Myoblasts and Myotubes in Primary Cultures Deprived of Growth Factors undergo to Apoptosis

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Abstract

Previously we demonstrated that programmed cell death (PCD) occurs in normal and *mdx* muscle after extempore spontaneus exercise. The results induce us to investigate the program of apoptosis during myogenesis *in vitro* and *in vivo*.

In the present paper PCD was studied during different myoblast culture, before and after the myoblasts fusion.

Our results indicate that myoblasts and to a lower extent also myofibers possess the machinery of apoptosis. During the peak of maximal proliferation, apoptosis reaches its maximum, but it is present also, although at very low percent (less than 1%), in the myofiber nuclei. Normally, during culture times cells that are going toward PCD, change their shape from elongated to round shape, they detached from gelatin-substrate and they floated on the culture-medium. These cells are positive for nick-end-labelling ApoTag, they show a contract, rosette-like nucleus stained with acridine-orange and they show an increase of ubiquitin at nuclear level when treated with anti-ubiquitin policlonal rhodamine-coniugated antibodies.

Key words: apoptosis, ubiquitin, myoblasts cell culture, myotubes.

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Introduction

Apoptosis, also called programmed cell death (PCD), had recently acquired a strategical importance in regulating many physiological and pathological processes. During development, apoptosis regulates the regular progression of developmental process in embryogenesis. In the adult life apoptosis keeps the cell number balance in proliferating tissues and, surprisingly enough, plays some role also in stable tissue [1]. Apoptosis is a physiological process, gene regulated, that induces cell death by a direct damage to DNA without provoking inflammation [2].

Programmed cell death had been demonstrated to occur not only in tissues with high cellular turnover but also in fully differentiated cellular structure as adult myofibers [3,4,5,6,7]. In normal and *mdx* dystrophic adult muscles, after estempore spontaneous exercise, very few myonuclei peripherally-located in normal and the centrally-located myonuclei of the small regenerating dystrophic fibers were positive to the nick-end labelling reaction [4]. Ubiquitin revealed with a specific immunoreaction was also increased at the nuclear level in the myonuclei positive for apoptosis (paper submitted).

The objective of the present study was to check how proliferation of myoblasts *in vitro* is regulated before fusion into myofibers. It is well known that during *in vivo* development, the first myoblast generation, primary myoblasts, although able to fuse, early disappear probably via apoptosis, and only secondary myoblasts are giving origin to definitive myofibers [8, 9].

In myoblast culture that mimics *in vitro* the *in vivo* myogenesis, both primary and secondary myoblasts might be present, but primary myoblasts might be eliminated via apoptosis. In any case, not all myoblasts are giving origin to mature myofibers, some are doomed to die via apoptosis. That cells detache themselves from the normal gelatin layer, change from elongated to round shape and float on the medium surface during culture time it is well known phenomenon to people working in this field. These floating cells loose their vitality and they are freely permeable to external molecules. In the present paper, these cells are demonstrated to be apoptotic and when treated with acridine-orange, their nucleus shows typical features of rosette shrinkage. Furthermore, we were able to show that the process of apoptosis is present at different degrees

during all culture times in controls and in cultures where growth factors (fetal calf serum) were withdrawed. The role of apoptosis when the highest grade of myoblast proliferation is in progress will be discussed.

Many studies are still needed to elucidate the complex physiological process of myogenesis that *in vitro* and *in vivo* combines both proliferation and cell death.

Methods

Primary muscle cell cultures

Skeletal muscle myoblasts were obtained from legs of Wistar newborn rats by enzymatic digestion with trypsin. After cell pre-plating to eliminate most non-muscle cells (fibroblast and endothelial cells), myoblast-rich fractions were cultured in plastic flasks, coated with gelatin (2% in PBS), at the density of 100,000 cell per cm^2 in DMEM supplied with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 ug/ml streptomicin and 2.5 ug/ml fungizone (complete medium). For immunocytochemistry cultures were grown on a glass coverslips [10]. After 24 hrs of culture, the cells were incubated in complete medium and incubated in serum free culture medium (DMEM supplied with 10% of BSA 1% in PBS) for 56 hrs. In some cultures, after 3 days the medium was changed and FCS was substituted with 10% of horse serum (HS) to favor a complete fusion of all proliferated myoblasts. After 5 days the serum concentration was reduced to 5% HS. On the 7th day the cells were treated as described above and cultivated for 56 hrs.

