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EXPRESSION OF A CELL DEATH MARKER (CLUSTERIN) IN MUSCLE TARGET FIBERS

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SUMMARY — We report, for the first time, the expression of immunoreactivity to clusterin in skeletal muscle. Clusterin, a protein probably related to the process of programmed cell death (apoptosis), was specifically very highly expressed in target fibers. All target fibers found in 50 muscle biopsy samples from a variety of neuromuscular disorders expressed a high concentration of clusterin in the middle of the targets. Clusterin was not expressed in any targetoid fibers or cores. Acute denervation, where targets are mostly seen, may be the beginning of apoptosis. Hence our findings support the concept that targets are harbingers of acute denervation.

KEY WORDS: skeletal muscle, target, clusterin.

Expressão de marcador de morte celular programada (apoptosis) em fibras musculares com «target».

RESUMO — Descrevemos, pela primeira vez, a expressão de imunorreatividade para clusterina em músculo esquelético. Clusterina, proteína relacionada ao processo de morte celular programada (apoptosis), encontrou-se especificamente muito aumentada em fibras musculares com «target» (FT). Todas as FT encontradas em 50 biópsias musculares de pacientes com diferentes doenças neuromusculares apresentaram alta concentração de clusterina no centro das FT. Clusterina não esteve semelhantemente aumentada em fibras com «targetóide» ou com «core». A alta expressão de clusterina em «targets» indica que FT podem ser o início de apoptosis e que há íntima relação entre FT e desnervação aguda.

PALAVRAS-CHAVE: músculo esquelético, fibras com «target», clusterina.

Clusterin, a recently cloned and sequenced gene [3] is a representative, and probably specific, marker related to processes of programmed cell death (apoptosis) in mammalian cells [2,9]. Originally characterized as a constitutively expressed gene product in mammalian Sertoli cells [3], the expression of clusterin is now also associated with apoptosis (ordered and active process of acute onset of cellular atrophy and death) in many human tissues [1].

Taking advantage of this recent discovery, we studied 50 samples of skeletal muscle in search of the expression of this protein in a variety of neuromuscular disorders. We have demonstrated a high expression of this gene in target fibers (TF).

MATERIAL AND METHODS

Muscle biopsy specimens obtained from 50 patients with a variety of neuromuscular disorders, diagnosed at Columbia University, (Table 1), were evaluated by immunohistoche-

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mistry using an antiserum against clusterin (generously supplied by Dr. C. Yan Cheng, Rockefeller University). As part of the diagnostic work-up, all samples had been analysed with routine paraffin-embedded stained sections and histochemistry, according to standard criteria5.

All skeletal muscles were immediately frozen in liquid nitrogen. For immunohistochemical study, specimens were oriented for transverse 10 micrometers-thick sectioning and mounted 2 per standard microscopic slide coated with 0.1% poly-D-lysine. Unfixed or fixed sections in acetone in a Coplin jar for one min at room temperature were washed in PBS for 10 min and then in sequence covered with a 1:10 dilution of normal goat serum in PBS for 10 min to block nonspecific binding sites in the section and incubated in a humidified chamber for 2 hours at room temperature with rabbit antiserum to clusterin diluted in different dilutions (1:50 to 1:500) in PBS. Slides were washed in PBS, covered with 1:20 dilution of goat anti-rabbit IgG FITC conjugate (Sigma) for 30 min, washed in PBS and coverslipped with a synthetic mountant.

Control sections were processed in an identical manner substituting a preimmune rabbit serum for the primary antibody.

To determine whether the observed staining was due to the anti-clusterin antibody activity, 30 microliters of the sera with anti-clusterin were absorbed by incubating with 3.5 micrograms of pure protein (clusterin) during a period of 8 hours. The immunoprecipitate was removed by centrifugation and the absorbed serum was tested for binding to sections of the skeletal muscle.

Table 1. Muscle specimens of a variety of neuromuscular disorders studied by immunohistochemical clusterin: 50 cases.

