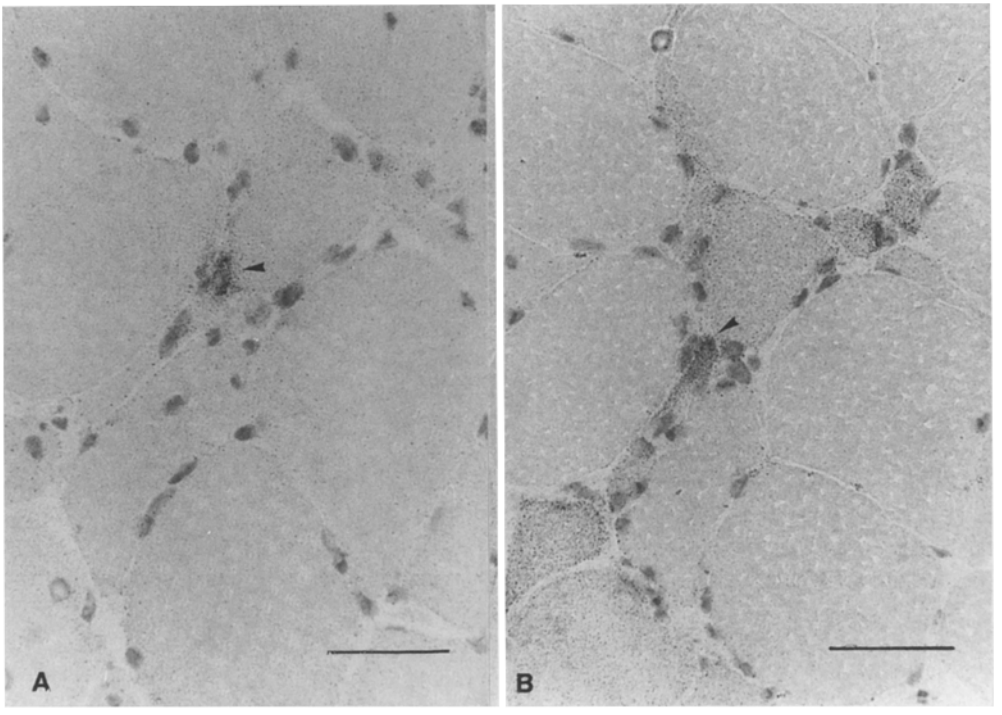

Fig. 1A, B Idiopathic polymyositis. **A** B-50-expressing small cell (*arrowhead*) corresponding to a satellite cell as seen in **B:** serial section prepared for detection of N-CAM. N-CAM immunoreactive satellite cell (*arrowhead*) (frozen sections, APAAP method) *Bar,* **A** 100 μm, **B** 90 μm

