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Skeletal Muscle Apoptosis: a Debated Issue Now Well Resolved in Favor of the Padua School of Skeletal Muscle. A Review

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ABSTRACT

In my research I have often found myself on the wrong side of the flow of international beliefs. I have generally been wrong and wasted my time and resources and my co-workers, but, sometimes, we have been on the right side. Such was the case with the role of apoptosis, also known as the programmed cell death, in biology and pathology of skeletal muscle tissue. Indeed, our original and pioneering findings have led to a change of direction in this research area. This role had been dismissed by the leading myologists, but using electron microscopy and molecular analyzes we demonstrated that accepted markers of apoptosis were present in mouse skeletal muscles two days after one night of voluntary running (up to 5 km during the first night). In a few years we have extended this fundamental observation to other experimental models in vivo and in vitro and in human cases of muscular dystrophies. In this paper I will give an overview of how the story began, but I must emphasize that Marzena Podhorska-Okolow and Marco Sandri deserve the highest praise for their most notable roles in the beginning and after, the roles and services that are still notable today.

KEYWORDS: skeletal muscle tissue, apoptosis, programmed cell death, skeletal muscle regeneration.

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Апоптоз скелетных мышц: обсуждаемый вопрос, который в настоящее время успешно решен в пользу Падуанской школы скелетных мышц. Обзор

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РЕЗЮМЕ

В своих исследованиях я часто оказывался не на той стороне потока международных убеждений. Как правило, я ошибался и тратил впустую свое время и ресурсы моих коллег, но иногда мы были на правильной стороне. Так обстояло дело с ролью апоптоза, также известного как запрограммированная гибель клеток, в биологии и патологии скелетно-мышечной ткани. Действительно, наши оригинальные и новаторские результаты привели к изменению направления в этой области исследований. Ведущие миологи отвергли эту роль, но с помощью электронной микроскопии и молекулярных анализов мы продемонстрировали, что общепринятые маркеры апоптоза присутствовали в скелетных мышцах мышей через два дня после одной ночи добровольного бега (до 5 км в течение первой ночи). Через несколько лет мы распространили это фундаментальное наблюдение на другие экспериментальные модели in vivo и in vitro, а также на случаи мышечной дистрофии у людей. В этой статье я расскажу о том, как начиналась эта история, но должен подчеркнуть, что Марцена Подгорска-Околоу и Марко Сандри заслуживают самой высокой оценки за их самые заметные роли в начале и после, роли и заслуги, которые заметны и сегодня.

КЛЮЧЕВЫЕ СЛОВА: скелетная мышечная ткань, апоптоз, запрограммированная гибель клеток, регенерация скелетных мышц.

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ABSTRACT

In my research life I have sometimes found myself on the wrong side of the stream of internationally published results. Often I was wrong and I wasted my time and my resources, but in a few cases I was on the right side. Either way, surfers say it is exciting to go against the tide. This typescript offer a short account of one of the few examples proving that sometimes I was right. In fact, it led to a change of direction towards an interesting area of research: muscle apoptosis, also known as programmed cell death. Rejected by leading myologists, we used electron microscopy and molecular analyzes to show that accepted markers of apoptosis were present in the mouse muscle two days after a night of voluntary running (up to 5 km during the first night). We extended this pioneering observation to other experimental models in vitro and in vivo and in human cases of muscular dystrophies [1–8]. For a recent general review of muscle cell apoptosis, please read: Kopeina G.S., Zhivotovsky B. [9], but now thousands of papers are listed in PubMed if one search for: skeletal muscle apoptosis. Thus, I was right.

Prof. Claudio Franceschi, an immunologist specialized in the study of centenarians, spent three years at the University of Padua, Italy working in a lab together with Paola Arslan, Marcello Cantini and a few pupils. On a spring day of 1994 he entered my lab asking for support to demonstrate that both myocardiocytes and skeletal muscle fibers may undergo apoptosis, a process that occurs every day in cells that die and regenerate continuously in labile tissues, e.g., skin epidermal cells and blood cells. The strange name describes the leaf fall that occurs each autumn in deciduous trees, with leaves falling to the ground to nourish the trees the following spring. Apoptosis is indeed a Greek word that describes the fall from the top of the tree (apoptosis means from high-fall). Though we know well now that the skeletal muscles (but not the cardiac cells, at least in large mammals) may die and regenerate, it was obvious for me that a kind of normal event must occur unrecognized in the tissue to explain the impressive potential of regeneration of the skeletal muscle tissue after a trauma or myotoxic injuries. Why not unrecognized cycles of death by apoptosis and regeneration?

