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Inhibition of Myoblast Differentiation by Polyamine Depletion with Methylglyoxal Bis(guanylhydrazone)

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Abstract: The role of polyamines in skeletal myoblast differentiation was investigated using the polyamine metabolic inhibitor methylglyoxal bis(guanylhydrazone)(MGBG). Concentrations of intracellular free spermidine and spermine increased 2 to 2.5-fold at the onset of myoblast fusion. The systhesis of actin, and creatine kinase activity both dramatically increased during myotube formation. However, MGBG at a concentration of 0.5 mM not only abolished the increase of intracellular free polyamines, but also reduced cell fusion to almost half the level of untreated cells, without noticeable morphological alteration. The production of actin, and creatine kinase activity were almost completely abolished by MGBG. The inhibition of myoblast fusion by MGBG was partially recovered with 0.1 mM of spermidine or spermine added externally. Results indicate that polyamines are necessary for normal myoblast differentiation. Since the first indication of myoblast differentiation is alignment of muscle cells and membrane fusion of adjacent cells, and since polyamine depletion completely inhibited the synthesis of actin, which might be associted with membranes, polyamine might be involved in myoblast differentiation through membrane reorganization events.

Key words: actin, methylglyoxal bis(guanylhydrazone), myoblast, polyamines.

Polyamines are ubiquitous polycationic metabolites in both prokaryotic and eukaryotic cells (Tabor and Tabor, 1984). Both prokaryotic and eukaryotic cells produce putrescine and spermidine, but spermine is confined to eukaryotic cells. Prokaryotes have a higher concentration of putrescine than spermidine, whereas eukaryotic cells have higher levels of spermidine and spermine than putrescine (Heby, 1981). Although the exact biological functions of these compounds are still not well understood, many studies have shown that polyamines are necessary for normal cell proliferation and differentiation (Heby, 1985; Kaczmarek and Kaminska, 1989; Pegg, 1988). It is already known that polyamines are directly and indirectly involved in the synthesis of DNA, RNA, and proteins (Heby, 1981 and 1985; Smith, 1985). More recently, several studies have shown their importance in membrane-associated biological activities, such as stabilization of membranes, interaction with phospholipids and membrane-bound proteins, and Ca^{2+} homeostasis through a Ca^{2+} channel (Koenig *et al.*, 1988; Schuber, 1989; Sjoholm, 1993; Tadolini, 1988). Since polyamines have a polycationic nature they can noncovalently interact with acidic phospholipids and proteins. However, their ionic nature is fundamentally different from inorganic cations, such Mg^{2+} and Ca^{2+}, because their positive charges are distributed at fixed lengths along the flexible carbon chain (Liquori *et al.*, 1967).

Skeletal muscle, consisting of multinucleated myotubes, is formed through fusion of mononucleated myoblasts. The differentiation of primary culture chick embryo myoblasts to myotubes is accomplished through several morphologically and biochemically distinct stages (Devlin and Emerson, 1978; Wakelam, 1985). Freshly isolated myoblasts multiply rapidly, but as soon as cells align side-by-side and end-to-end they cease division and undergo membrane fusion. At approximately the same time as the onset of cell fusion, the cells cease DNA synthesis and initiate elaboration of the specialized proteins associated with skeletal muscle contraction e.g., actin, myosin, and troponin (Allen *et al.*, 1978; Paterson and Strohman, 1972). Even though creatine kinase is not a muscle specific contractile protein, it amounts to 10~20% of total muscle proteins and is synthesized selectively during myoblast differentiation (Watts, 1973).

This study is interested in the importance of poly-

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amines during myoblast differentiation using the well known inhibitor of polyamine metabolism methylglyoxal bis(guanylhydrazone)(MGBG). MGBG is a potent inhibitor of S-adenosyl methionine decarboxylase (SAMDC) which is one of the rate limiting enzymes in polyamine metabolism. Membrane fusion, the activity of creatine kinase, and synthesis of actin during myoblast differentiation were studied through the depletion of intracellular polyamines.

