

## Expression of Bcl-2 Family Proteins in Recovering and Regenerating Muscles

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### Abstract

Bcl-2 proteins are known to control, either positively or negatively, the apoptotic process. The main objective of the research group is the study of the role of components of the Bcl-2 family not only in the apoptosis, but also during the skeletal muscle recovery from denervation-induced atrophy following reinnervation. Immunofluorescence and electron microscopy analysis of recovering fibres indicate that components of the Bcl-2 family are expressed within regions characterized by intense anabolism. Therefore, it emerges the possibility that Bcl-2 proteins, known modulators of apoptosis, may also act in other cell processes.

**Key words:** apoptosis, Bcl-2 proteins, muscle anabolism, muscle recovery, muscle regeneration.

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Apoptosis is a highly coordinated mechanism of cell death adopted to control tissue cell status (by eliminating unwanted or superfluous cells) in normal and pathological conditions. Stimulated cells respond by activating a genetically controlled cell suicide machinery that leads to apoptosis [8, 9]. Several pro- and anti-apoptotic intracellular elements able to control the apoptotic process have been so far identified. A number of pathways leading to apoptosis have been identified, with, as a common element, the activation of a family of cysteine proteases collectively known as caspases [5]. In the mitochondrial cell death pathway members of the Bcl-2 family play fundamental roles [15]. In fact, recruitment of pro-apoptotic members, such as Bax, activate caspases and apoptosis, while anti-apoptotic members protect the cell from apoptosis [15]. However, some aspects related to the actual role and mechanisms of action of Bcl-2 family proteins are still controversial [3, 15].

Recently, several workers have demonstrated significant apoptotic DNA fragmentation in skeletal muscles from animal models [21] and human muscular dystrophies [24, 25]. From these studies, it appears that apoptosis always preceded necrosis, even though, at later stages, necrosis appears to be the main cause of cell death. Consistent with this observation, it is now suggested that apoptosis occurs in only part of the multinucleated muscle fibre as a "segmental" process removing the neglected regions without perturbing the entire fibre [1]. This appears the case particularly dur-

ing muscle atrophy in which nuclei and cytoplasm loss are parallel events [6].

However, many questions concerning the apoptotic processes in the skeletal muscle tissue still remain open, especially considering the mechanisms adopted, the way unnecessary nuclei are removed, which and how apoptotic elements are recruited. Moreover, based on our recent findings of some apoptotic symptoms in the muscle overworked in an extended position [4], it appears of interest to investigate whether in normal mature skeletal muscle some apoptotic processes is activated even without the intention to provoke cell death. In fact, we found indications that some apoptotic processes could be adopted by skeletal muscle as a physiological mechanisms to adapt its structure and dimension to new functional demands [4]. Therefore, our group is interested to investigate whether the apoptotic pathway is activated also in skeletal muscle undergoing intensive anabolic processes as during muscle recovery after atrophy and in the regenerating muscle.

### Experimental protocols

As an experimental model of high anabolism, we utilize skeletal muscles recovering from denervation-induced atrophy by self-reinnervation. In such muscle, most of the fibres are simultaneously subjected to intensive anabolic processes. This experimental model has been used several times in the past for biochemical and morphological examination [10, 13]. In addition, skeletal muscle recovering after eccentric exercise is very convenient model to study muscle regeneration [19]. In

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fact, eccentric exercise is well known to cause severe injury to muscle fibres. Within the damaged regions, regeneration starts very soon from satellite cells, so that in few days myotubes are formed beginning a period of high anabolic metabolism.

For the experiments we utilize soleus muscles from 3-month old Wistar female albino rats. Denervation is produced under ether anaesthesia either by cutting or crushing the sciatic nerve, to investigate muscle atrophy or recovery of the denervated muscle following reinnervation, respectively [13]. Muscles from the contralateral leg and from legs of non-operated animals normally serve as controls. Eccentric exercise of the soleus muscle is produced by keeping the muscle in an extended position by immobilization of the ankle joint in a plastic tube at an angle of 90°, while the sciatic nerve is continuously stimulated for 5 h by pulses of 0.3 ms duration at 20 Hz frequency, as previously described [4, 12]. Stimulating electrodes are implanted on the sciatic nerve, under pentobarbital and ether anaesthesia, few days before the experiment. As controls, untreated muscles, muscles stimulated without joint immobilization and muscles immobilized without stimulation are typically used.

Muscles are quickly excised immediately after decapitation (under ether anaesthesia), attached to plastic rods, as previously described in detail [11] and immediately subjected to further procedures. For ultrastructural studies, muscle fixation, embedding and sample preparation were applied as previously described in detail [11]. Ultra-thin sections are prepared on an LKB Ultratome R III and inspected with JEM 1200 EX or JEM-100B electron microscopes.