On the other hand, only a severe trauma and ischemia or genetic muscle diseases were accepted as causes of death and regeneration of the muscle fiber of our muscles [10, 11], not functional events like those occurring in runners.

However, we had previously studied the impressive damage and regeneration that occurs in mice, which are released (offered an exercise wheel) after months or years of sedentary life in small cages. The mice follow their instincts and run up to 5 km during the first night, exhibiting extensive muscle damage and regeneration during the following week. It was in my mind the ideal experiment to

study skeletal muscle apoptosis during the first two days after running [1].

Starting from the first in vivo observations, we then demonstrated also in vitro that a stressed muscle tissue presents the well-known ultra-structural and molecular markers that characterize and allow to quantify apoptosis in labile tissues (blood cells and epidermal cells of skin and internal mucosae) [2–8].

In the spring of 1995 I organized the first world meeting on the role of apoptosis in the development, damage and repair of skeletal muscle and heart in mammals in Abano Terme (balneological resort in Padova, Italy). In addition to Italian and international clinical neurologists and cardiologists, the event was attended by molecular, cellular and structural myologists. We have successfully presented our results on skeletal muscle apoptosis in dystrophic mice after a night of spontaneous running in freewheeling cages [12]. We subsequently published two reviews [3, 7] that helped establish muscle apoptosis as an important topic also for human muscle biology and pathology [13]. A dream had come true.

Sudden Spontaneous Exercise Increases Apoptosis of Dystrophin Deficient Muscle

An apoptosis or a programmed cell death is an active multistep process characterized by morphological, biochemical and molecular events, which requires coordinated regulation of specific genes [14]. This program of cell suicide plays a major role in development, in tissues with high cellular turnover and contributes to the pathogenesis of several human diseases [15]. In vitro experiments on normal and dystrophin deficient myoblasts [1, 16] add information on regulation of myoblast proliferation, differentiation and death during regeneration of the skeletal muscle, but a little information is available on the role of apoptosis in adult muscles. Some observations come from studies on myocardium, since it can display apoptosis after ischemia and reperfusion [17, 18]. Accordingly, consistent with other published results, we showed apoptosis in the skeletal muscles of adult mdx mice in vivo [1, 16, 19–21].

One of the first genes up-regulated during programmed cell death is the ubiquitin gene [22]. In mammalians, different conditions of muscle wasting reveal an increased expression of ubiquitin [23]. To determine whether ubiquitin plays a role in progressive damage of dystrophic muscle we studied myofibers of mdx mice after a light spontaneous exercise. Sedentary mdx mice and congenit BALB/c mice were used as controls.

Light mycroscopy of muscles of dystrophic mice, both al rest and exercised, shows foci of muscle injury with inflammatory cells, small regenerating myofibers and myofibers with centrally located myonuclei, while muscles of sedentary BALB/c mice present homogeneous welldefined fibers with peripheral myonuclei. After immunoreaction with an anti-ubiquitin antibody BALB/c myofibers appeared to show poor reaction due to low level of ubiquitin expression in physiological conditions, while some cytoplasmic stain distinguishes between slow and fast fibers. Myofibers of sedentary mdx mice present a positive reaction in some peripherally-located nuclei, and in small regenerated myofibers.

On the other hand, in mdx muscle after exercise many centrally located myonuclei are positive both in small regenerating and in mature myofibers, while the foci of inflammation are negative. The high turnover of ubiquitin and the 24 hr of rest after exercise exclude that ubiquitin is induced in parallel with Heat Shock Proteins by the stress due to exercise per se. On the other hand, it is well documented that ubiquitin is tightly bound to histones or to some other proteins of the nuclear matrix after the DNA damage.