Materials and Methods

Cell culture

Myoblast cultures were prepared from the breast muscle of a ckick embryo according to O'Neill and Stockdale (1972) with minor modifications as described by Hah *et al.* (1993). Briefly, cells from a 12-day old embryo were prepared with 0.05% trypsin. Trypsinzed cells were washed and resuspended in Eagle's minimum essential medium supplemented with 10% horse serum, 10% embryo extract, and 1% antibiotics. After fibroblasts were removed by preplating on a collagen treated culture dish, 1×10^6 cells/ml were incubated at 37℃ in 95% air and 5% CO_2 for 24 h. On day 2, the concentration of embryo extract was reduced to 2% for the remaining period of incubation by replacing the whole medium. MGBG was added to the initial plating.

Fusion index

After washing with Hank's balanced salt solution, cells were fixed for 5 min in a mixture of 95% ethanol, 40% formalin, and concentrated acetic acid (20 : 2 : 1). Fixing was followed by washing and staining with hematoxylin for 5 min, for microscopic observation. The fusion index was expressed as a percentage of the number of nuclei within myotubes to the total number of nuclei.

Measurement of creatine kinase activity

Cells were washed twice with PBS and suspended in 0.05 M glycylglycine (pH 6.75) then sonicated for 30 sec. The homogenate was used as the enzyme source for the creatine kinase assay, according to the method of Shainberg *et al.* (1971). The change in absorption spectrum at 340 nm was recorded on a spectrophotometer. The rate of NADH formation at 30℃ was determined from the linear region of the reaction curve. One enzyme unit was calculated as the amount of enzyme which catalyzed the formation of one mole of NADPH per min at 30℃, pH 6.75. The rate of ATP formation from ADP and creatine phosphate was determined by coupling the reaction to hexokinase and glucose-6-phosphate dehydrogenase (Oliver, 1955; Nielsen and Ludvigsen, 1963). The reaction mixture contained 0.8 mM NADH, 10 mM magnesium acetate, 3.3 mM glucose, 1 mM ADP, 10 mM AMP, 0.5 U/ml each of glucose-6-phosphate dehydrogenase and hexokinase, 15 mM creatine phosphate, and 0.1 M glycylglycine (pH 6.75). A control assay was performed without creatine phosphate in the same manner.

Polyamine extraction and quantitation

Extraction was done by a method adapted from Flores and Galston (1982). After washing twice with 0.15 M KCl, cells were treated with 6% ice-cold $HClO_4$ for 30 min at 4℃. After sonication for 10 sec, the homogenate of $HClO_4$ treated cells was microcentrifuged for 20 min (a small portion of the homogenate was saved for protein assay). The pellet was saved for actin analysis by SDS-PAGE. The supernatant was benzolated for free polyamine analysis, according to Redmond and Tseng (1979). Polyamines from the cells, along with the standards, were analysed on a Bondapak C18 reversephase HPLC column (Waters Chromatography, Massachusetts, USA) at 254 nm.

Actin analysis by SDS-PAGE

The $HClO_4$ extracted pellet was washed twice with a 1 : 1 mixture of ethanol-ether and resuspended in distiled water for further SDS-PAGE. SDS-PAGE was carried out by the method of Laemmli (1970) on 10% acrylamide slab gel. After electrophoresis, the gel was stained with Coomassie blue, and scanned at 570 nm.

Protein assay

Protein contents of cultures were measured with Coomassie brillant blue by the protein assay of Bradford (1976). Bovine serum albumin was used as a standard.