For histological and immunohistochemical studies, muscles are snap-frozen in isopentane cooled in liquid nitrogen and stored at -70°C. Cryostat transverse sections (8 µ) are typically utilized and treated, as previously described [4], with selected antibodies, such as, for example, antibodies specific for several components of the dystrophin complex (Novocastra, Newcastle upon Tyne, UK), desmin (Cell Signaling Technology) embryonic myosin heavy chain (generous gift of S. Schiaffino), Bax and Bcl-xl (Santa Cruz Biotechnology).

### Results and discussion

Present research project is based on preliminary observations obtained in skeletal muscle of a possible alternative, side, action of components of the Bcl-2 family, in non-apoptotic processes and in particular during period of intense anabolism. For such study we are utilizing two distinct experimental models: 1) skeletal muscle recovering from denervation-induced atrophy by self-reinnervation and 2) skeletal muscle recovering after eccentric exercise.

As previously described, when the rat sciatic nerve is crushed, denervation atrophy of soleus muscle progresses for about 2 weeks, as it occurs cutting the nerve. Then, nerve regeneration determines the re-establishment of muscle innervation, so that muscle recovery takes place

ensuing the rescue of most normal muscle characteristics in few months [10, 13]. In the planned experiments, we check carefully the characteristics of denervation atrophy and muscle recovery, both by electron and light microscopy. The characteristic signs of denervation atrophy observed at the 14<sup>th</sup> day in soleus muscle after sciatic nerve crushing are indistinguishable, at the same date, from those in the muscle where denervation was produced by cutting the sciatic nerve. For example, just to mention few aspects that are important for the general interpretation of our work, we report the uniform diminution of fibre diameter, the presence of folding of sarcolemma, the substantial decrease of mitochondria number (only sparse dark mitochondria are typically seen among myofibrils and in subsarcolemmal space), the narrow subsarcolemmal space and diminished proportion of myosin-to-actin filaments (Figure 1a). The last phenomenon, together with the decreased muscle content of myosin, is characteristic of soleus muscle denervation atrophy, as we documented earlier [10, 13]. In summary, these data confirm the effectiveness of muscle denervation after nerve crushing.

According to previous work, the processes of muscle recovery after re-innervation are intensive between the 20<sup>th</sup> and 35<sup>th</sup> day after sciatic nerve crushing, when muscle fibres considerably increased in size [10, 13]. Under electron microscope, among other features of the recovery, it is typically observed: the accumulations of polyosomes and, endoplasmic reticulum and granular material within subsarcolemmal regions of numerous muscle fibres (Figure 1b). Within these regions, it is noted the presence of mitochondria, endoplasmic reticulum, Golgi-system vesicles, lysosomes, free myosin and actin filaments, and few small irregular myofibrils, resembling those observed in the developing muscle. Consistently, haematoxylin-eosin staining demonstrates the presence of basophilic rings in the sarcolemmal regions of many muscle fibres (data not shown). Thus, protein synthesis appeared to be particularly intensive within subsarcolemmal areas of the recovering muscle fibres.

However, it worth mentioning the occurrence of some heterogeneity in the dimension of fibres within particular muscle regions, and of neighbouring fibres within the same region. Namely, numerous fibres were increased in size, while the size of others was still comparable to that found in denervated muscles. Nevertheless, all fibres, either large or small, appear well preserved with good outlined sarcolemmal regions, as documented by both morphological and immunohistochemical analysis (Fig. 2a). The observed heterogeneity in fibre diameters suggests the occurrence of non uniform reinnervation and, thus, non simultaneous beginning of recovery of muscle fibres. The larger fibres are obviously more advanced in recovery.

Surprisingly, numerous large muscle fibres of the recovering muscle show strong reaction with antibodies specific for Bcl-2 family proteins, both anti- and pro-

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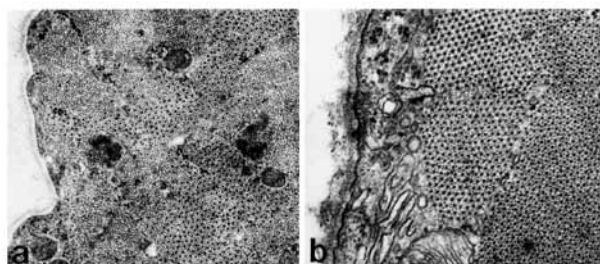


Figure 1. Electron micrographs of transverse sections from soleus muscle at the 14<sup>th</sup> (a) and 20<sup>th</sup> day (b) after crushing the sciatic nerve. (a) It can be noted the folding of sarcolemma, the presence of irregular contractile structure, the substantial reduction of the myosin-to-actin filaments ratio, and the presence of few small and dark mitochondria. (b) Within the subsarcolemmal space it can be seen the accumulation of polysomes, Golgi system vesicles and granular material, while the contractile structure looks regular. Magnification: (a) 27 000x and (b) 35 000x.