We obtained two samples of myoblasts and myofibers that could be tested for apoptosis: cells grown in complete medium (sample 1, control), cells grown in serum free medium (sample 2, experimental).

Immunocytochemistry

Cell cultures were washed twice in PBS, fixed with cold acetone for 10 minutes, air dried and incubated for 60 min at 37° C with either mouse anti-rat desmin monoclonal antibody (mAb) (Boehringer), diluted 1/5 in 1% BSA. The coverslips were then washed twice with PBS and incubated with rhodamine coniugated rabbit anti-mouse Ig (diluted 1/100 in 1% BSA) for 60 min at 37° C respectively. Negative controls were performed by omitting the first antibody. Coverslips were washed three times in PBS, mounted in Elvanol and observed under an epifluorescence microscope (Zeiss).

Acridine orange staining

Cell cultures were washed twice in PBS, fixed with acetic acid and methanol (1/3) for 10 min at room temperature. Then coverslips were stained with orange acridine (5 ug/ml

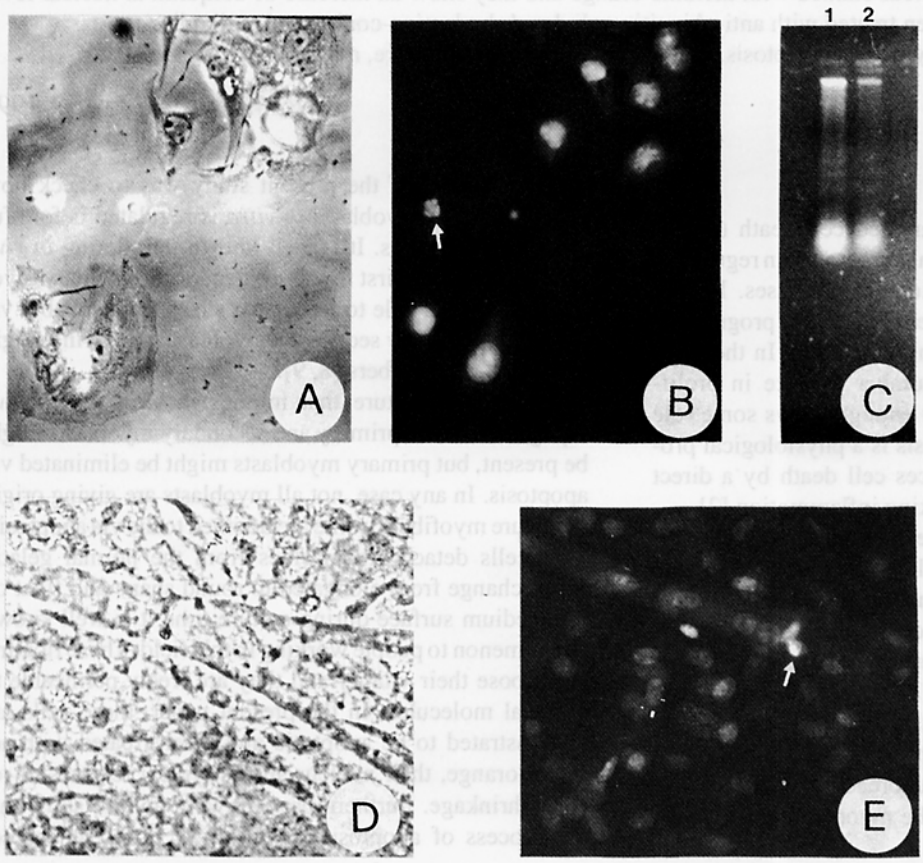

Figure 1. Myoblast cell culture at 80 hrs in serum free medium. A, contrast phase and B, Acridine orange staining. A round myoblast is detaching from the normal gelating layer and presents a condensed rosette like nuclei after staining. C, DNA analysis of floating cell from complete medium (lane 1) and from serum free medium (lane 2). D, Myotube at contrast phase and E, after Acridine orange staining. A condensed myonuclei (arrow) is present inside myotube.

in PBS) for 5 min, washed two times in PBS. Slides were then observed immediately under fluorescent light using a yellow green filter. Apoptotic nuclei are expressed as percentage of total nuclei counted.