Results

Inhibition and recovery of fusion

Fusion of myoblasts began on day 2 and became more prominent on day 3. However, the fusion index never reached 100% during the remaining period of the culture. In the MGBG treated culture, fusion was markedly inhibited, as shown in Fig. 1. The fusion index with 0, 0.25, and 0.5 mM MGBG was 75.8, 48.7, and 39.4%, respectively, at day 5. At concentrations of higher than 0.75 mM most myoblasts showed neither proliferation nor fusion. Since MGBG at a concentration of 0.5 mM did not cause any noticeable cell damage or growth inhibition, the same concentration was used for the remaining of the experiments. The

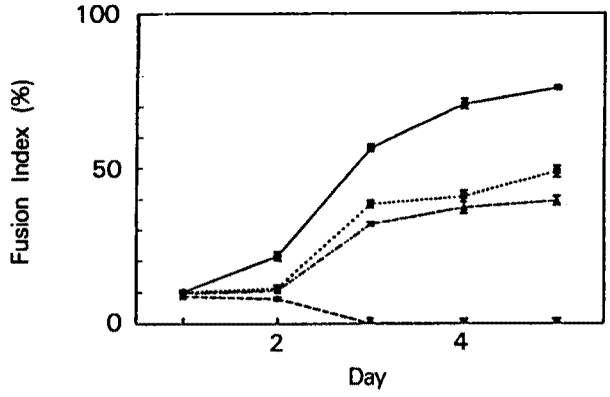

Fig. 1. Myoblast fusion index in the cultures treated with 0 (○–○), 0.25 (■–■), 0.5 (▲–▲), and 0.75 mM (●–●) MGBG (data from the average of three exp). MGBG was added at the initial plating.

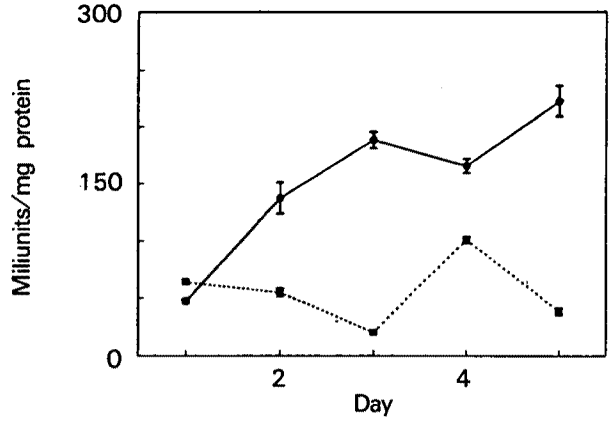

Fig. 3. The effect of MGBG on creatine kinase activity. (●–●), control; ■–■, 0.5 mM MGBG (data from the average of three exp).

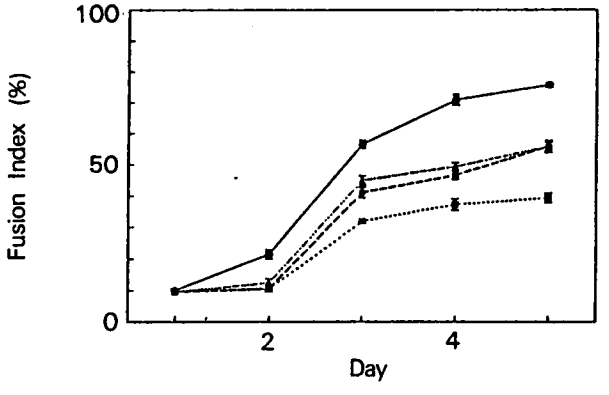

Fig. 2. Recovery of myoblast fusion in the presence of externally added 0.1 mM spermidine (▲–▲) and 0.1 mM spermine (●–●) along with 0.5 mM MGBG. (○–○), control; (■–■), 0.5 mM MGBG alone. Polyamines were added at 24 h culture (data from the average of three exp).