When the slides were processed for in situ analysis of DNA fragmentation, numerous myonuclei in exercised muscles of mdx mice were positive for apoptosis. As we described, muscles of sedentary mdx mice show 2–3 % of apoptotic myonuclei while BALB/c muscles are negative [2]. The increase of the percentage of positive myonuclei for ubiquitin in mdx muscles after exercise correlates with the increased number of apoptotic myonuclei.

When DNA analysis by pulsed field gel electrophoresis is performed on isolated myonuclei the results reveal that: i) No DNA fragments are detectable in BALB/c muscles; ii) some fragments at 200 kb and at 50 kb are present in muscles of sedentary mdx mice in good correlation with the 2–3 % of apoptotic myonuclei found with Apo-Tag kit; and iii) an increased amount of DNA fragments are detected in muscles of mdx mice after a sudden spontaneous exercise together with a smeared pattern of DNA, which suggests a complete digestion of DNA typical of the necrotic process.

The possibility that inflammatory cells contributed to DNA fragmentation is not excluded, but a myonuclear origin of the DNA fragments is suggested by the presence in myonuclei of apoptotic features detected by in situ nick-end labelling and by electron microscopy. Normal myofibrillar fields around apoptotic nuclei distinguish myonuclei from nuclei of satellite cells, endothelia, fibroblasts and eventual invading macrophages. In 15 % of nuclei of mdx muscles after exercising, electron microscopy detects typical features of apoptosis with a condensed chromatin around myonuclear membrane.

Massive activation of proteases is one candidate in triggering cell apoptosis and it is implicated in nuclear proteins catabolism and in lamin-DNA fragmentation. Which protease system it is associated with is not yet known; ubiquitin may be one candidate. Ubiquitin binding proteins for successive degradation, influences life of several important proteins for apoptosis such as p53, cmyc, BAG-I, and a relationship between ubiquitin and DNA fragmentation was clearly shown. When the distribution of ubiquitin and ubiquitin-conjugated proteins was investigated by SDS-PAGE and Western blot in supernatants and myofibrills of muscle homogenates, low level of free ubiquitin is constantly shown in all studied muscles, which is in good agreement with the published data. This observation could be related to the ceased expression of stress proteins two days after exercising, since shock and other stressors cause only a transient increase in free ubiquitin. In the soluble fraction of exercised mdx muscle we detect an increased content of ubiquitin-conjugated proteins compared with muscles of both mdx and BALB/c mice at rest: the exercised mdx muscles contain at least ten times of the amount present in the muscles of sedentary mice. Similar results are obtained in the myofibrillar fractions. The highest level of ubiquitination is detected in mdx mice after exercise. Densitometry of ubiquitin-reacting bands shows that ubiquitin

linked to contractile proteins increased two-three times in comparison with the ubiquitin amount of the mdx and BALB/c sedentary mice muscles. On the other hand, in situ analysis suggests that exercise-induced ubiquitin is preferentially linked with the nuclear proteins. This has been associated with DNA damage and could be important for fragmentation of histones or nuclear matrix proteins, as lamin, or for changes of the nuclear structure during the apoptotic process. Also, some myoplasm proteins were labelled indicating that proteinase activity is generalized. The widespread expression of ubiquitin and its capacity to link with multiple nuclear and cytoplasmic proteins suggests a major role in regulating apoptosis and other mechanisms of muscle damage. Recent in vitro studies underlay the role of cell death in regulating myoblast proliferation and fusion and this could be relevant in regenerating myofibers of mdx mice, in particular after exercise. On the other hand, in vivo apoptotic myonuclei were found in mature myofibers indicating a pathogenetic role of the mechanisms of programmed cell death in exercise-induced muscle damage in dystrophynopathies. The secondary pathogenetic processes by which a lack of dystrophin/dystrophin associated glycoproteins leads to progressive muscle degeneration in muscular dystrophies is an open issue. A number of possible mechanisms have received attention: changes in plasma membrane permeability, a specific defect in muscle intracellular free calcium homeostasis, and a decreased mechanical stability of the muscle plasma membrane and of the sarcomers. It is general expectation that exercise-induced damage plays a role in the myodystrophic process and that modifications of the training programs of muscles may have some importance in influencing muscle degeneration in patients with muscular dystrophies. It is well known that exercise in an unaccustomed muscle provokes mild injury, soreness and lactic acid accumulation. Our observations that a sudden spontaneous running in unaccustomed animals increases the number of apoptotic myonuclei in differentiated muscle fibers of adult mdx mice shed a light on the pathogenesis of the post-exercise muscle injury. We suggest that exercise-induced damage or fatiguing exercise itself activates the program of cell suicide in mdx muscle possibly because of unbalanced calcium homeostasis or because of an increased generation of reactive oxygen species during reperfusion. Muscle cells initiate the apoptotic process activating the process of DNA fragmentation and the protease system. Only some myofibers reach the final steps of apoptosis, i.e., chromatin condensation and apoptotic body formation. In spite of the clear difference between sedentary and exercised mdx mice observed, myonuclei showing apoptotic features by electron microscopy were one/half of positive myonuclei for both ubiquitin and in situ DNA end-labeling.

