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Actin Cytoskeleton as a Target for 2-Chloro Adenosine: Evidence for Induction of Apoptosis in C2C12 Myoblastic Cells

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Recent results for various cell types have shown that adenosine is capable of inducing relevant cytophatological alterations which can eventually lead to apoptosis. No data are available regarding the involvement of adenosine in apoptosis of muscle cells. In this work, we studied the effect of the relatively hydrolysis-resistant adenosine analog 2-chloro adenosine on a cultured myogenic cell line, C2C12, which is able to differentiate *in vitro,* leading to the formation of syncythia, giant multinucleated cells called myotubes. Results indicated that 2-chloro adenosine induces apoptotic cell death in both myoblasts and myotubes; this was preceded by a derangement of actin microfilaments, which represent the main cytoskeletal component and play a pivotal role in the physiology of such cell line. The time-dependency of cytoskeletal alterations suggested a causal relationship between these changes and the induction of apoptosis. These results implicate adenosine as an endogenous regulator of apoptosis in muscle cells and validate this cell model system as a useful tool for studying human muscle cell pathologies.

Apoptosis is a highly regulated process by which a damaged or unwanted cell kills itself (1). Great interest and a wealth of information have been generated on this ubiquitous process that plays roles in development, reproduction, aging, immune function, normal cell-homeostasis and disease (2). However, the biochemical events associated with apoptosis in a particular cell type as that represented by vertebrate muscle cells have not been characterized so far. Histochemical studies have proposed cell death as a normal developmental event in both proliferating myoblasts and in postmitotic muscle fibers (3-5). On the other hand, several lines of evidence have also suggested a major role for apoptosis as the first step in some degenerative muscolar diseases such as Duchenne Muscular Dystrophy (DMD) (5, 6), congenital muscular distrophy (7) and acute infantile spinal muscular atrophy (8). However, the endogenous molecules responsible for regulation of muscle cell apoptosis are totally unknown.

In this work we have tested the hypothesis that the nucleoside adenosine may act as an endogenous trigger of muscle cell apoptosis. The rationale for this hypothesis stems from the following evidence: i) as a result of abnormal catabolism of purines in DMD (namely, an accerelated degradation of ATP coupled to a greatly decreased adenylate kinase activity), the levels of adenosine are markedly increased in both blood and muscles of patients (9, 10); ii) the exposure of various cell types (astrocytes, thymocytes, lymphocytes and various immortalized cell lines) to excessive adenosine concentrations triggers cell death by apoptosis (for review see 11); iii) in another genetic metabolic disease, the adenosine-deaminase (ADA)-immuno-deficiency syndrome (12), the lack of another enzyme involved in the purine cascade (ADA) results in accumulation of adenosine to toxic levels, with consequent early and massive deletion of thymocytes and dramatic immunodeficiency. This study was specifically undertaken to evaluate the subcellular effects of adenosine in muscle cells by utilizing the relatively metabolically stable adenosine ana-

