Role of Apoptosis in Muscle Disorders

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Abstract

Mononucleated cells from multicellular organisms self-destroy when no longer needed in organogenesis or when damaged. They do this by activating genetically controlled machinery that lead to apoptosis. Apoptosis has been described in developing and in adult human skeletal muscle. Alterations in the pathways that regulate myoblasts proliferation/differentiation processes lead to the induction of apoptosis during ontogenetic and regenerative myogenesis. Fully differentiated syncytial cells make skeletal muscle tissue in adults. In this case, apoptosis seems to start from segmental area of myofiber often producing loss of a single myonucleus. Tough apoptosis has been shown to occur in the skeletal muscle, the role played in neuromuscular disorders and the pattern followed in developing and adult muscle cells are far from being clear. The bcl2/bax system is active in muscle when apoptosis occurs, but conflicting results are reported on the role played by Fas/FasL and caspases systems. Some of the caspase cascades seem to be inhibited in adult myofibers, but others are activated in disease.

The role of apoptosis in such diverse pathological processes as tumour growth, immune response and neurodegeneration suggests that its regulation by drugs will become important to the medical community. A number of compounds have been used to inhibit or enhance some of the fundamental mechanisms of apoptosis. This knowledge is paving the path toward applications to treat muscular disorders.

Key words: anti-apoptotic agents, apoptosis, Bax, Bcl-2, caspases, Fas, FasL, muscular dystrophy, pro-apoptotic, skeletal muscle, therapy.

Basic Appl. Myol. 9 (6): 301-310, 1999

Apoptosis is a specific form of cell death, which in terms of tissue kinetics may be considered a mechanism that counterbalances the effect of cell proliferation. Apoptosis have been underestimated for decades, since the apoptotic cells shrink and are rapidly phagocytised by neighbouring cells. Time-consuming electronmicroscopy could only reveal this kind of programmed cell death. The first studies on apoptosis derived from the description of the developmentally regulated death of larval muscles [25]. In spite of these pioneering studies, myologists investigated this kind of myofibre death in skeletal muscle of adult mammals only in the last few years.

Disregulation of apoptosis has been implicated as a fundamental pathogenetic mechanism in a variety of diseases. Apoptosis plays an important role in the turnover of rapidly dividing tissue and decrease in the rate of apoptosis might have severe pathological consequences and might facilitate the growth of tumours. Immunologists have developed a strong interest in the apoptotic process, as it can regulate the development of an appropriate immune response and the host's response to viral infection. Indeed, many viruses circumvent the host cell's attempt to activate the apoptotic machinery, providing time for the virus to replicate. Neuronal apoptosis is now thought to play a significant role in the pathology of a number of degenerative diseases of central nervous system as well as in the periphery (for example, various neuropathies and retinal degeneration). During the last five years evidence had been accumulated that sarcomeric muscles (cardiomyocytes and skeletal muscle fibres) are also prone to apoptosis. This review highlights recent developments in the understanding of muscle cell death as well as new insights into the roles

played by apoptosis in neuromuscular disorders. Understandings of these mechanisms are providing new therapeutic strategies.

Necrosis and Apoptosis

Cells from multicellular organism self-destroy when no longer needed or when damaged. They do this by activating genetically controlled cell suicide machinery that leads to apoptosis. Survival in all cells from multicellular animals also depends on the constant repression of this suicide program by signals from other cells [51]. "Necrosis", the traditional description for cells that have ceased to demonstrate viability, is no longer believed to be the only term synonymous with a non-functional and non-viable cell. Necrosis has been associated with conditions that lead to cell membrane damage, osmotic imbalances, ion fluxes, cell swelling and death. The passive influx of Ca^{2+} is the hallmark mechanism of necrosis, and expression of new RNA is not required for the necrotic process [48]. Necrosis accounts for numerous destructive changes associated with pathological conditions such as energy deprivation, trauma and inflammation.