apoptotic ones. This reaction was concentrated within subsarcolemmal regions of the fibres (Fig. 2b). In parallel, given the particular importance of caspase-3, -7 and -9 in the apoptotic processes [2, 7-9, 15], we examine the expression of active caspases in recovering fibres. To our surprise, preliminary experiments show that the reaction with antibodies specific for activated caspases is always negative in the serial sections of the muscle fibres reactive for the Bcl-2 family proteins. In addition, no morphological features of apoptosis is detected in the recovering muscles. Diversely, denervated atrophying muscle fibres are stained by anti-caspase antibodies; in the same fibres also some staining with antibodies specific for Bcl-2 family proteins is evident. These initial findings deserve more in depth study, especially in the light of recent results showing the expression of apoptosis-related proteins in neurogenic disorders [20].

The above mentioned observations, concerning the recovering muscle, are made on the 19-35<sup>th</sup> day after crushing the sciatic nerve. During the following weeks, the diameter of muscle fibres became more uniform, and most of the muscle characteristics resemble those of control muscle. Importantly, the reaction with antibodies specific for Bcl-2 family proteins is no longer noted.

Expression of components of the Bcl-2 family in the recovering muscle is in some way a puzzling phenomenon. Proteins of the Bcl-2 family are regulators of the apoptotic machinery [9, 18], and, to our knowledge, this is the first time it is reported their possible involvement in non-apoptotic processes. In the recovering muscle, which is highly anabolic and growing in mass, apoptosis can be relatively excluded, especially considering that Bcl-2 proteins are particularly evident within fibre regions of very intensive anabolic processes. Therefore, it appears that expression of the Bcl-2 proteins is associ-

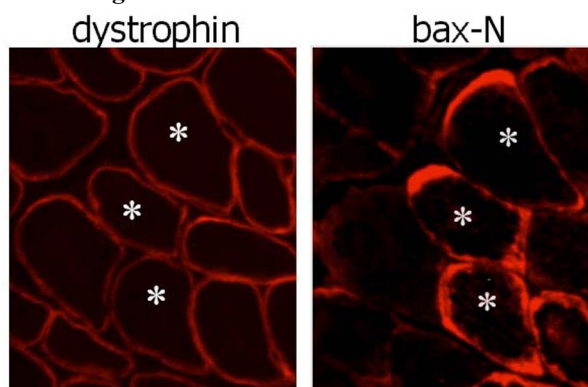


Figure 2. Immunofluorescence analysis of the soleus muscle recovering after 32 days of sciatic nerve crushing. Serial cryostat transverse sections were stained with antibodies specific for dystrophin (left panel) and bax-N (right panel). Indicated by asterisks are three fibres with normal dystrophin distribution at sarcolemma and intense sub-sarcolemmal staining for bax-N.

ated with the anabolic situation of the cell. Based on our preliminary findings, it appears, however, premature to speculate on this possible novel function of Bcl-2 family proteins, even though identification of new members of the Bcl-2 family, which number and knowledge of their selective functions is still growing [14, 23], would offer new insight on this aspect.

To obtain additional information about the “diverse” role of the Bcl-2 family, we are performing also experiments on the regenerating muscle after eccentric exercise. As mentioned above, sustained eccentric exercise, i.e. repeated contraction during which a muscle is extended is known to cause severe injury of the muscle fibres. In the damaged regions, recovery of muscle is usually denoted by diffuse regeneration [16, 19]. Three days after eccentric exercise we found groups of myotubes, as identified by the presence of embryonic myosin and desmin. Within myotubes, proteins of the Bcl-2 family are also detected. Their expression within myotubes is also very puzzling, especially considering that no signs of apoptosis are appreciable. Therefore, again, the explanation for the presence of Bcl-2 family proteins in myotubes could be their involvement in non-apoptotic processes.

### Perspectives

Taking together, our preliminary results suggest that components of the Bcl-2 family are expressed in non apoptotic fibres during the recovery both after denervation atrophy and after eccentric exercise muscle fibre damage. They appear in the subsarcolemmal regions of the muscle fibres recovering after denervation atrophy, where intensive anabolic processes occur. These proteins are also expressed in new formed muscle fibres that are not going into apoptosis. Therefore, components of the Bcl-2 family, normally considered as modulators of apoptotic processes, appear also to play important role, at least in skeletal muscle fibres, in non-apoptotic cell proc-

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esses. To our knowledge, this is first demonstration of a non-apoptotic function of components of the Bcl-2 family.

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