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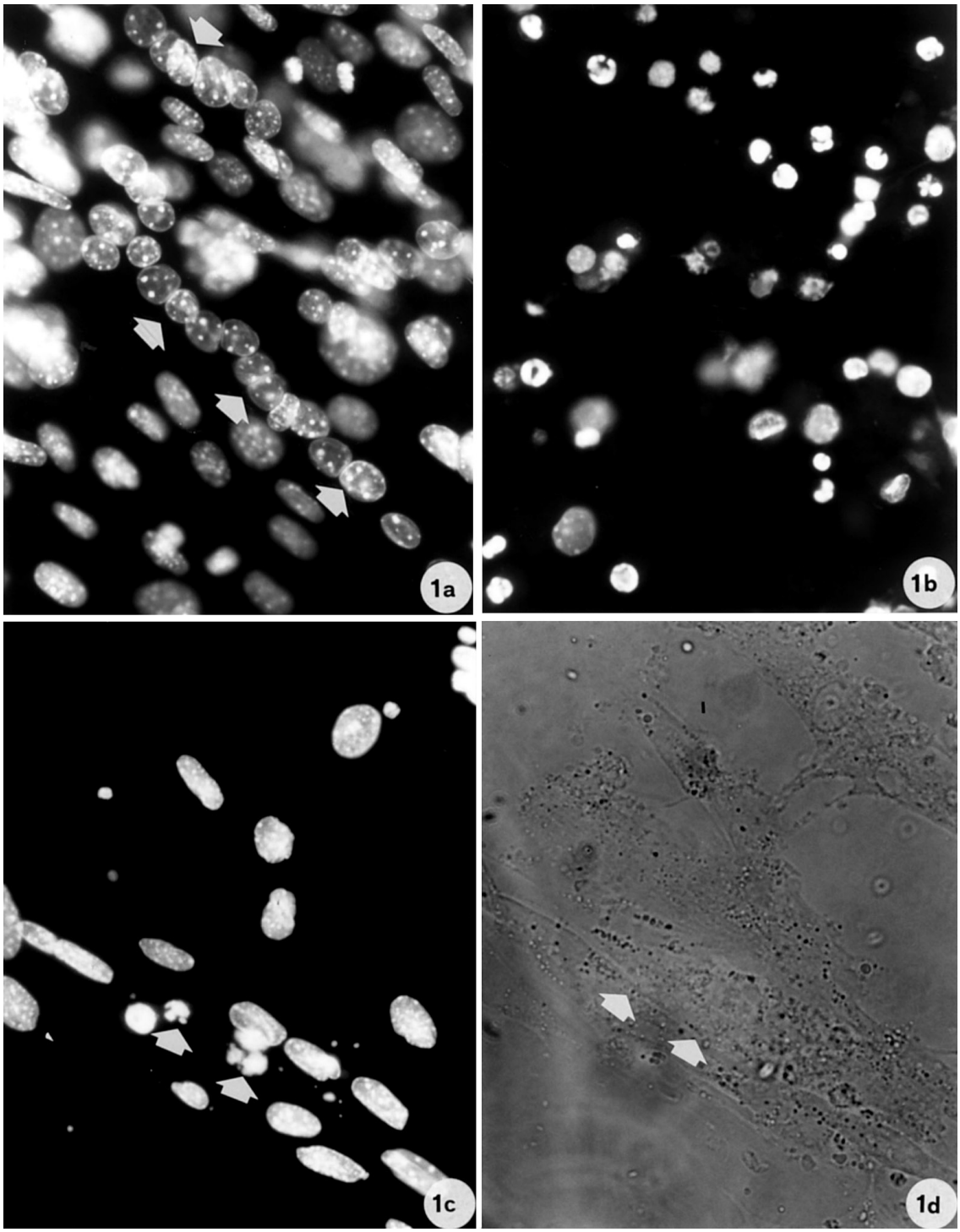

FIG. 1. 2CA-induced apoptosis as detected by nuclear staining with Hoechst chromatin dye 33258. Control myoblasts and myotubes (arrows) (a); floating cells, apoptotic myoblasts (b). Note the nuclear clumping and chromatin superaggregation. Adhering myotubes with isolated apoptotic nuclei (c) and corresponding brightfield (d). Magnification: 1500×.

logue 2-chloro adenosine (2CA) on the myoblastic cell line C2C12. Such experimental model was chosen since our current understanding of the cellular and molecular mechanism involved in myogenic cell death has been gained through the use of *in vitro* models (7, 13). Moreover, C2C12 are widely used to study *in vitro* mus-

