Apoptotic Myonuclei Appear in Adult Skeletal Muscles of Normal and *mdx* Mice after a Mild Exercise

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Abstract

It is well known that muscle damage may be induced by eccentric exercise, but foci of real myonecrosis appear few days later after the exercise. In the present paper we show that normal and dystrophic mice muscles, after all-night spontaneous running in a wheel-cage, present DNA strand breaks that can be interpreted as the initiation of an apoptotic process. Apoptosis was found both in postural muscle (slow, fatigue-resistant soleus) and in fast muscle (fatiguable EDL and tibialis anterior), but real myonecrosis follows only in slow myofibers with prevalent oxidative metabolism. Apoptosis was absent both in normal non-runner and in dystrophic non-runner mice muscles. Apoptosis may be correlated to disturbance of blood flow or to production of reactive oxygen species during reperfusion. The observation that myonecrosis follows DNA damage only in slow myofibers supports the hypothesis that DNA damage is probably due to a non-compensated over-production of reactive oxygen species able to produce an irreversible injury only in myofibers where the oxidative metabolism is prevalent.

Key words: apoptosis, damage, eccentric, exercise, injury, mdx, mouse, muscle, myonecrosis, skeletal.

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Apoptosis, also called "programmed cell death" [7], is a specific form of cell death that plays an important role in development, growth regulation and diseases. Apoptosis in thymus, where it has been extensively studied, is considered either a lack of T-cell positive selection in the cortex area or a true negative selection in thymus medulla [18]. Apoptosis occurs during embryonic development and in adult, particularly in tissues undergoing reversible expansion, in which cell number homeostasis is under hormonal control. The morphological and biochemical events in apoptosis had been extensively studied, but little is known about the stimuli that prime cells for apoptosis. Priming is reversible and may be part of a strategy in the regulation of cell populations, since cells primed for apoptosis are all doomed to die unless rescued by specific growth factors.

More clues to the nature of the apoptosis triggering are coming from the literature, i.e. increase in cytoplasmic Ca^{2+}-concentration, protein kinase C activation, Ca^{2+}-Mg^{2+} dependent and independent endonucleases activation etc. [1, 5, 12]. Apoptosis follows triggering immediately, but the time between priming and triggering may vary [13].

Few information are available about apoptosis in fully differentiated cells, but massive muscle cell elimination by apoptosis has been described in acute infantile spinal muscular atrophy [6]. Interestingly, in post-natal development of dystrophin-deficient muscles apoptosis seems to precede any detectable necrotic change and to be present during dystrophic pathology also in adult life [19].

Using a terminal deoxynucleotidyl transferase (TdT) mediated end *in situ* labelling of DNA in tissue sections

we were able to demonstrate that under a mild muscle exercise induced by spontaneous running in a cage-wheel for one night normal and *mdx* adult mouse muscles show an appreciable amount of labelled myonuclei (apoptotic nuclei) when compared with non-runner mouse muscles, indicating that eccentric exercise was followed by signs of nuclear DNA damage and acute inflammation.

Materials and Methods

Adult C57Bl and *mdx* mice of both sexes were used. They were individually housed in a wheel-cage for one night and let them spontaneously running until the day after. After two days in normal cages the "runner" mice and the sedentary controls (which were not wheel-caged) were sacrificed. Muscles were excized from C57Bl mice and *mdx* mice: the left muscles were rapidly frozen in liquid nitrogen; 5-6 μm sections were cut in cryostat. The right muscles were paraffin embedded. Cryostat sections were air-dried, fixed in acetone. All sections were incubated in 2% H_2O_2 diluted in Tris buffer-saline, pH 7.4 to neutralize endogenous peroxidase (quenching). To detect cells with DNA strand breks we modified the method ApopTag (Oncor) as follows: sections were washed once with 0.5 μM cacodylate, pH 6.8, for 1' at room temperature, then 54 μl of 0.5 mM TdT, 0.05% BSA, 0.15 M NaCl (working strength TdT-enzyme reaction buffer) with 2-4 μM digoxigenin-conjugated dUTP were added and incubated 1 hr at 37°C. After washing in Tris saline three times, sections were incubated with 5 μg/ml Fab antibody specific for digoxigenin conjugated with horseradish-peroxidase HRP in Tris-saline, and then washed three times in PBS. Bound HRP was detected with 0.05% of the substrate 3'-diamino-benzidine (DAB, Sigma) in 0.17 M sodium acetate, pH 5.2, plus 0.01% H_2O_2, staining buffer. Incubation was continued until development of the color. Sections were then washed for 5'. Sections were lightly counterstained with haematoxilin and eosin or methyl-green for nuclei, dehydrated and mounted. Sections were then photographed under a Leitz Axioscope microscope [2, 16]. In every experiments positive and negative controls were run. Positive controls were thymocytes incubated with dexamethasone for 3 and 6 hrs. In negative controls TdT enzyme was omitted.