Agarose gel electrophoresis

The formation of oligonucleosomal DNA fragments (0.2-1.2 kbp) was assessed by conventional agarose gel electrophoresis. DNA isolation was performed on floating cells. DNA was phenol/chloroform extracted, ethanol-precipitated, and resuspended in 10 mM Tris-HCl, pH 8, 10 mM EDTA, pH 8 (TE). The extracts were subjected to electrophoresis in a 2.2% agarose gel and stained with ethidium bromide.

Results

Primary muscle cells were cultured in a complete and in a serum free medium and analysed for apoptosis. Both the conditions had shown cells with a round shape that were detaching from the gelatin layer and floating in the medium (Fig 1A). Immunocytochemistry confirmed that these cells were positive with anti-desmin (data not shown) and recently we have shown that these cells were myoblasts (manuscript submitted). Acridine-orange staining showed a nuclear morphology typical for apoptosis (Fig 1B). A condensed, orange shining, rosette like feature was detected in both conditions of cell culture. The same type of cell presented a positive reaction for *in situ* nick end labaling (data not shown). Then floating cells were collecetd, DNA extracted and subjected to electrophoresis on agarose gel. The DNA fragmentation in a nucleosomal ladder was detected (Fig 1C) confirming the morphological data. Difference was found between complete and serum free medium, an higher amount of floating cells were detected in myoblasts treated with growth factor. The same cells commited to apoptosis displayed a nuclear posistive reaction for ubiquitin (data not shown) confirming our recent findings on the role of ubiquitin in muscle apoptosis (manuscript submitted). Myofibers were analysed with acridine orange staining in conditions of cell culture identical to the previously described. We detected, but only in the serum free treated, few myonuclei displaying a condensed chromatin typical of the apoptotic process (Fig 1D-E).

Discussion

Recent literature data have shown that myoblasts in primary cultures from mdx muscle explants [5], have a variable percent of apoptotic myonuclei, but not when cultures are deprived of fetal calf serum (FCS). In the present paper we confirm the presence of apoptotic myonuclei in myoblasts in primary cultures derived from neonatal rat muscles, either in the presence or in the absence of growth factors. The presence of apoptosis during the most active myoblast proliferation, is demonstrated, by the increased number of floating, apoptotic cells in both experimental conditions and it might be seen as a process of autoregulation of the cellular number, as shown also by DNA ladder.

The observation that also myofibers, although at a very low extent, might show nuclei with clear apoptotic morphological features (Fig.1, panel E), as revealed by Acridine orange staining, confirm that, certainly silent under normal condition, the complex machinery of apoptosis is present in differentiated muscle fibers. Recent findings have demostrated in adult normal and dystrophic myofibers the presence of apoptotic myonuclei [4].

An unsolved question arises, however, from the data of the recent scientific literature [5, 6, 7], mostly performed *in vivo*: are single myofiber nuclei that undergo to apoptosis or is the entire myofiber that is apoptotic and what is observed is only a piece of the muscle fiber? The Fig. 1, panel E, of the present paper seems to indicate that a single nucleus of the fiber (arrow) might present apoptotic features within an environment of normal nuclei. If this observation is correct, a normal fiber might have one or very few nuclei apoptotic without being the entire fiber apopotic.

On the other hand, *in vitro*, a myoblast, already committed to apoptosis, still alive, might have fuse into the fiber and then continue the apoptosis programm inside the fiber.

Studies are still in progress to understand better the program of apoptosis before and after the myoblast fusion in primary cultures.

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