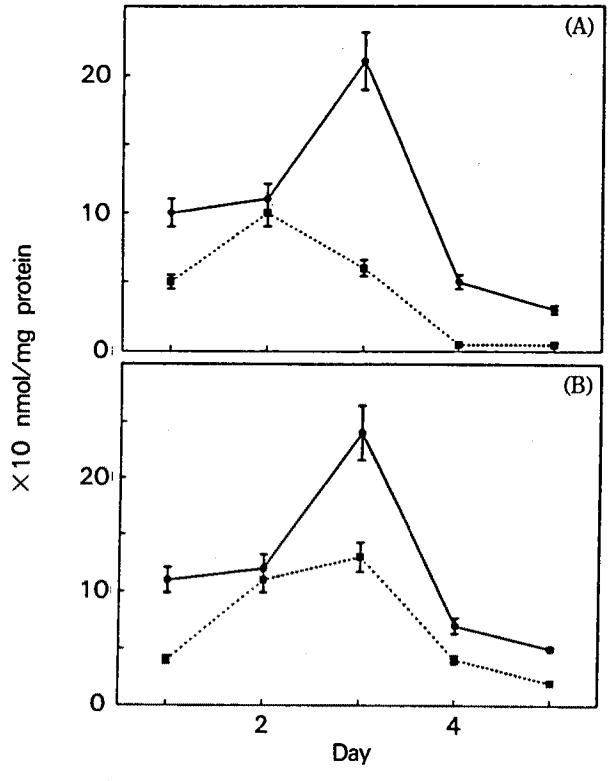

Fig. 4. Changes of intracellular free spermidine (A) and spermine (B) in the presence of 0.5 mM MGBG (■–■). (●–●), without MGBG (data from the average of three exp).

inhibition of fusion by MGBG could be recovered, partially, by externally added polyamines (Fig. 2). When 0.1 mM of either spermidine or spermine was added to the cultures along with 0.5 mM MGBG, the fusion index increased to approximately 56%, compared to 39.4% without polyamines. However, polyamines administered at a concentration of 0.1 mM could not induce full recovery from MGBG inhibition.

Creatine kinase activity

As shown in Fig. 3, the activity of creatine kinase, which is one of the markers of myoblast differentiation, increased gradually and reached a maxmium level on day 3. However, the addition of MGBG almost completely abolished creatine kinase activity throughout the culture.

Levels of polyamines during differentiation

The concentrations of polyamines in myoblasts were in the range of other eukaryotic cells; e.g. trypanosomes (Bacchi *et al.*, 1977), filamentous fungi (Paulus *et al.*, 1982; Wallis, 1966), mouse skin cells (Hibasami *et al.*, 1988 and 1989), CHO cells (Beninati, 1988), and human leukemia cells (Hibasami *et al.*, 1989). As was found in most other eukaryotic cells, the concentration of putrescine was much lower than other polyamines. In this study the concentration of putrescine was too low to achieve an accurate measurement. Its concen-

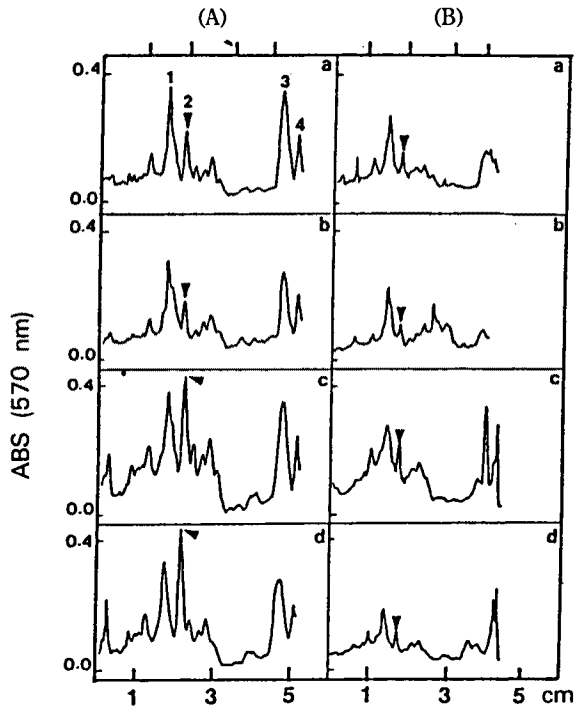

Fig. 5. Densitometric scanning of SDS-slab gel. A, control; B, with 0.5 mM MGBG. a, b, c, and d represent 1, 2, 3, and 4 day culture , respectively. Arrow head represents actin. Bars on top indicate molecular weight standards (66, 45, 29, 14.3 kDa from the left).