Apoptosis was originally distinguished from necrosis on the basis of its morphology [20]. The term was introduced by Kerr et al. [21] to describe a common series of morphological changes that accompany the death of cells in many tissues. The ultrastructural features of apoptosis suggested active cellular self-destruction, the cardinal events being chromatin compaction and segregation, rapid cellular condensation, budding to produce membrane-enclosed apoptotic bodies containing intact organelles, and disposal of these by neighbouring cells without the occurrence of inflammation. Subsequent molecular studies supported the distinctive nature of apoptosis, characteristic features being new gene expression and then internucleosomal cleavage of nuclear DNA. The digested DNA, when size-fractionated by electrophoresis, produces a "ladder" appearance consisting of multiples of 180 bp of DNA fragments [68]; this is in contrasts with the random DNA degradation observed in necrosis. Within the category of cell death, not all dying cells display the ultrastructural features of standard apoptosis and numerous examples to this fact have been reported in both invertebrate and vertebrate developmental biology research [24]. Finally, in the last years researchers showed that not all the necrotic cells started to dye following the necrotic pathway. Now it is well documented that, when it is impossible for the cell to observe the apoptotic program, the cell undergoes necrotic cell death even if the primary signal was for apoptosis. There is general agreement that apoptosis is an active, energy-requiring process. Cellular ATP level is an important determinant for cell death, either by apoptosis or necrosis [40]. When there is a severe drop in cellular ATP, controlled cell suicide ceases and necrosis occurs. So it is not surprising that apoptosis and necrosis could have the same pathway of activation. Trimerisation of Fas receptor could induce apoptosis or necrosis depending on the possibility to activate caspases [64]. Similar results were obtained using TNF [23]. Moreover apoptosis and necrosis may account for cell death in the same tissue, e.g., during infarction apoptosis and necrosis coexist. The severity of ischaemia and the time and degree of reperfusion determine what process is prevalent [16]. A particular cell undergoes one or the other type of cell death depending on the degree of injury and on the possibility to activate a gene expression programme.

Apoptosis of Myoblasts, Myotubes and Early Myofibres in Developing Skeletal Muscle

Like thymocyte and labile epithelia, myoblast is a stem cell stimulated to re-enter in the cell cycle. Its healthy is important for its destiny: whether it undergoes differentiation and fusion into a myotube or death before fusion. Consequently it is not surprising that damaging agents (Growth Factor withdrawal, DNA damage, oxidative stress, alteration in cell cycle process and in cellular metabolism) induce activation of the apoptosis. We recently reviewed inducers and effectors of apoptosis in replicating myoblasts [43].

During differentiation, myoblasts follow sequential events, which end with the fusion into myotube, a cell type more resistant to sublethal damage than proliferating myoblast.

It was first observed in insects that developing muscles undergo apoptosis [1]. A related type of fragmentation occurs in embryonic skeletal muscles of birds [28], and mammals [19], including humans [13]. Apoptosis in embryonic muscles is a well-known event [67,14]. Electron-microscopic studies of neonatal muscle degeneration induced by bupivacaine, notexin or cold showed apoptosis in rats younger than 5 days and necrosis in rats older than 7 day. This suggests that the mechanism of muscle cell death in response to injury depend on cell maturity [19]. If apoptosis and necrosis are clearly defined by morphological criteria, apoptosis seems to be a stereotyped cellular response to injury of immature muscles.

Observations of human embryos indicate that muscle apoptosis can appear at different stages of the development. At the myotube stage undesired cells are eliminated by apoptosis, thus so reducing the final number of muscle fibres. Between 20-24 weeks of gestation, single fibres are removed from the cluster, which is ultimately responsible for the final muscle cell size and shape. Loss of muscle cells as a result of enhanced apoptosis has been observed also in pathological muscle tissue in early postnatal life. In neonates with fatal form of Werdnig-Hoffmann disease partially immature muscle cells undergo deletion by apoptosis (see denervation and atrophy). As in many other tissues apoptosis is governed

by a developmental programme and is considered a process by which excessive immature cells are eliminated [43].

Apoptosis of Adult Myofibers in Animal Models of Muscular Disorders

In much degenerative and metabolic diseases, muscle fibres die without a marked inflammatory response and apoptosis could accomplish this, as we just have seen to occur during myogenesis.