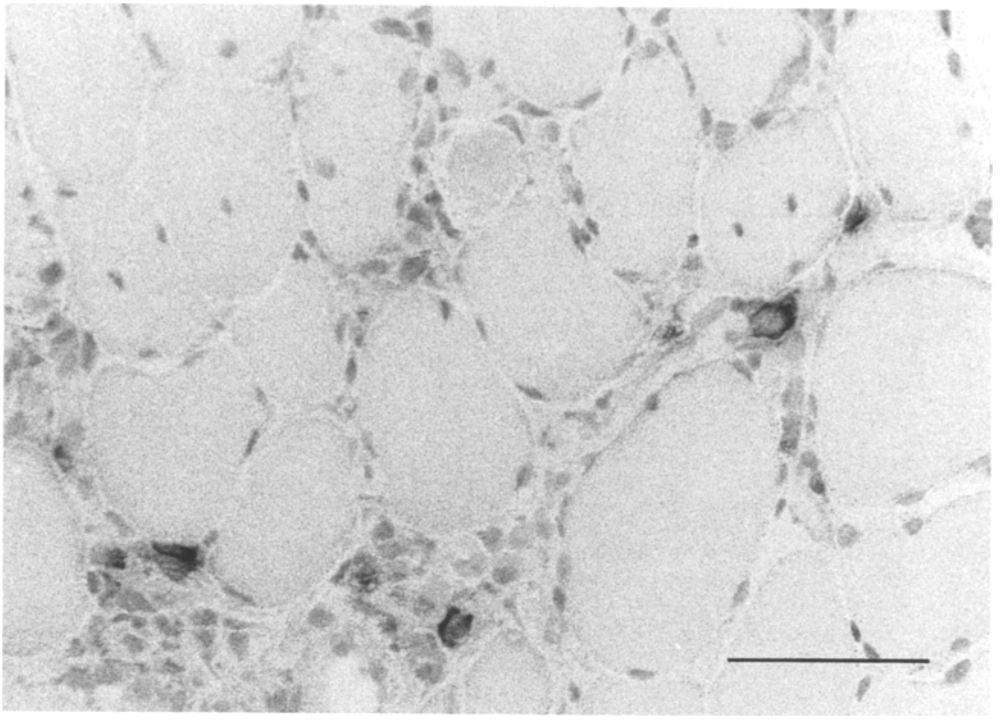

Fig. 2 Idiopathic polymyositis. Small round B-50-expressing myotube-like fibers (frozen section, APAAP method). *Bar,* 100 μm

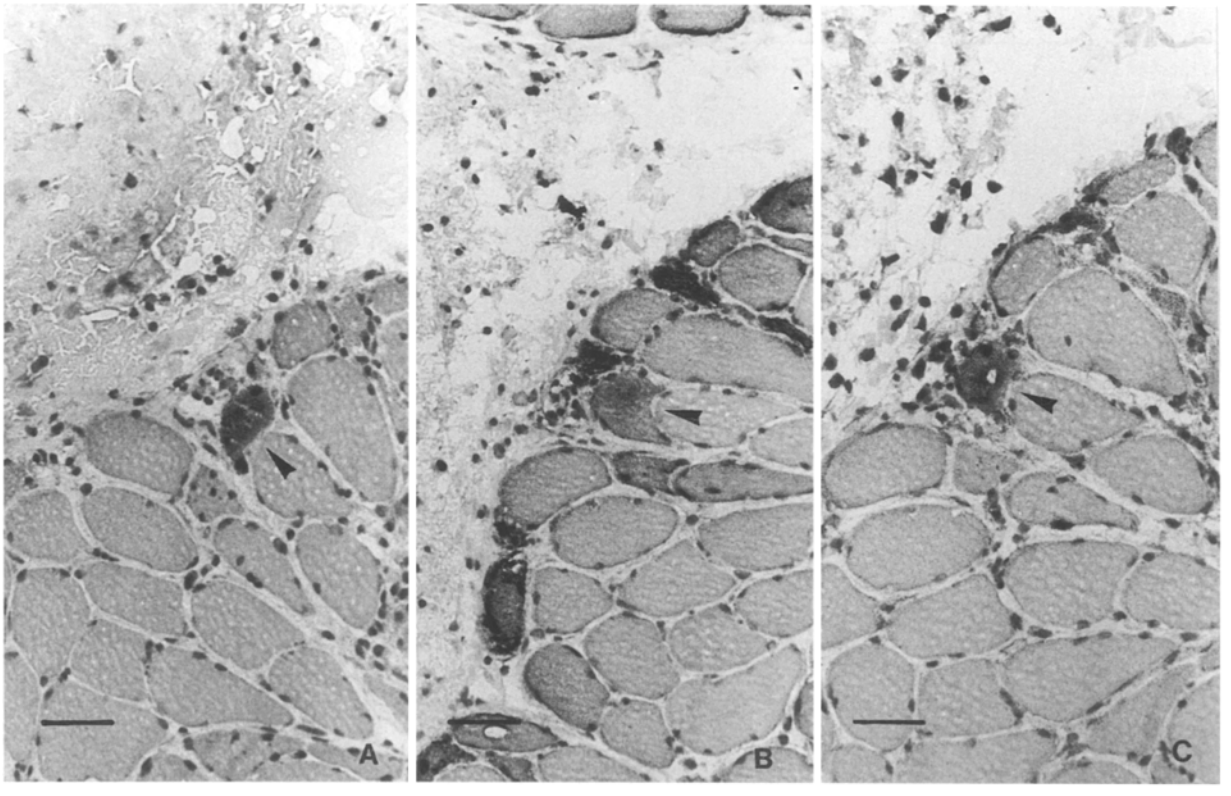

Fig. 3A–C Dermatomyositis. Serial sections. (**A**) A small perifascicular muscle cell with strong B-50 immunoreactivity (*arrowhead*) corresponding to a regenerating fiber as seen in the serial sections prepared for detection of N-CAM and cytoskeletal protein vimentin (**B, C**). **B** Fiber from **A**, expressing N-CAM (*arrowhead*). Note that N-CAM-positive fibers are more abundant than in **A**, with only one B-50-reactive cell. **C** Fiber from **A, B**, with vimentin immunostaining (*arrowhead*) (frozen sections, APAAP method). *Bars,* **A, B** 60 μm, **C** 50 μm