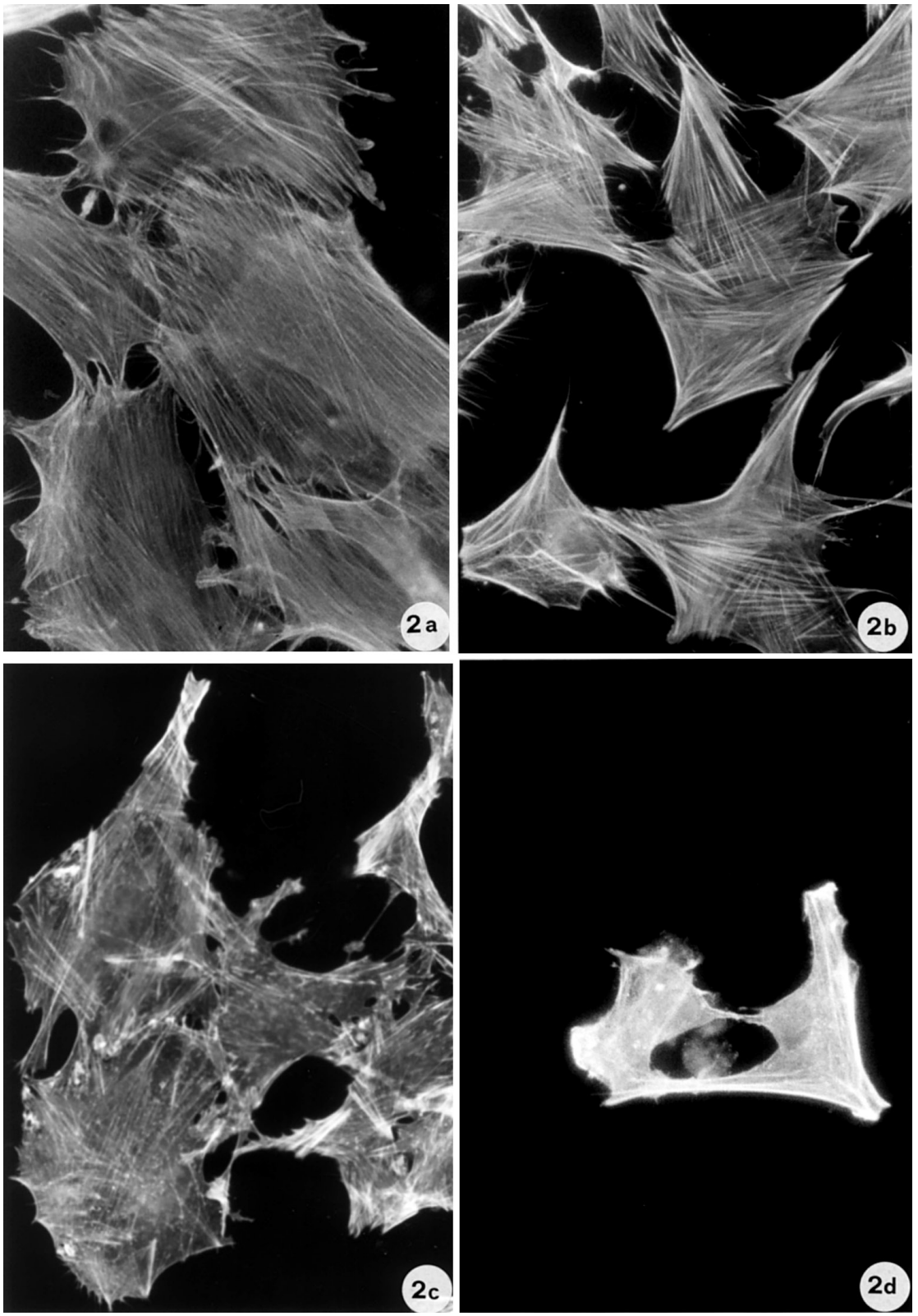

FIG. 2. Time-dependent cytoskeletal changes induced by 2CA in myoblasts. F-actin microfilaments as appear in control cells (a) and after 24 h (b), 48 h (c), and 72 h (d) of exposure to 2CA. Note the marginalization and rearrangement of filaments in (b); the loss of stress fibres in (c) and the collapse of actin network in (d). Magnification: 1500×.