Atrophy

A skeletal muscle fibre consists of many successive segments, each controlled by a nucleus residing in that part of fibre. Because growth and hypertrophy require the addition of nuclei to fibres [9, 41], it is of interest to determine whether atrophy causes a decrease in myonuclear density [17]. The role of apoptosis in eliminating myonuclei during development of muscle atrophy has been studied in hind limb unloading [1]. Apoptosis was detected by TUNEL assay and the double staining TUNEL/laminin allowed distinction of apoptotic myonuclei from interstitial cells. The number of TUNEL positive myonuclei is significantly higher in hindlimb-suspended than in control rats. Along the same fibre there are also TUNEL negative nuclei. Using confocal microscopy on isolated myofibres, 1 nucleus over 400 is altered. Combined treatments with growth hormone, insulin-like growth factor I and exercise training attenuate apoptosis and significantly decrease the number of fibres with morphologically abnormal nuclei. The relationship with some pro- and anti-apoptotic products has been studied in long-term denervation [59]. Denervated muscle constantly a display high numbers of apoptotic myonuclei, while reinnervated muscles show a distinct decrease of primarily elevated DNA-cleavage, resembling normal controls. Necrosis of muscle fibres is absent. Nearly all denervated muscle fibres show a strong expression of bax, while at the same time the anti-apoptotic bcl-2 and bcl-xL are expressed in 25% of fibres (mainly atrophic fibres).

By analysing the effect of experimentally induced heart failure in rat skeletal muscle we revealed a significant appearance of muscle atrophy paralleled by an increase in apoptotic myonuclei. We also detected a higher level of apoptosis in interstitial cells (mainly endothelial cells) suggesting an imbalance in myofibre nutrition, which triggers the preferential synthesis of fast-type isomyosins. In these animals apoptosis is paralleled by an increase in the circulating levels of TNF [65].

Exercise-induced muscle damage

Skeletal muscle damage occurs following excessive exercise [23]. The degree of damage appears to be largely dependent upon the nature of the predominant contractions. Repeated contractions in which the muscle undergoes lengthening contractions (eccentric activity) are considerably worse than those involving predominantly shortening (concentric) or isometric activity. These data imply that different types of activity cause muscle damage with different mechanisms [3]. After a night of spontaneous wheel running apoptotic myonuclei increase both in normal and adult mdx mice [25]. Western blot shows an increase of ubiquitin-conjugated proteins both in the soluble fractions and in the myofibrillar fractions of exercised mdx [47] and in human muscles two-days after high-force eccentric exercise [27].

DNA analysis and electron microscopy analyses confirmed these observations. Electron microscopy shows in fact the typical features of apoptosis with condensed chromatin around the myonuclear membrane [25, 47]. Eccentric electrostimulation of rat soleus and extensor digitorum longus at low frequency for 4-24 h damages numerous muscle fibres. Simultaneously several preserved fibres were not stained or were only discontinuously stained by the anti-dystrophin and anti-dystroglycan antibodies. Apoptotic nuclei, detected by TUNEL, are found within these myofibres [5]. The time-course study of post-exercise apoptosis in normal mice confirms the presence of peculiar apoptotic features in myonuclei at the end of a night of wheel running. Apoptotic nuclei are present both in myofibres and in endothelial cells of intramuscular capillaries [38].

Dystrophinopathies and related disorders

Dystrophin, the protein product of the human Duchenne muscular dystrophy (DMD) gene, is a minor component of the muscle membrane cytoskeleton and exists in a large oligomeric complex tightly associated with several surface glycoproteins. The dystrophin-glycoprotein complex appears to provide a linkage between the extracellular matrix component merosin and the sarcolemmal actin membrane cytoskeleton. In addition, the extracellular glycoprotein alpha-dystroglycan was shown to be a novel type of laminin/merosin receptor, which therefore links the dystrophin-glycoprotein complex to the basal lamina of the muscle periphery [35]. The detailed function of the glycoprotein complex is currently unknown, but it appears that a loss of part of the glycoprotein complex alone is sufficient to cause dystrophic degeneration of muscle even in the presence of normal dystrophin content [34].