Amyotrophic lateral sclerosis	6
Central core myopathy	2
Charcot Marie Tooth	1
Corticosteroid myopathy	2
Chronic inflammatory demyelinating polyneuropathy	1
Dermatomyositis	1
Duchenne muscular dystrophy	4
HIV associated myopathy	2
Inclusion body myositis	2
Lipid storage myopathy	2
Mononeuropathy multiplex	1
Multifocal conduction block	2
Myoglobinuria	2
Mitochondrial myopathy	7
Polymyositis	5
Neuropathy related to tumor	4
Neuropathy of unknown cause	1
Scleroderma	1
Stiff man	1
Tubular aggregates myopathy	1
Uremia	1
Vasculitis	1

Sections were examinated with a fluorescence microscope (Zeiss) and a confocal microscope. Photomicrographs were taken with a Nikon camera using Ektachrome P800/1600 film for color slides (Kodak). Color prints were developed from these slides and kept as the permanent record.

RESULTS

Immunostaining in muscle fibers with anti-clusterin antibody was seen in all 50 cases, but the expression of clusterin was markedly increased in 4 samples, 1 with amyotrophic lateral sclerosis, 2 with chronic neuropathies, and 1 with polymyositis. All these 4 specimens depicted target fibers. Clusterin was expressed mostly in the center of the muscle fibers (Fig. 1) that harbored the target, previously evidenced with NADH-tr (Fig. 2). The intensity of staining remained strong at dilutions of up to 1:500, and was most intense after acetone fixation for one minute at room temperature. Such marked pattern of staining was found only in target fibers, and was seen mainly in normal or slightly atrophic muscle fibers, but rarely in totally atrophic fibers. In two cases that presented with a large number of TF, seen in small groups, there was a broad variation in the size of the targets, but the immunoreactive pattern with anti-clusterin was the same in all fibers of the same specimen. Studies of these findings with a confocal microscope (that permits the analysis of different levels in the same section) detected a uniform staining in the center of the TF at all levels.

TF that showed an advanced stage of secondary degenerations *8*, seen in one of the patients with a chronic neuropathy, had an immunoreactive staining weaker than in the ones without such abnormality. No immunoreactivity was detected in the middle of the target of fibers depicting secondary degeneration (Fig. 3).

All other structural abnormalities found in our material, that included, targetoid fibers, central cores, moth-eaten fibers, cytoplasmic bodies, ringbindens, ragged-red fibers, rimmed vacuoles, necrotic fibers, tubular aggregates, and regenerating fibers, showed no reaction with this antiserum (Fig. 4).

Color reaction was not observed in any negative control sections.

Pre-absorption with pure clusterin removed all binding activity.

COMMENTS

The present study is the first to demonstrate the expression of clusterin in skeletal muscle. The most striking finding was the high expression of this protein in target fibers.

Target fibers (TF), first reported in human skeletal muscle by Engel [6], are characterized by myofibers having three concentric zones (central, intermediate and outer). TF are detectable by phosphotungstic acid-haematoxylin, histochemical analysis or electron microscopy. There is considerable morphological and histochemical information about TF, but their morphogenesis and significance are still debated [8]. TF are generally interpreted as indicators of recent denervation [7], long-standing incomplete denervation [10], or reinervation [4].

The markedly increased immunoreactive clusterin in TF indicates that these structures are related to a process of apoptosis, and may represent an early sign of denervation. The expression of clusterin in TF seemed to be synchronous since the intensity and pattern of staining of the antibody was the same in all targets seen in each of the cases. The synchronic response of the skeletal muscle is another indicator that TF are probably related to denervation. We assume that denervation leads to apoptosis and one of the forms that apoptosis may be expressed in muscle is as targets. TF (and apoptosis) start in the center of the muscle fiber leading to degeneration of the myofibrils in the direction of the periphery of the fiber. As the process continues, targets «degenerate» and the process of apoptosis, that had started at the center of the targets, follows a course yet to be determined. The immunostaining pattern of the TF shown in Fig. 3 seems to support this theory — after the demarcation of the central area, the expression of the protein becomes absent in the middle of the fiber, while the periphery continues to show immunoreactivity. In addition, weaker immunoreactivity in TF presenting secondary degeneration, suggests that the expression of clusterin decreases with time after the lesion.