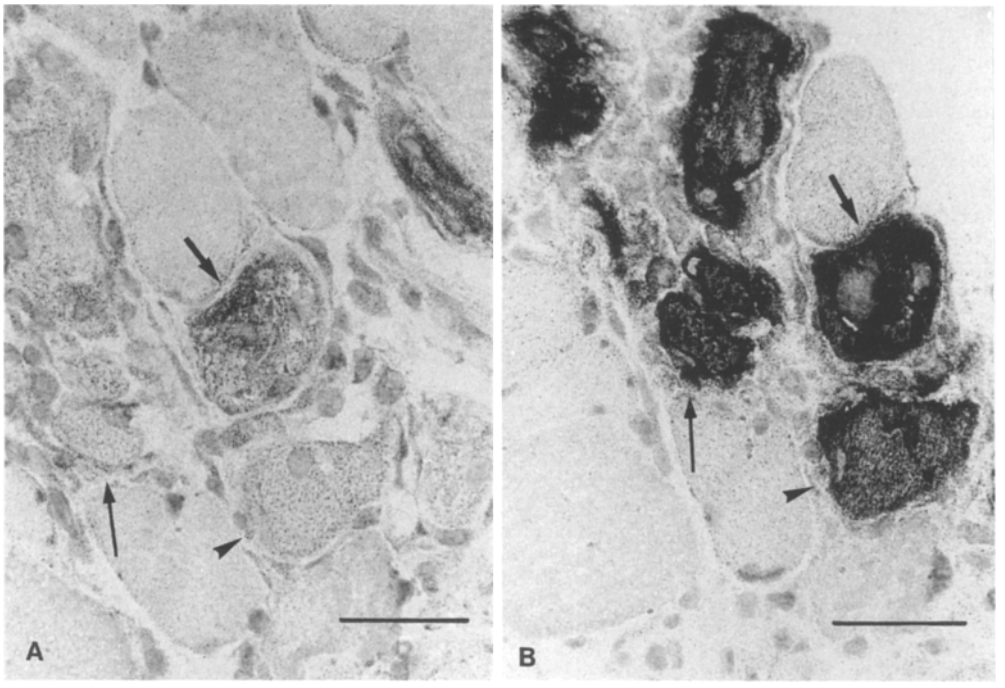

Fig. 4A, B Idiopathic polymyositis. Serial sections. **A** B-50-immunoreactive myofibers of variable size, some with large vesicular nuclei corresponding to regenerating fibers as seen in the serial sections prepared for detection of N-CAM (Fig. 5B; arrows and arrowheads indicate same fibers). (**B**) Fibers from **A** with strong N-CAM immunoreactivity. Note that N-CAM is more strongly expressed than B-50; APAAP method). *Bars*, 80 μm

