Adenosine-Induced Apoptosis of C2C12 Myoblastic Cells

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Abstract

To assess a possible role for adenosine in muscle cell apoptosis, we have studied the effects of its hydrolysis-resistant analogue 2-chloro adenosine on a myogenic cell line, C2C12, which can differentiate *in vitro* to giant multinucleated cells (myotubes). Exposure of cultures to the adenosine analogue resulted in time-dependent apoptotic cell death of both myoblasts and myotubes. Adenosine may therefore represent an endogenous regulator of pathophysiological apoptosis in muscle.

Key words: myogenic cells, adenosine, apoptosis.

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Programmed cell death (apoptosis) plays a key role in development, reproduction, aging, immune function, normal cell-homeostasis and disease [8]. In muscle, apoptosis has been proposed as a normal developmental event in both proliferating myoblasts and in postmitotic muscle fibers [6, 13, 15]. Signs of apoptosis have also been detected in several muscular degenerative diseases such as Duchenne Muscular Dystrophy (DMD) or its animal models [11, 14, 15], as well as in loaded muscle after prolonged exercise [12]. The endogenous molecules responsible for regulation of muscle cell apoptosis are totally unknown.

In this work we have tested the hypothesis whether the nucleoside adenosine may act as a regulator of apoptosis in muscle cells. Adenosine has indeed been shown to trigger apoptosis of a variety of other different cell types [1]; moreover, it may play a role in DMD-associated progressive degeneration of muscle, since, as a result of abnormal metabolism of purines, this disease is characterized by markedly increased adenosine levels in both blood and muscle of patients [2, 4]. It is therefore possible that, in a similar way to another genetic metabolic disease, the adenosine-deaminase (ADA)-immunodeficiency syndrome [5], where excessive adenosine accumulation results in massive death of thymocytes, also in DMD-like diseases, accumulation of endogenous adenosine may represent a novel pathogenetic pathway in muscle degeneration. A specific study was hence undertaken to verify the ability of the hydrolysis-resistant adenosine analogue 2-chloro-adenosine (2CA) to induce apoptosis of C2C12 myocytes, a cell line which is widely used to study *in vitro* muscle differentiation program. In fact, in a similar way to embryonic myoblasts, if grown in the presence of low concentrations of mitogens, these cells withdraw from the cell cycle, express muscle-specific structural features and fuse into multinucleated myotubes [9].

Methods

1) Cell culture, myotubes differentiation and treatments

C2C12 cells were grown in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum (GIBCO), 100 units/ml penicillin and 100 mg/ml streptomycin in a humidified atmosphere of 5% CO2 and 95% air at 37°C.

Experiments were performed on cells that had undergone no more than 20 passages in culture. For experiments, cells were removed from flask by trypsinization and seeded (1 x 10^5 cells) into 35 mm culture dishes. To induce cell differentiation, myoblasts were seeded on glass cover slips at concentration of 400,000/ml in DMEM supplemented with 2% horse serum (GIBCO). After 24 h, spindle-shaped cells in the first stages of differentiation to myotubes were observed. Experiments were carried with cells grown for 72 h in differentiating medium. Treatments were performed by direct addition of 2CA to the culture medium (final concentration: 100 μM, on the basis of previous studies, [1]).

2) Evaluation of apoptosis

Apoptosis was assessed after 48-72 h treatments in both cells floating in the culture medium and in adhering cultures by light fluorescence microscopy after nuclei labelling with the Hoechst 33258 chromatin dye, as previously described [7].

Results and Discussion

Exposure of cultures to 2CA induced cell detachment and apoptosis, both phenomena reaching the maximum whithin 72 hours. Figure la shows the appearance of nuclei in myogenic C2C12 cultures treated with 2CA for 48 h and still adhering to the substrate. In these treated myotubes, the simultaneous presence of normal and apoptotic nuclei (the latter being characterized by chromatin aggregation) is evident (see arrows in Fig. la and 1b). These morphological features were totally absent in control cultures, where all myotubes had nuclei with normal appearance (data not shown). When floating cells of 2CA-treated cultures were analyzed, the typical nuclear features of apoptosis (i.e., clumping and/or aggregation of chromatin) were detectable in the whole population (Fig. 1c).