In conclusion, exercise-induced muscle damage in mdx mice suggests new roles of ubiquitin related to nuclear events, and it provides evidence for a new and provoking pathogenesis in dystrophinopathies, which could open new pharmacologic strategies in managements of exercise-induced muscle damage and muscle dystrophies.

The view that apoptosis precedes necrosis in the death of dystrophin-deficient muscle fibers of the mdx mouse, an animal model presenting mild muscular dystrophy, has been well substantiated [3, 7]. Additionally, apoptotic myonuclei have been reported to increase in dystrophin-deficient mice 2 days after sudden spontaneous runn [1, 2, 12]. To investigate the role of apoptosis in human muscular dystrophy, the muscles of 11 patients of different ages with Duchenne muscular dystrophy were analyzed for apoptosis [4]. Muscle apoptosis was evaluated by terminal deoxynucleotidyl transferase test and expression of bcl-2 and bax was examined by immunohistochemistry. Very rare in normal muscles of age-matched controls (less than 0.1 %), apoptotic nuclei have been detected in dystrophic muscles, particularly at the interstitial level. Furthermore, dystrophin-deficient myofibers with centrally located nuclei (regenerating myofibers?) showed a positive reaction for DNA fragmentation. A mosaic pattern of bcl-2 / bax-positive myofibers characterized the dystrophic muscles, so the relative proportion of pro- and anti-apoptotic proteins differs between muscle fibers in correlation with the presence of apoptotic myonuclei. In the interstitium, apoptotic cells were identified as macrophages and activated satellite cells. This was the first worldwide study to show an apoptotic process in the adult muscle fibers of patients with Duchenne muscular dystrophy [4]. It added an additional pathogenetic mechanism, shedding new light on muscle damage and its progression in dystrophinopathies.

Apoptosis has been detected in several muscle diseases, including severe dystrophin deficiency [3], but apoptotic mechanisms are not fully described in diseases of adult skeletal muscle [7]. Studying patients with Duchenne muscular dystrophy (DMD) and facio-scapulohumeral dystrophy (FSHD) we have shown an increase of apoptotic myofibers and of bax and bcl-2-positive myofibers [8]. A positive correlation was found between apoptotic nuclei and bax expression. Caspase expression was analyzed by RNase protection. DMD muscles expressed caspase 8, 3, 5, 2, 7 and Granzyme B mRNAs. Low transcription levels of caspase 6, 3 and Granzyme B were detected in FSHD patients. Tissue levels of the caspase 3 protein were significantly correlated with apoptotic myonuclei and with bax expression. Caspase 3 activity was increased in all DMD cases, while FSHD samples were heterogeneous. Caspase transcription was not detected in normal skeletal muscle.

These data indicate that human skeletal muscle fibers during the dystrophic process modulate the expression of caspases and that caspase 3 is involved in the death and progression of myofibers, opening new perspectives in pharmacological treatments of dystrophinopathies, such as the use caspase inhibitors.

ADDITIONAL INFORMATION

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Data Access Statement. The data that support the findings of this study are available on reasonable request from the corresponding author.

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