The mdx mouse lacks muscle dystrophin and provides an animal model for the study of Duchenne muscular dystrophy. Characteristic changes occur in the phenotypic expression of the disorder during the life cycle of the dystrophin-deficient mdx mouse. There is little evidence of muscle degeneration during the first two weeks of age in this animal model, but at 14-21 days of age a period of acute muscle degeneration occurs. This is followed by important muscle regeneration so that at 40 days of age almost all muscle fibres undergo at least one

cycle of degeneration and regeneration. Degeneration of dystrophic skeletal muscle has generally been accepted to occur by necrotic pathways, but several studies suggest that during the phase of acute muscle degeneration in the mdx mouse, apoptosis precedes necrosis. After pioneering observation of single-strand DNA breaks in nuclei of a sub-population of cells within regenerating skeletal muscle of the dystrophic mdx mice [8], we and other authors have recently shown the occurrence of apoptosis in skeletal muscle of adult mdx mice by TUNEL method and DNA ladder analysis. The presence of apoptosis in mdx mice is estimated between 0.5 and 2% of total nuclei [44,60,27,50].

With western-blott techniques the expression of proteins with functions related to apoptosis (ubiquitin, c-myc, bcl-2 and bax) was also studied. Both bcl-2 and bax are expressed in mdx and control mouse muscle at all ages, although the level of Bax decreases with age. No changes in the pro- and anti-apoptotic products were found. The findings may account for the onset of muscle degeneration at 14 days in the mdx mouse. However, the decreased levels of bax protein in older mice may help to explain the apparent stabilisation of skeletal muscle in mdx mice older than 40 days [36]. All in all ubiquitin, c-myc and bax expression are altered at different ages in both groups of mice and these proteins may be involved in the active phase of the disease in the mdx mouse [63].

Mutations in genes encoding α, β, γ and δ- sarcoglycans or α2 chain of the basement membrane component merosin cause various forms of muscular dystrophy. Mice lacking these genes for the laminin 2 chain are characterised by growth retardation and severe muscular dystrophy and die within 5 weeks of age. Histological observations reveal that myofibre degeneration begins no later than 9 days after birth. Degeneration results in fibre size variability and proliferation of endomysial connective tissue at day 21-28. Compared to the muscles of the wild type, a remarkable number of TUNEL positive nuclei are present, and DNA ladder is detected [32].

In another study, analyses of integrins show an abnormal expression and localisation of α7β1 integrins in myofibers of merosin-deficient mice, but not in dystrophin-deficient and or sarcoglycan-deficient animals. Correction of merosin deficiency through cell transfection restores the normal localisation of α7β1 integrins as well as myotube survival. Over-expression of the apoptosis-suppressing molecule bcl-2 also promotes the survival of merosin-deficient myofibres but doesn't restore a normal expression of α7β1 integrins. The block of β1 integrins in normal myotubes induces apoptosis and reduces dramatically their survival [61]. Mice lacking γ-sarcoglycan gene show pronounced dystrophic muscle changes early in life. The loss of γ-sarcoglycan produces secondary reduction of β- and δ-sarcoglycan with partial retention of α-sarcoglycan and ε-sarcoglycan. On the other side mice show normal dystrophin content and localisation, demonstrating that myofibre degeneration occurs independently from dystrophin alterations. Furthermore, β-dystroglycan and laminin are left intact, implying that the mechanical link of myofibres is unaffected by γ-sarcoglycan deficiency. Apoptotic myonuclei are abundant in skeletal muscle lacking -sarcoglycan. TUNEL positive nuclei are most commonly seen in degenerating areas but are also seen in intact or partially degenerating fibres [15]. Vital staining with Evans blue dye reveals that muscle lacking γ-sarcoglycan develops membrane disruption as previously observed by Matsuda and co-workers in dystrophin-deficient animals [27]. The presence of TUNEL-positive myonuclei in intact, dystrophin positive muscle fibres suggests that sarcoglycan deficiency leads to apoptosis as an early event in the dystrophic process.