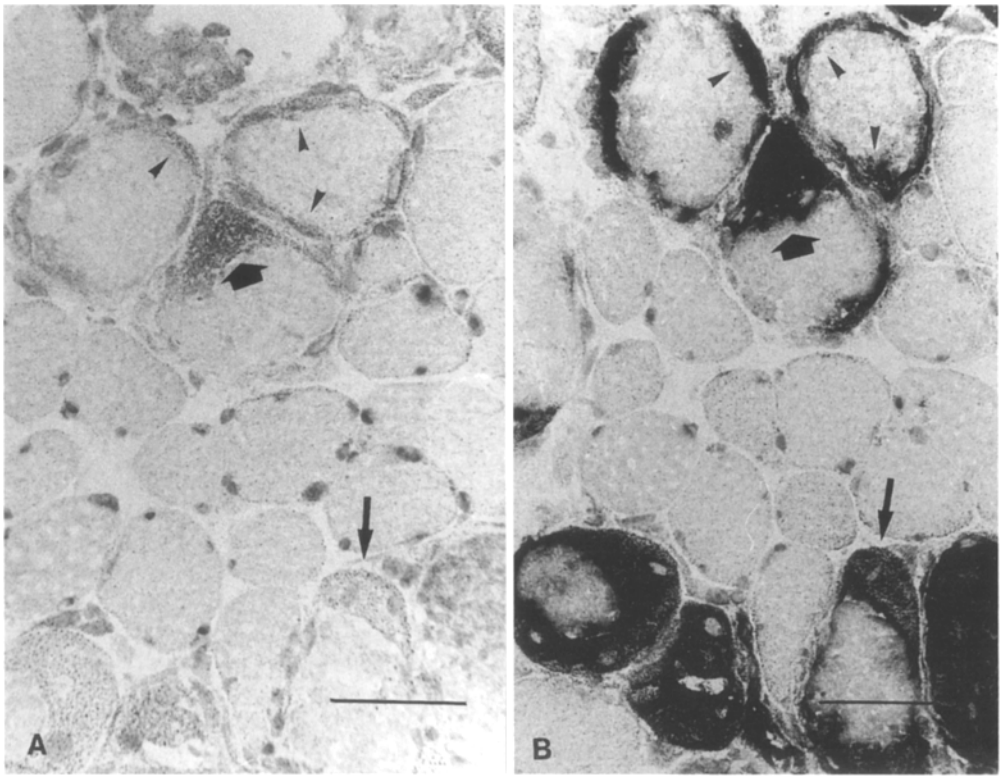

Fig. 5A, B Dermatomyositis. Serial sections. **A** B-50 immunoreactive regenerating fibers located in cap fashion on preexisting muscle fibers (*arrows*). Additionally, muscle cells show B-50 reactivity in a somewhat stripped peripheral distribution (*arrowheads*) corresponding to activated satellite cells as seen in the serial section prepared for detection of N-CAM in **B:** fibers from **A**, showing intensive immunoreactivity for N-CAM (frozen sections, APAAP method). *Bars*, 90 μm

distribution (Fig. 5A), indicating activated satellite cells or myoblasts. As shown in serial sections, B-50 immunoreactive cells coexpressed N-CAM (Figs. 1B, 3B, 4B, 5B, 6B) and the cytoskeletal protein vimentin (Figs. 3C, 6C). Fibers, consistently B-50 negative but N-CAM positive, were also abundant in polymyositis, dermatomyositis, lymphocytic vasculitis and interstitial myositis (for an example see Fig. 3B). Frequently, N-CAM was more strongly expressed than phosphoprotein B-50 (Figs. 4–6). In interstitial myositis, owing to the mild myopathic changes and therefore sparse regenerating fibers fewer specimens, showed phosphoprotein B-50-expressing myocytes than in polymyositis, dermatomyositis, lymphocytic vasculitis and Duchenne dystrophy (Table 2). In