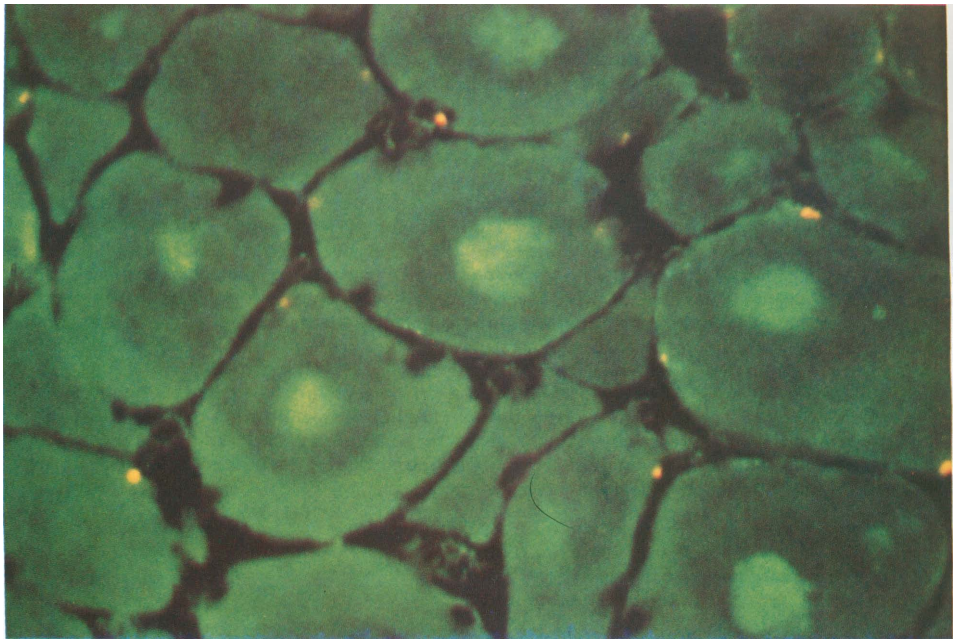

Fig. 1. Direct immunofluorescence stain with clusterin in a case with target fibers. Strong immune reactive deposits of clusterin are located in the center of the muscle fiber closely related to target muscle fiber as seen with NADH-tr at Fig. 2.

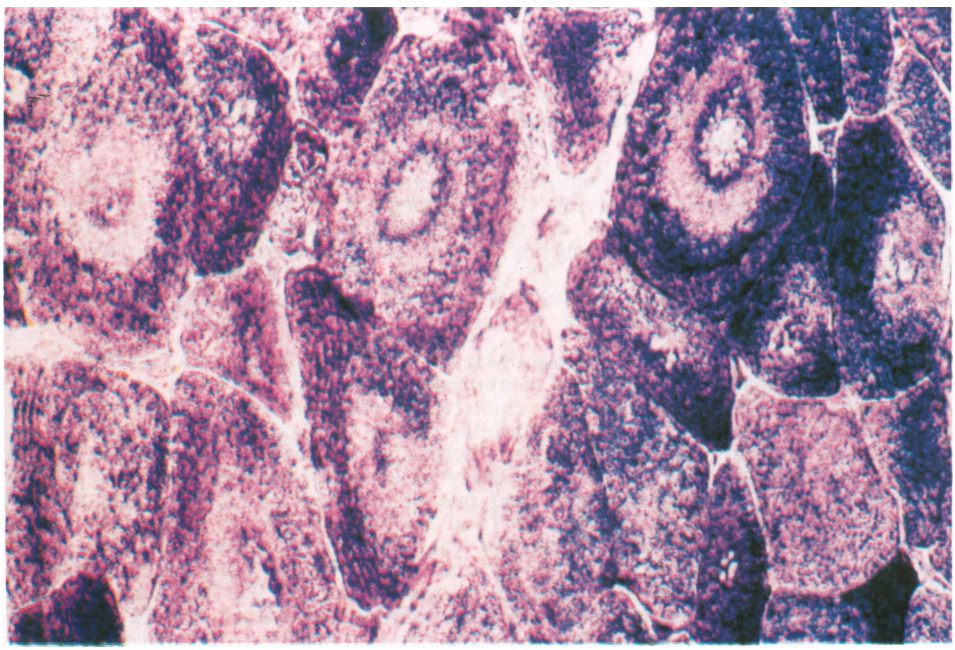

Fig. 2. Cryosection of the skeletal muscle stained with NADH-tr with many targets fibers. Note the three concentric zones: central zone (zone 1 - target); a loosened intermediate zone (zone 2) with a diminution of fibrillar structures; and a normal or slightly changed peripheral zone (zone 3).