Primary cell cultures from control and mutant mice show no differences when plated on laminin-1. However, the phenotypes of the two populations are very different when plated on fibronectin. Mutant myoblasts adhere poorly, show little spreading and display a round morphology. Assay for apoptosis reveals that α5-expressing cells show similar percentages of annexin-positive cells on fibronectin or laminin-1, while α5-deficient cells show a four fold increase in annexin-positive cells for fibronectin compared to laminin-1.

Apoptosis of Myofibres in Adult Human Skeletal Muscle

Denervation, disuse, ischaemic atrophy and Spinal Muscular Atrophy

As described in animal models, muscle atrophy seems to be supported by myonuclear loss, which occurs with massive loss of cytoplasmic proteins [58]. In amyotrophic lateral sclerosis single or groups of small atrophic muscle fibres as well as clusters of piknotic nuclear clumps were detected. Type grouping, necrosis and myophagocytosis were never seen. TUNEL-positive myonuclei (44±16%) appear predominantly in atrophic fibres. All muscle specimens revealed an increased expression of bcl-2, bax and ICE. Bcl-xL was detected only in one case. While bcl-2 and ICE were only expressed in atrophic fibres, immunoreactivity for bax was also detected in some normotrophic fibres. There was no expression of Fas. Similar results were obtained analysing muscle tissue of patients with polyneuropathy. TUNEL positive fibres (14±9%) were mainly localised in atrophic fibres. In all specimens, atrophic myofibres expressed bcl-2 and bax, while ICE was detected in 50% of the samples. Fas was never detected and bcl-xL was detected only in one sample. Control muscles were not stained for bcl-2, bax, bcl-xL, ICE, Fas.

Spinal muscular atrophy (SMA) is a degenerative disease, predominantly of the lower motor neurones. One pathogenetic hypothesis invokes degeneration and loss

of motor neurones with neurogenic muscle atrophy due to deficient levels of the neuronal apoptosis inhibitory protein (NAIP) [42]. A case of a child who died 8 weeks after birth from acute infantile form of SMA has been reported. Electron microscopy revealed, between numerous necrotic fibres, the presence of cell fragments within the cytoplasm of healthy fibres [14]. The cellular fragments were identified as apoptotic bodies. Apoptotic muscle cells showed nuclei with abnormally convoluted morphology and with nuclear chromatin aggregated in dense masses. Myofibrils and sarcoplasmic organelles were compacted in dense masses. Subsequent studies revealed in SMA patients higher level of TUNEL-positive myonuclei than in controls. There were no differences among different SMA subgroups [57]. Migheli and co-authors analysed two patients with SMA I (3-5 months) and two patients with SMA III (15-19 years). In SMA I cases, most nuclei of atrophic fibres, as well as scattered nuclei of normal fibres, were TUNEL-positive. In SMA III cases, TUNEL staining showed frequent positive nuclei (2.3-3% of total myofibre nuclei) in subsarcolemmal location, not necessarily related to atrophic muscle fibres [31]. Pro- or anti-apoptotic proteins have been recently studied: the findings show a constant expression of bax in early-onset SMA atrophic as well as normo- and hypertrophic muscle fibres. ICE, bcl-2 and bcl-x are only expressed in atrophic fibres, predominantly in late-onset SMA. Bax expression is correlated with defective innervation of myofibres indicating an upregulation of N-CAM [46]. These findings suggest that expression of apoptosis-protecting factors such as bcl-2, is needed to neutralise high bax-levels, while lack of their expression in defective innervation will secondary promote muscle fibres death.