Results and Discussion

TdT-mediated dUTP end-labelling detects DNA strands breaks in cells presumably undergoing apoptosis. Thus, unlike normal cells, apoptotic nuclei incorporate exogenous nucleotides (dUTP) in the presence of TdT. When amplified with a three step staining procedure, ApopTag method readily detects apoptotic cells in cryostat muscle sections. In preliminary experiments we noted that under conditions suggested by the producer the percentage of positive myonuclei in normal non-runner mice muscle was so high that it was logical to classify the results as experimental artifacts. The protocol was therefore optimized by us using thymocytes and developing muscles, i.e. well-known cells and tissues that under determinate conditions present apoptosis. Under the new experimental conditions normal adult non-runner mice muscles become negative, while a large number of myonuclei became positive to ApopTag in both *mdx* and normal runner mice. Under these conditions, in normal and dystrophic non-runner mice muscle, ApopTag staining was practically undetectable (results not shown), while in normal and dystrophic runner mice (Fig. 1b and Fig. 1d) more than 50% of myonuclei showed DNA strand breaks. In Figure 1 the results obtained in normal and *mdx* running muscles are shown. In particular, the peripheral myonuclei of normal runner mice stained with hematoxilin-eosin (Fig. 1a) are positive for apoptosis detected with ApopTag method (Fig. 1b). The *mdx* runner mice muscles shows foci of chronic inflammation with the presence of leucocytes infiltration and regenerated myofibers with centrally located myonuclei (Fig. 1c). Fig. 1d shows that inflammatory cells (leucocytes with green nuclei) are negative for apoptosis while centrally located myonuclei are positive for apoptosis (brown).

The percentage of positive myonuclei (more than 50%) for ApopTag in *mdx* and normal runner mice muscles raises the problem of the significance of the phenomenon because the percentage of positive myonuclei largely exceeds the percentage of the slow-type myofibers that are well-known to be prone to undergo to necrosis after such a mild exercise like the spontaneous running in a wheel-cage.

In contrast with report recently published by Tidball et al. [19] no difference was found between normal and dystrophic mice muscles either in runner or in non-runner animals indicating that, at this age, both normal and dystrophic muscles had reached a cellular homeostasis, that in terms of frequency of cell death is about the same.

To exclude artefact, ApopTag staining was checked with agarose gel-ethidium bromide eleectrophoresis and/or SDS-PAGE followed by silver stain, techniques which reveal DNA laddering or fragmentation (manuscript in preparation). In muscles were myonuclei were positive for Apoptag staining, DNA ladders were detectable after 30' minute of incubation of excized muscles at 37°C in 5% CO_2-air atmosphere.

Muscles in adult animals must be considered fully differentiated cells and apoptosis, might occur as a consequence of a variety of causes. Regeneration after injury, if takes place, starts from satellite cells [11, 17], substituting the loss of myofibers. It is well known that exercise in a unaccustomed muscle provokes lactic acid accumulation, soreness and mild-injury. Smith [17] suggested that in adult muscle the events observed after an eccentric muscle exercise, as the running in a wheel, that in humans are the so-called delayed onset muscle soreness (DOMS), reflect the typical sequence of acute inflammation. In particular, the proposed sequence of events was divided in 24 hrs time lapses after exercise and comprises accumulation of neutrophils within the first 24 hrs, release of lysosomal