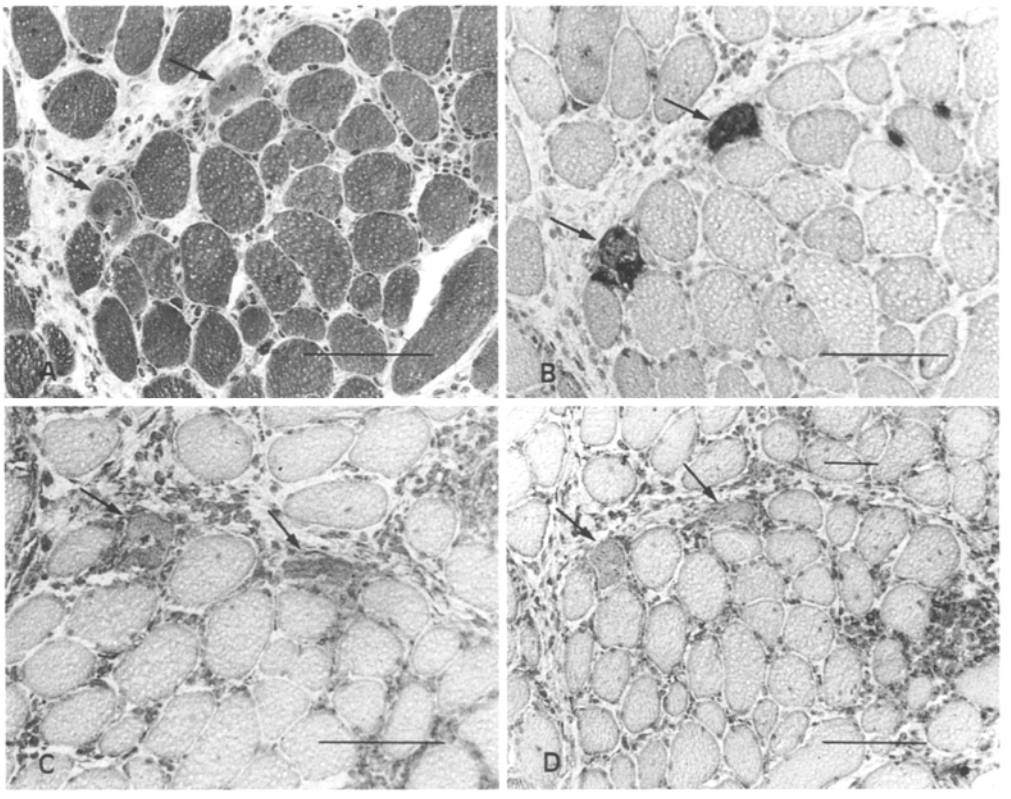

Fig. 6A–D Lymphocytic vasculitis. Serial sections. **A** Hematoxylin-eosin stain, **B** N-CAM and **C** cytoskeletal vimentin immunoreactivity. Perifascicular basophilic fibers (*arrows*) disclose N-CAM and vimentin-immunoreactivity indicating regeneration. **D** Phosphoprotein B-50-immunoreactivity. The regenerating fibers in **A, B** coexpress phosphoprotein B-50 (frozen sections, APAAP method). *Bars,* 110 μm

dermatomyositis and lymphocytic vasculitis we saw many more B-50-positive fibers in areas of perifascicular atrophy. The phenotype analysis of inflammatory cells in idiopathic polymyositis showed CD8-, CD68-, and occasional CD4-positive cells. We found some intact myocytes invaded by CD8- and CD4-positive cells. Numerous mononuclear cells expressed MHC class II antigens; there was expression of MHC class I antigens on myocytes. In dermatomyositis, in lymphocytic vasculitis and, less commonly, in interstitial myositis, CD4-, CD68- and some CD8-positive mononuclear cells were seen mainly perivascularly in the perimysium. Perimysial and endomysial mononuclear cells expressed MHC class II antigens. In dermatomyositis and interstitial myositis, a complement-mediated angiopathy was seen on vessels immunohistochemical (C5b-9).

The two biopsies from patients with Duchenne muscular dystrophy disclosed almost identical patterns of myopathology. There was some liposclerotic change. The scatter of fiber diameters is increased. Internal nuclei were more frequent than normal, but not very abundant. The necrotic fibers in cross-sections appeared singly or in small groups. These necrotic fibers were round and partly appear dense (opaque hypercontracted fibers). About 15% of the muscle fibers were of immature and probably regenerating type 2C, often arranged in groups and showing N-CAM, vimentin and phosphoprotein B-50 immunoreactivity. Phenotyping of inflammatory cells revealed some interstitial CD4- and sparse CD8-positive cells. Furthermore, CD68-positive cells were abundant.

In inflammatory myopathies, there were scattered occurrences of small round and angulated fibers in which the cytoplasm assumed a uniformly bluish color with H&E. These cells have vesicular nuclei. In polymyositis there was no difference between the distributions in perifascicular and in intrafascicular areas, but in dermatomyositis and lymphocytic vasculitis the basophilic fibers were found more often in areas of perifascicular atrophy. Some of the basophilic fibers coexpressed N-CAM, vimentin and phosphoprotein B-50 (Fig. 6A–D). In interstitial myositis, basophilic fibers coexpressing N-CAM, vimentin and phosphoprotein B-50 were very sparse in keeping with the mild myopathic changes. In Duchenne dystrophy, basophilic fibers are often arranged in groups and sometimes coexpress N-CAM, vimentin and phosphoprotein B-50. Fibers, consistently B-50 negative but N-CAM positive, were also abundant in Duchenne dystrophy.

In muscle biopsies from patients with neurogenic muscular atrophy, most small angulated fibers expressed N-CAM intensively in a diffuse cytoplasmic distribution. Like normal muscle tissue, however, phosphoprotein B-50 was not detectable, nor was there any vimentin immunoreactivity in myocytes. Immunostaining showed some CD4- and CD68-positive interstitial cells.

Controls for staining specificity with non-immune immunoglobulin instead of the first antibody showed no specific immunostaining.

Discussion

Our study demonstrates that phosphoprotein B-50 is expressed in human skeletal muscle. In inflammatory myopathies and muscular dystrophy, B-50 staining occurs in satellite cells, myoblasts, myotubes and small fibers. B-50 immunoreactivity was demonstrated in a diffuse cytoplasmatic distribution. Light-microscopic means cannot help us to decide whether B-50 is associated with the cytoskeleton or other subcellular structures. In biopsies of non-weak control muscles and neurogenic muscular atrophy there was no B-50 immunostaining.