Inflammatory myopathies

In muscle biopsies from 5 patients with polymyositis, 4 with inclusion bodies myositis and 3 with dermatomyositis, the proportion of Fas-positive muscle fibres ranges form < 1% to 50% and is higher in polymyositis and inclusion bodies myositis than in dermatomyositis [4]. A high percentage of Fas-positive fibres co-expressed bcl-2 (63-100%). No sign of apoptosis was detected using TUNEL method in myonuclei and only <0.1% of inflammatory cells showed DNA fragmentation. The Fas-positive fibres are smaller than the Fas-negative ones and less than 10% of the Fas-positive co-expressed the regeneration marker CD56 (N-CAM). In ten patients affected by polymyositis analysed by using immunohistochemistry for Fas only four were studied for apoptotic DNA fragmentation [18]. Fas-positive fibres were 10.3±13.6% and no apoptotic nuclei were detected either inside myofibres or in the interstitium. RT-PCR for FasL transcript was performed in two cases and negative results were obtained. Migheli and colleagues showed DNA fragmentation and positive staining for CPP32 and ICH-1L in inflammatory cells of two cases of dermatomyositis and two cases of polymyositis [31]. Similar results were obtained by another group, which studied 6 cases of polymyositis revealing apoptosis only in interstitial cells (1-3%) [36]. Another study analysed 21 patients with autoimmune inflammatory myopathies: 9 dermatomyositis, 8 polymyositis and 4 inclusion bodies myositis [10]. No features of muscle fibre apoptosis such as DNA fragmentation or expression of apoptosis-related proteins were detected. Inflammatory cells show numerous TUNEL positive nuclei and expression of apoptosis-related proteins (bax, bcl-2, ICE). Apoptosis of myofibres (1.8%, 0.7%, respectively) has been detect in 2 patients with polymyositis and in one patient (1.1%) affected by dermatomyositis. In these cases of inflammatory myopathies, for the first time, a correlation between FasL/Fas system and muscle apoptosis has been observed [52].

Myopathies with mitochondrial defects

Myopathies with heterogeneous clinical features have been correlated with distinctive changes in the number and morphology of muscle mitochondria [26]. It is highly likely that myopathies are at least in part due to mitochondrial ROS production [53]. Mitochondria play a major role in the activation of apoptosis through the release of activating factors as APAF1 and AIF [16]. In spite of recent results suggesting that cytosols from skeletal muscle biopsies from healthy human volunteers lack the ability to activate caspases by a cytochrome c-mediated pathway [6], there is increasing interest on the possibility that apoptosis plays a role in myopathies with mitochondrial defects.

In muscles of patients with mitochondrial respiratory chain defects, the TUNEL method showed a high proportion of apoptotic myonuclei in all the 10 patients with mitochondrial myopathies and in one with multiple acyl-CoA dehydrogenase deficiency [33]. Recently a review on mitochondria in neuromuscular disorders has pointed out that muscles with mt DNA deletions have ubiquitin, bcl-2 and bax expression in ragged-red fibres [55]. The same authors also show TUNEL-positive myonuclei in ragged-red fibres, positive for the succinate dehydrogenase staining but negative for the cytochrome oxidase staining. Primary defect of Coenzime Q10 is characterised by exercise intolerance, mitochondrial myopathy, myoglobinuria, epilepsy and ataxia. After CoQ10 therapy, lipid accumulation disappears, CoQ10 and mitochondrial enzymes levels normalise, accompanied by an impressive proliferation of mitochondria. Assay for apoptosis reveals that TUNEL-positive nuclei are 30% and 50% in the CoQ10 deficient pre-therapy biopsies, and disappear after therapy [36].