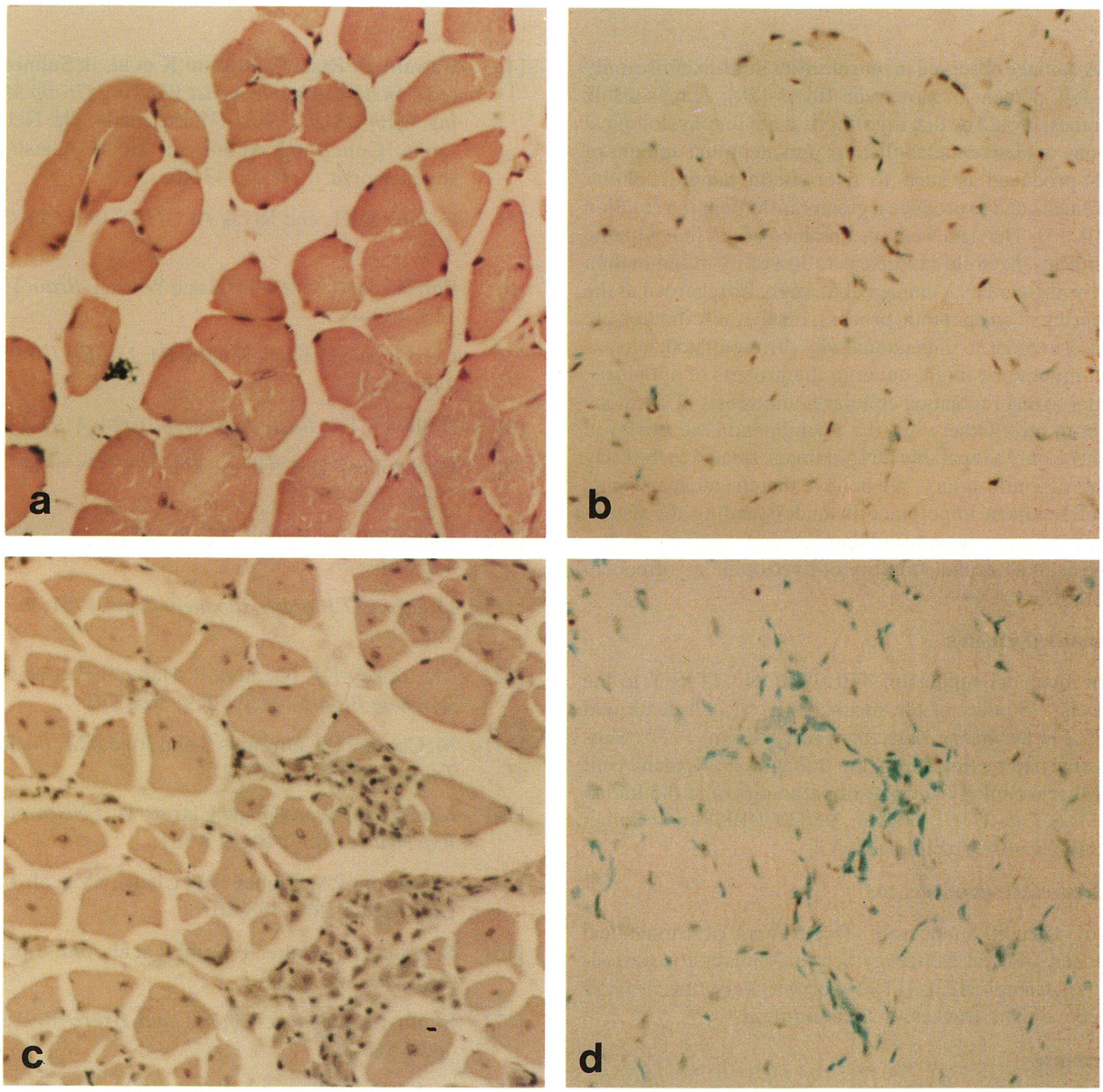

Figure 1. In situ detection of apoptotic myonuclei with a modified ApopTag method in sections of skeletal muscles of running normal and mdx adult mice. Positive myonuclei appear brown. a: hematoxilin-eosin stain of a muscle section of a runner normal mouse; b: ApopTag positive myonuclei (brown) in the presence of counterstain with methyl-green (green) in a muscle section serial to that of panel a (runner normal mouse); c: hematoxilin-eosin stain of a muscle section of mdx *runner mouse; d: ApopTag positive myonuclei (brown) in the presence of counterstain with methyl-green (green) in a section serial to that of panel c (mdx runner mouse). Note the central location of ApopTag-positive myonuclei.*

enzymes in the time lapse of 48 hrs, release of inflammation mediators until the 72nd hr and signs of repair beginning at approximately 72 hr [3, 8].

The data here presented shed a light on the pathogenesis of the post-exercise muscle injury, since apoptosis, detectable after the exercise, precedes any sign of necrosis, indicating that DNA damage is present while inflammation is in progress [4]. In line with a recent report that apoptosis precedes necrosis in dystrophin-deficient muscle of young mice [19], in *mdx* adult muscle, apoptosis was present after eccentric exercise but the number of apoptotic nuclei seems not different when compared to the number of nuclei of normal muscle after exercise. The observed DNA damage, probably the initiation of an apoptotic process, was presumably due to an accelerated muscle metabolism during exercise or to the production of reactive oxygen species (ROS) at the mitochondrial or endoplasmic reticulum level, not compensated by normal glutathione cycle and/or cellular ROS scavengers. Of particular interest is the observation that a real myonecrosis follows the

DNA damage observed in muscle after mild-exercise, only in a few percent of slow-type fibers [20]. A reasonable hypothesis could be that myofibers, were for physiological reasons oxidative metabolism is prevalent, the amount of ROS produced is such to overwhelm natural cellular scavengers and to produce a greater inflammation reaction [9, 10, 15]. The data we have obtained are in line with the hypothesis that mild exercise is followed by inflammation and by nuclear DNA damage that might be regarded as the triggering of an apoptotic process. Further information are needed in order to understand why differentiated cells, as adult myofibers, might undergo the process of apoptosis, similar to that regulating cellular homeostasis of hormone target tissues. Otherwise, the phenomenon we observed might be only a reparable DNA damage related to the early events of a mild-injury. We believe that the nuclear events are of foremost importance in understanding the pathogenesis of exercise-induced damage and supports the hypothesis of a peculiar fragility of dystrophic myofibers to normal-life stress.

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