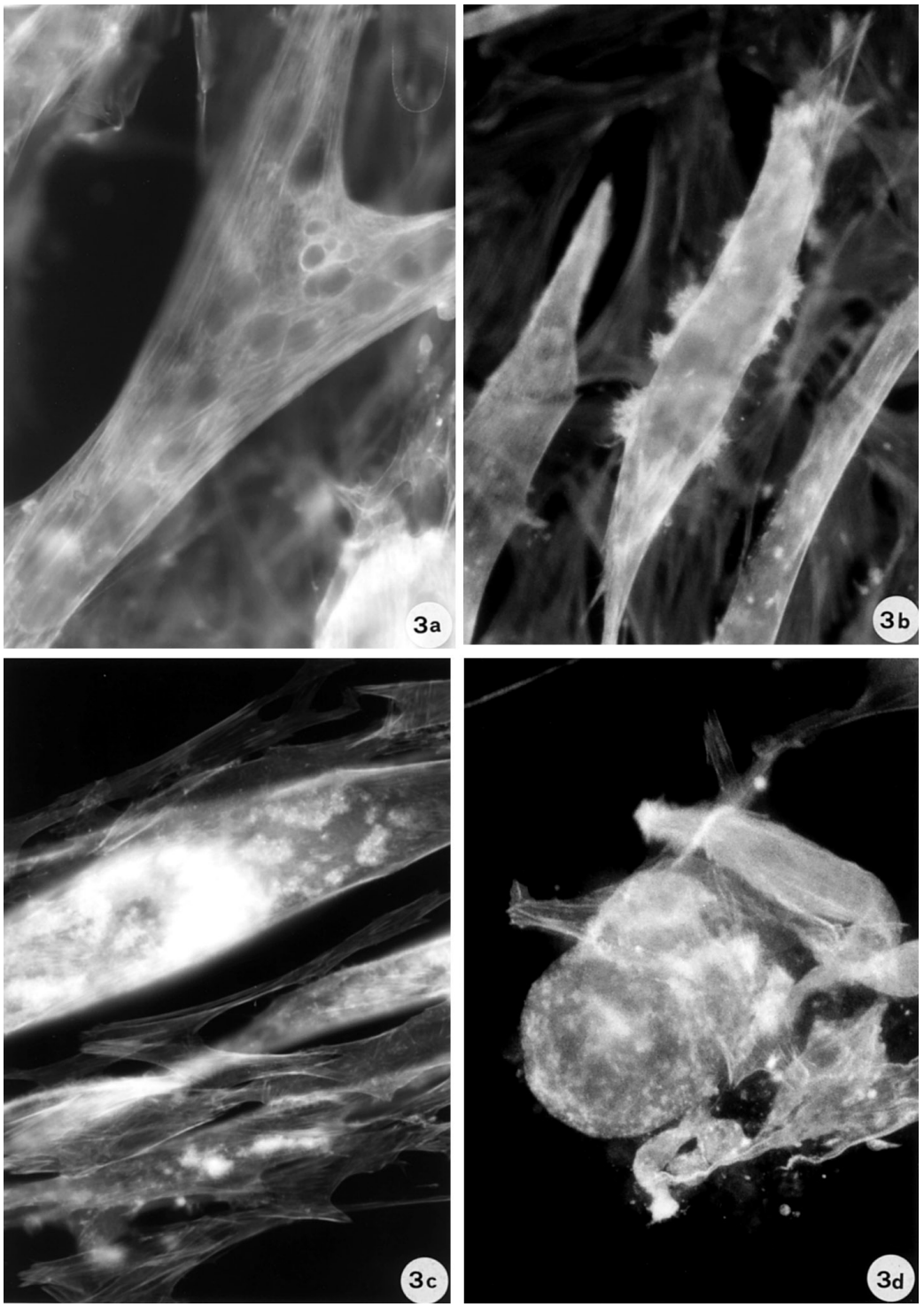

FIG. 3. Time-dependent cytoskeletal changes induced by 2CA in myotubes. F-actin microfilaments as appear in control cells (a) and after 24 h (b), 48 h (c), and 72 h (d) of exposure to 2CA. The loss of stress fibres and membrane ruffling is well visible in (b). Actin filament fragmentation appears evident after 48 h (c) while a complete network collapse is detectable after 72 h in (d). Magnification: 1500×.

cle differentiation program. Like embryonic myoblasts, C2C12 can undergo differentiation after shifting the culture to medium containing low concentrations of mitogens. During this process myoblasts withdraw from the cell cycle, express muscle-specific structural features and fuse into multinucleated myotubes (14).