Dystrophinopathies and related disorders

In contrast with the well described process of apoptosis occurring in muscles of human foetuses, until recently there were no electron microscopy observations of the occurrence of "standard" apoptosis in muscular dystrophies, in particular Duchenne and Becker. The presence of apoptosis in human muscle pathology is based on new molecular approaches [4]. Negative results were also reported in another study [18]. Similar results were obtained on muscles affected by spinal muscular atrophy [31]. Similar results were also obtained in samples of patients affected by myotonic dystrophy. Tews and Goebel [56] studied nine patients affected by Duchenne muscular dystrophy (4.7±1 years old), four patients affected by adhalin-deficiency (8.8±0.9 years old) and one affected by merosin-deficiency (5 years old) using TUNEL and immunohistochemistry for bcl-2, bax, ICE, bcl-xL. In dystrophin-deficient muscles 10.3±1.5% of intact, non-necrotic muscle fibres revealed DNA fragmentation, TUNEL-positive nuclei were also detected in numerous muscle fibres during phagocytosis and regeneration as well as in mononuclear cellular infiltrates. Morphological criteria supports the evidence that apoptosis occurs in nuclei of myofibres, myoblast and invading macrophages. The level of bcl-2 expression (from 1% to 25%) is higher than previously reported. All muscle fibres expressing bcl-2, bclxL and ICE are in a necrotic and regenerating state; within these lesions numerous TUNEL-positive nuclei were detected. Adhalin-deficient muscles show DNA fragmentation in 12.4±4.3% of non-necrotic myofibres and to a higher degree in groups of necrotic and regenerating myofibres. Pro- and anti-apoptotic products show a similar pattern of expression of dystrophin-deficient samples. In merosin-deficient muscles, 11.3% of non-necrotic myofibres revealed TUNEL positive nuclei. We analysed muscles from 11 patients with Duchenne dystrophy for a total of 12.000 fibres. Patients were selected from preclinic newborn (9 months) to 8 years old (mean = 5.4±0.7 years). Using a direct method to reveal DNA fragmentation (fluorescein-modified nucleotides) combined with immunohistochemistry for laminin to localise apoptotic nuclei, we found a significant difference between normal and dystrophic muscles [45]. Although very rare in normal muscles (less than 0.1%), apoptotic nuclei were detected in dystrophic muscles reaching a peak of 2.1%. The majority of apoptotic nuclei are located in the interstitium. Quantitative measurements revealed that the process is present in few dystrophin-deficient myofibres (0.35±0.3%). However the patients age seems to be an important factor: the highest amount of TUNEL-positive myonuclei is found in 4- to 7-year-old subjects. Newborn, preclinic patients show very few apoptotic fibres (0.07%) if compared to normal subjects (almost 0%) according to previous results [45]. A mosaic pattern of bcl-2 /bax-positive myofibres characterises dystrophic muscles and the difference in the expression of pro- and anti-apoptotic products correlates with the presence of apoptotic myonuclei. Several apoptotic cells in the interstitium are identified as inflammatory cells while others are recognised as activated satellite cells by immunoreaction against MyoD. Electron microscopy confirmed the presence of satellite cells with the peculiar features of apoptosis [45]. Myonuclear apoptosis, correlated to calpain 3 deficiency, was found in deltoid muscle biopsies of limb-girdle muscular dystrophy type 2A [2].

Perspective of Apoptosis-Based Treatments of Muscular Disorders

The key role of apoptosis in diverse physiological and pathological processes suggests that the pharmacology of apoptosis will become important to medical community. The mechanism of the agents could be divided in two broad classes: those that act extracellularly and those that interact with intracellular mediators of apoptosis. Compounds that modulate intracellular pathways can be further divided into inhibitors and enhancers of intracellular death activators and of death effectors.

Death effectors, such proteases, which can then lead to nuclear and cellular fragmentation, especially mediate morphological and molecular processes. Prior to the activation of these death effector pathways, the life and death balance of the cell is modulated by the complex interaction of death activators. Once the balance swings towards death, the death effectors start to disassemble the cell systematically. In the absence of these signals, it has been proposed that the default option for the cell is to commit suicide by apoptosis [51]. For example, adhesion to the extracellular matrix (ECM) by integrins is required for survival by many cell types.

Cell survival is determined by the appropriate exposure to growth factors. Two examples of the death cytokines are Fas ligand and tumor necrosis factor (TNF), which bind to nerve growth factor/TNF superfamily of receptors [30]. Inhibiting the actions of death cytokines, such as TNF, is an exciting way forward to inhibit apoptosis in a number of cell types. It seems likely that the protection is mediated by inhibition of adenylate cyclase following dual activation of Group II and Group III receptors [29]. Small-molecule inhibitors of this pathway already exist, and some of the pharmacology is already known. Most of the evidence supporting the link between ceramide generation and induction of apoptosis involves the activation of a particular isoform of SMase termed acid SMase. Subsequent activation of kinases and proteases either sequentially or in parallel depends on the nature of stimuli and cell type, or both [37,53]. Some of these pathways are thought to occur early in the apoptotic process to prime the cell for death and to activate the death effectors. Members of the proto-oncogene family that includes Bcl-2-like proteins are in-

volved in the control of apoptosis in a range of different cell types. The identification of selective inhibitors of the Bax-like proteins is likely to be crucial to understand the function of these proteins, as well as for the development of commercial compounds. Available inhibitors have been used in various cell-based models looking at the effects of ceramide and its metabolites, or both, as inducers or blockers of apoptosis [16].