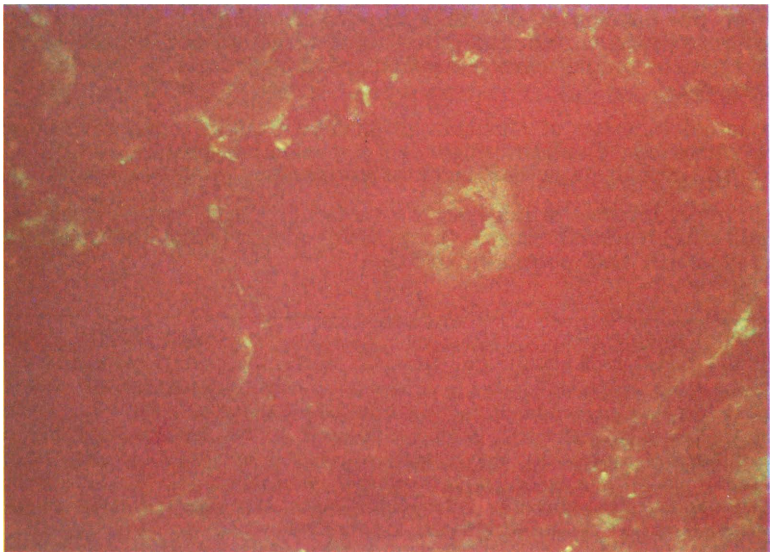

Fig. 3. Target fiber with secondary degenerative changes. Note absence of immunoreactive clusterin in the center of the fiber.

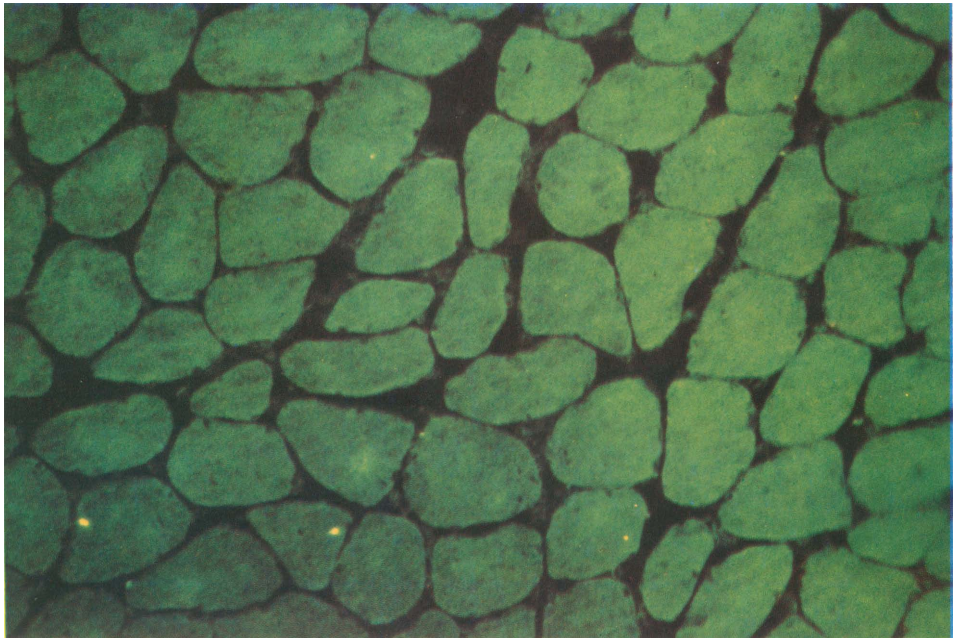

Fig. 4. Direct immunofluorescence stain with clusterin in a case with core fibers. No reactive deposits of clusterin are seen in the center of muscle fiber.

The expression of clusterin in TF, but not in targetoid fibers or in core fibers, structures that are similar by electron microscopy, indicates that these structures do not develop at the same time as TF, and do not share a common mechanism of appearance. On the other hand, targetoid/core fibers could appear at another moment of the denervation/reinervation process, when clusterin is no longer expressed.

The actual function of clusterin in skeletal muscle remains to be clarified. If TF are reversible, clusterin might be a protective protein, if not, clusterin would also be, in muscle, a harbinger of apoptosis.

Further prospective studies at the molecular level, with experimental target-like lesions in skeletal muscle, may better characterize the relationship between TF, clusterin and peripheral nerve.

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