On serial sections, an immunohistochemcial reaction for the detection of B-50, N-CAM and vimentin occurs in identical cells. The assumption that cells exhibiting B-50 immunoreactivity are regenerating ones is supported by these findings, particularly as N-CAM and vimentin are excellent markers for muscle regeneration [5, 16, 28].

As in the nervous system [19, 33, 34], phosphoprotein B-50 may play a part in differentiation and development of human skeletal muscle. This is underlined by the fact that B-50 is expressed together with N-CAM and vimentin. The fact that in intact mature muscle virtually no B-50 immunoreactivity could be detected also lends credence to the assumption that B-50 is probably a key molecule in the morphology of muscle growth. The observation that B-50 is enriched in axonal growth cones, where it is associated with the membrane cytoskeleton [25], could be another strong argument for a role of B-50 in the construction of muscle cells. Concerning this, in 1994 Heuss et al. [14] found B-50 immunoreactivity on the inner face of the plasma membrane in hypotrophic type I fibers in congenital muscle fiber disproportion. It is the distribution of B-50 in developmentally disordered myocytes in particular that allows an analogy with the corresponding results of Moos et al. in 1990 [25] for growing axons. Otherwise, it is uncertain whether developmentally disordered myocytes mimic fetal development or regeneration of muscle. Thus, in regenerating muscle, because of the diffuse cytoplasmic expression, phosphoprotein B-50 may have a different role than in the nervous system.

With regard to N-CAM, a change in isoforms during myogenesis coincides with myoblast fusion [6, 7, 24] suggesting that the shift in NCAM isoforms is part of the developmental program in skeletal muscle. Thus, it seems that both phosphoprotein B-50 and the transmembrane molecule NCAM fulfill functions in the developing and regeneration processes. Our observation that N-CAM expression is more abundant in regenerating muscle than B-50 means a stage-dependent expression of B-50 in myocyte regeneration. Therefore, N-CAM and phosphoprotein B-50 probably have supplementary roles. The finding that N-CAM frequently is more strongly expressed than phosphoprotein B-50 probably shows a higher translational level of N-CAM.

Looking at previous literature concerning expression of B-50 in muscle tissue, it seems that our findings contrast with the results of Hesselmans et al. in 1989 [13]. In developing muscle they found B-50 in human intramuscular nerves and neuromuscular junctions, but not in myocytes. A possible explanation for this difference could be that they used a polyclonal affinity-purified rabbit anti-B-50 antibody, in contrast to our monoclonal mouse anti-B-50 antibody. Concerning growth morphology, it seems unlikely that a distinction need be made between the pattern of developing muscle fibers and that of regenerating ones, because fetal developing muscle is growing muscle and it is assumed that regeneration recapitulates fetal development. Furthermore, Verhaagen et al. [34] reported high B-50 immunoreactivity in nerve fibers that invade muscle and in newly formed neuromuscular junctions. However, in our specimens of neurogenic muscular atrophy in amyotrophic lateral sclerosis there was no B-50 immunostaining. Because these authors studied experimental denervation and reinnervation in rats one cannot compare their results in affected muscle with our material. And there was no evidence in our cases of neurogenic muscular atrophy in amyotrophic lateral sclerosis of reinnervation by collateral sprouting, when studied by conventional enzyme-histochemistry.

Furthermore, in our cases of neurogenic muscular atrophy, small angulated fibers stain intensively with the N-CAM antibody. As shown in serial sections this fibers show no vimentin immunoreactivity. This corresponds to the findings of Illa et al. [16] and Müller-Felber et al. [26] on the expression of N-CAM in denervation. Müller-Felber et al. [26] studied experimental denervation and reinnervation in rabbits, and Illa et al. [16] demonstrated N-CAM in denervated fibers in amyotrophic lateral sclerosis and in the postpolio syndrome, but neither group studied indicators of regeneration such as vimentin expression. The lack of vimentin immunostaining in neurogenic muscular atrophy can be explained by the fact that vimentin is known to be expressed in regenerating fibers [5] and in our cases of neurogenic muscle atrophy no signs of regeneration (basophilia) were detected. Our results confirm the conclusion reached in previous studies [16, 26] that denervated myocytes can be identified by demonstrating N-CAM expression.

Finally, phosphoprotein B-50 expression in myopathy suggests that further immunohistochemical research, including in-situ hybridization and immuno-electron microscopy, should be carried out to study this growth-associated molecule in myopathies.

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