Intracellular death effectors as targets of apoptosis pharmacology

Apoptosis is an event that requires disassembly of the cellular scaffolding, and proteolysis is clearly necessary for this to occur. Protease inhibitors of different specificity's, including serine and cysteine protease inhibitors, have been shown to inhibit apoptosis suggesting that apoptosis might require the participation of several classes of proteases [22].

Involvement of caspases in apoptosis of sympathetic neurones has been revealed by studies that have shown protection by crmA and p35, two viral inhibitors of these proteases, and tetrapeptide inhibitors. Caspase 1 was the first member of a family of cysteine proteases with the distinguishing feature of a near-absolute specificity for aspartate in the S1 subsite. Subsequently, further homologues of the caspase family were identified and for example, caspase-3, that has one of the greatest differences in sequence homology to caspase 1, has been implicated as a key mediator of apoptotic death in a number of different cell types.

Consequently, the pharmacological potential of calpain inhibitors is an area of developing interest. In summary, various groups of proteases can participate in the process of apoptosis.

Directions of Future Research

Beside the well know cases of apoptosis of myoblasts, myotube and young myofibres, this review summarised the evidence that conclusively demonstrate how and when adult myofibres disappear by apoptosis. It remains to be determined if some contradictory results in human neuromuscular disorders have to be ascribed to different causes. Sampling is a problem, since dystrophic muscles are usually poor in muscle fibres, and so the number of counted myonuclei could be too small to detect phenomena occurring at low-rate such as apoptosis. Of course, methods are of foremost importance. Indeed, in cell culture the TUNEL reaction labels from 20% to 90% of the cells with apoptotic morphology, depending on the method of fixation and permeabilisation used. Differential incidence of apoptosis in sedentary and exercised muscles could explain the differences in terms of different population of subjects. In myofibers of patients with highest TUNEL positive myonuclei there is a decreased expression of bcl-2 relative to bax, which promotes cell death. In control subject and in new-born pre-clinic patient the ratio was totally converted: a similar expression or an excess of bcl-2 relative to bax was found, which results in myofibre survival. Age seems to be a relevant factor in our series, and, together with the few cases studied by others, may explain negative results previously published.

On the other hand, in Duchenne Muscular Dystrophy events are scarce when compared to apoptosis in mdx mice. This raises the question of a species-specific event in mice. A second hypothesis is that the relatively low apoptosis detect in human dystrophic muscle could be part of the causal mechanisms, which allow progression of muscle damage, contrary to mdx mouse in which a burst of apoptosis could limit progressive muscle "death" and favour muscle regeneration and hypertrophy. Whatever the case, results are needed to weight apoptosis in progression of muscle dystrophies, and to recognise its role in either minimising or exacerbating muscle damage in different animal models and human dystrophies. Whether or not the results of our exercise experiments in normal and mdx mice are extended to human dystrophies, clinical implications in terms of secondary pathogenetic mechanisms, and muscle training are obvious.

At basic research level, we begin to understand the role and mechanism of apoptosis muscle cells. This could open new therapeutic approaches to preserve muscle function or increase muscle bulk. One clear direction is the experimental use of specific inhibitors of the caspase family in order to block apoptosis. On the other side, the resistance of normal adult myofibres to apoptosis could be used for over-expressing those genes, which inhibit the immune response against allografts and could prolong survival of transgenic myofibres.

Acknowledgements

The financial support of TELETHON-ITALY to the project "Role of apoptosis of myofibers, satellite cells and endothelia in exercise-induced muscle damage and in progression of muscular dystrophies (n. 968)" is gratefully acknowledged. Supported in part by funds from the Italian C.N.R. to the Unit for Muscle Biology and Physiopathology, and from the Italian Ministero per l'Università e la Ricerca Scientifica e Tecnologica (MURST) (contract n. 9806102428).

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