TABLE I

Induction of Apoptosis by 2CA in C2C12 Cells
(% of Total Cell Number)

	Adhering cells	Floating cells
Control (48 h)	0	10
2CA (48 h)	11.6	81
Control (72 h)	4.5	3
2CA (72 h)	[a]	61[b]

[a] Not detectable since most cells were detached.
[b] Secondary necrosis was detectable in remaining cells.

We demonstrate that 2CA does induce apoptosis of myogenic cells (both mononucleated resting myoblasts and polynucleated differentiated myotubes) by a mechanism that targets the microfilament system.

MATERIALS AND METHODS

Cell cultures, myotube differentiation, and treatments. C2C12 cells were grown in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum (GIBCO), 100 IU/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Experiments were performed on cells with no more than 20 passages in culture. To induce cell differentiation, myoblasts were seeded on glass coverslips at the concentration of 4×10^5/ml in DMEM supplemented with 2% horse serum (GIBCO). Spindle-shaped cells in the first stages of differentiation to myotubes were observed after 24 h of incubation. Experiments were carried out with cells grown for 72 h in differentiating medium. Treatments with 100 μM 2CA were performed by direct addition to the culture medium. Such a concentration was chosen on the basis of previous studies performed with this adenosine analogue (11). Cytoskeleton analysis and apoptosis evaluation were conducted after 24, 48 and 72 h of 2CA incubation.

Cytoskeleton analysis. Controls and treated C2C12 were fixed with 3.7% formaldehyde in PBS (pH 7.4) for 30 min at room temperature. After washing in the same buffer, cells were permeabilized with 0.5% Triton X-100 (Sigma) in PBS for 5 min at room temperature. Cells were stained with fluorescein-phalloidin (Sigma) at 37°C for 30 min. Finally, after washing, all the samples were mounted with glycerol-PBS (2:1) and observed with a Nikon Microphot fluorescence microscope.

Evaluation of apoptosis. Controls and treated cell surnatants were seeded on glass coverslips coated with polylysine. After adhesion to the glass surface, cells were fixed with 3.7% formaldehyde in PBS pH 7.4, for 10 min at room temperature. Cells still attached to the substrate of culture flasks were washed in PBS and then fixed as described above. All samples were then permeabilized with 0.5% Triton X-100 (Sigma) in PBS for 5 min at room temperature. Cells were then incubated with Hoechst 33258 chromatin dye (Sigma) for 30 min at 37°C. After washing, all samples were mounted with glycerol-PBS (2:1) and observed with a Nikon Microphot fluorescence microscope. Quantitative evaluation of apoptotic cells was performed by counting 500 cells at high magnification (500×). Student's *t* test was used to evaluate statistical differences ($p<0.05$ was considered as significant).

RESULTS

In order to verify whether the 2CA is capable of inducing apoptosis in C2C12 cells, we carried out time-course experiments by analyzing both cells in the surnatants and cells still adhering to the substrate. In agreement with other literature data obtained in different cell models (11), exposure to 2CA induced cell detachment and apoptosis, both phenomena reaching the maximum within 72 hours. Figure 1a shows the normal appearance of nuclei in myogenic C2C12 cultures adhering to colture substrate. Note the aligned nuclei characterizing a myotube (arrows). Typical morphological features of apoptotic cells, mainly consisting in the clumping and/or aggregation of chromatin, were detectable in floating cells (Fig. 1b). In cells still adhering to the substrate (Fig. 1c), chromatin clumping (arrows) was also appreciable in differentiated myogenic cells, i.e. in myotubes, where normal and modified nuclei coexist (Figure 1d, brightfield corresponding to figure 1c).