A quantitative analysis of these apoptotic figures has also been performed. Statistically significant time-dependent differences were detected between control cells and 2CA-treated samples. In particular, upon exposure to 2CA for 48 h, 81.0% of cells detached in the culture medium showed apoptotic nuclei, compared to only 10.0% in the control group. Accordingly, at this time-point 11.6% of adhering cells in 2CA-treated cultures were apoptotic, whereas no apoptotic figures were found in adhering cells of control samples. These effects were even more evident after a 72 h exposure to the adenosine analogue, when signs of secondary necrosis were also found in treated cultures (data not shown).

These results demonstrate that, in a similar way to other cell types, myogenic cells are susceptible to adenosine-induced apoptosis both in undifferentiated and differentiated state. It can be speculated that, in a similar way to the ADA-deficiency syndrome [5], the abnormal accumulation of adenosine known to occur in this pathology may contribute to a progressive deterioration of muscle cells and, eventually, lead to cell death. Preliminary data of our laboratory also suggest that induction of apoptosis by 2CA occurs through an early derangement of actin microfilaments [10], which represent the main cytoskeletal component and play a pivotal role in the physiology of 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 [17]), a protein that plays a major role in the survival of myotubes [16]. All genetic disorders affecting a component of this system lead to an increase of myofibers death and, ultimately, to a degenerative pattern of muscle cell [3]. Therefore, the elucidation of the nature of adenosine-induced cytoskeleton damage and of its possible role in regulation of myogenie cell death may provide new important insights into the pathophysiology of degenerative muscle diseases.

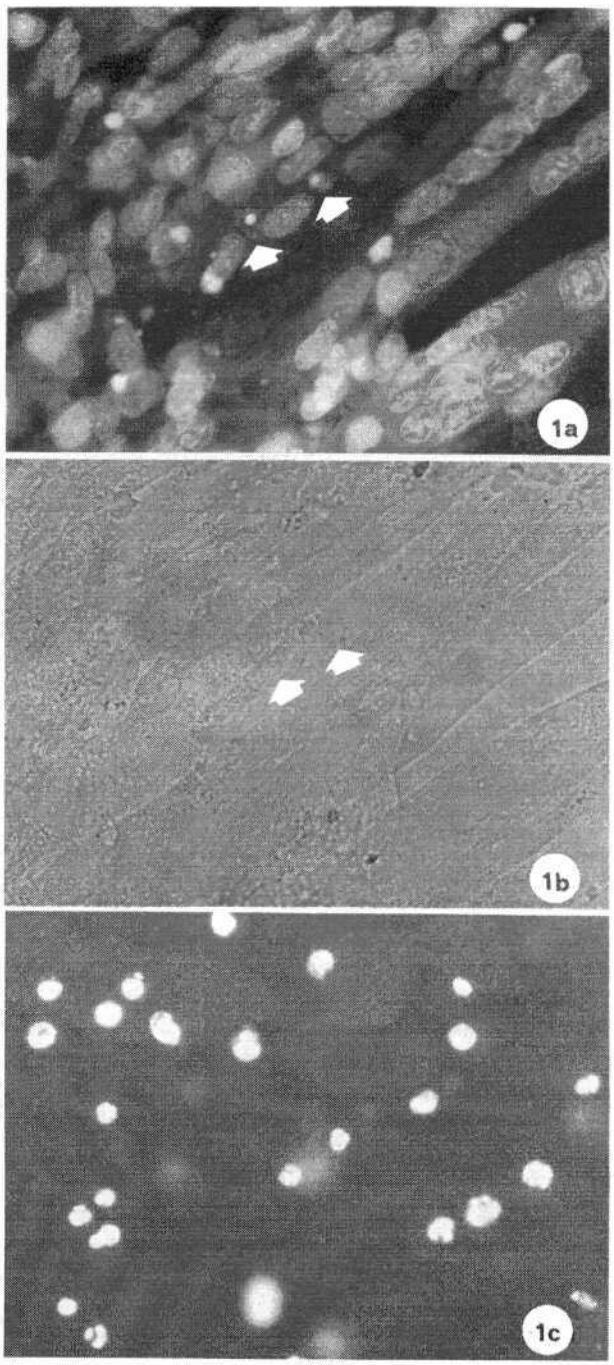

Figure 1. 2CA-induced apoptosis in C2C12 cultured cells. Adhering cells stained with the chromatin dye Hoechst 33258 (a) and corresponding brightfield (b). Floating cells (c). Magnification: 1500x.

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