A quantitative analysis of these apoptotic figures has also been performed and the results obtained are shown in Table I. Significant time-dependent differences have been detected between control cells and 2CA-treated samples. Considering floating cells, whereas only 10% of cells in controls underwent apoptosis, upon exposure to 2CA for 48 h, this type of cell death accounted for more than 80% of the cell population ($p<0.01$). Accordingly, 11.6% of adhering cells in treated cultures were apoptotic, while total absence of apoptotic figures was found in control samples ($p<0.05$). These effects were even more evident after a 72 h exposure to the adenosine analogue.

Since the importance of the cell cytoskeleton has recently been recognized in apoptotic cell death (1, 15), a specific analysis on the actin microfilament system was carried out. The time-course of the actin changes induced by 2CA is reported in Figures 2 and 3. Untreated myoblasts showed well organized stress fibers (Fig. 2a) whereas exposure to 2CA for 24 h (b), 48 h (c) and 72 h (d) caused a time-dependent rearrangement of the actin network (b) with loss of stress fiber integrity (c) and, finally, complete derangement and collapse of microfilamentous structures (d). Similar actin changes were observed in differentiated myotubes (Fig. 3). Control myotubes are characterized by a well organized network of parallely arrayed actin fibers (a). Alteration of stress fibers was detected as early as 24 hours of exposure to 2CA (b), a change which became more appreciable after 48 hours of treatment (c) with the complete desappearance of actin structures. Finally, after 72 hours, small clumps of fragmented actin filaments were visible in shrinked, detaching myotubes (d).

DISCUSSION

The major findings of the present study on C2C12 myogenic cells are that 2CA induces: i) actin cytoskeleton disorganization and derangement in both myo-

blasts and myotubes and ii) a massive cell death by apoptosis in myoblasts and, less frequently, in myotubes. *In vivo,* apoptotic nuclei were observed in differentiated fibers of several muscular degenerative diseases (5, 8, 16) as well as in loaded muscle after prolonged exercise (17). Some *in vitro* studies on programmed cell death in myogenic cells has also been reported (7, 10, 18). In this work we report that, as already demonstrated in other experimental systems (for review, see 11), adenosine is a trigger for apoptotic cell death in myogenic cells both in undifferentiated and differentiated state. Specific extracellular membrane receptors for adenosine have been described in muscular cells (19, 20); however, as already demonstrated in other cell types, also in myocytes adenosine-induced apoptosis may encompass both receptor-independent mechanisms (e.g., upon entry into cells, adenosine acts intracellularly on DNA, mitochondria, cytoskeletal elements and/or other organelles and molecules critically involved in cell survival), and receptor-dependent mechanisms (beside acting locally, adenosine generated by muscle metabolism may diffuse to neighboring cells and activate extracellular membrane receptors involved in induction of cells death). The elucidation of the exact contribution of these mechanisms to adenosine-induced apoptosis in myogenic cells will be the matter of future studies. Involvement of specific membrane receptors would be particularly intriguing, since a possibility for the pharmacological modulation of apoptosis in muscle, via specific agonists and antagonists, could be envisaged. In addition, we also show that early dissolution of actin filaments precedes the appearence of typical apoptotic features, suggesting a causal relationship between F-actin disorganization and adenosine-induced apoptosis in myogenic cells. In muscle cells, actin cytoskeleton binds to a complex of proteins, the dystrophin-associated proteins, that in turn bind to the laminin (for review see 21), a protein that plays a major role in the survival of myotubes (7). All genetic disorders which affect a component of this system, lead to an increase of myofibers death and, ultimately, to a degenerative pattern of muscle cell (22). Accordingly, the cleavage of membrane-associated actin by ICE-family protease seems to be one of the first steps in constitutive apoptosis (1, 15). Our results provide thus strong support for a role of adenosine as an endogenous regulator of apoptotic cell death also in myogenic cells and raise the hypothesis that, in a way similar to the ADA-deficiency syndrome (12), the abnormal accumulation of adenosine may represent a novel pathogenetic pathway in muscle diseases contributing to a progressive deterioration of cells and, eventually, to cell death.

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