tration was about 10 times less than spermidine and spermine. The concentrations of both spermidine and spermine rapidly increased at the onset of cell fusion on day 3, but they dropped almost to the baseline level thereafter (Fig. 4). In the culture treated with 0.5 mM MGBG, both spermidine and spermine showed minor increases compared to the control, then decreased to the baseline level, or below. At the time of active cell fusion on day 3, the intracellular amounts of spermidine and spermine were 0.21 and 0.24 mol/mg protein. However with MGBG, spermidine and spermine amounts dropped to 0.06 and 0.13 mol/mg protein.

Synthesis of actin

The synthesis of actin showed a close correlation with cell fusion (Fig. 5). Among the several major proteins identified from SDS-gel, protein peak 2 was identified as 46 kDa actin by Western blotting (Hah *et al.*, 1993). In the culture without MGBG, actin did not increase until day 2 but showed an abrupt increase at day 3. The ratio of the areas of peak 1 vs. 2 increased from 1 : 0.27 on day 2 to 1 : 0.89 on day 3 and 1 : 0.1.06 on day 4. However, no actin stimulation was detected in the culture treated with MGBG when the ratio of the peak areas was compared (1 : 0.21 on day 2 and 1 : 0.36 on day 3). Protein peaks 1, 3, and 4 did not show any significant change.

Discussion

Polyamines were required for optimum growth in all cells tested. In most cells the requirement for polyamines was absolute. Many studies have shown that rapidly growing cells have higher levels of polyamines than slowly growing or quiescent cells, but when quiescent cells are stimulated, polyamines usually increase before an increase in DNA, RNA, or protein content (Tabor *et al.*, 1976; Janne *et al.*, 1978; Williams-Ashmar *et al.*, 1980).

In the myoblast culture, cells treated with 0.5 mM MGBG did not exhibit any noticeable morphological alteration, but showed a samll growth delay. Polyamine depletion resulted in poor myoblast fusion. At day 5 the fusion index with MGBG dropped to almost half of the index value of the untreated culture. Membrane fusion is one of the earliest events during myoblast differentiation. Myoblast fusion is triggered through a series of signal transductions (David and Fitzpatrick, 1993). An influx of Ca^{2+} is necessary for myoblast fusion, and it occurs right before fusion (David and Higgenbotham, 1981). The Ca^{2+} influx is deeply associated with protein kinase C and membrane protein phosphorylation in myoblast differentiation (David and Fitzpatrick, 1993). Polyamines may serve as intracellular messengers which provoke the opening of voltage sensitive Ca^{2+} channels (Iqbal and Koenig, 1985; Goldstone *et al.*, 1986). Spermidine and spermine play important roles in the regulation of Ca^{2+} homeostasis (Jensen *et al.*, 1987; Nicchitta and Williamson, 1984). As an inhibitor of SAMDC, MGBG can deplete intracellular levels of both spermidine and spermine. The MGBG treatment causes depletion of intracellular free polyamines, especially spermidine and spermine in myoblasts (Fig. 4). At the onset of myoblast fusion, spermidine and spermine increase rapidly. The concentrations of polyamines decreased or just barely increased in cultures treated with MGBG. Therefore, the depletion of spermidine and spermine hinders the normal process of membrane fusion in myoblasts in as yet unknown ways.

The earliest event of membrane fusion is the contact of two membranes, and polyamines can make complexes with membrane proteins or phospholipids by bridging the surface charges of the two adjacent membranes. The formation of complexes was supposed to help membrane aggregation by reducing the repulsive forces between the two membranes (Schuber, 1989). Therefore, externally added spermidiné or spermine should reduce the mutual electrostactic repulsion and result in membrane aggregation (Tadolini *et al.*, 1986). This study supports this hypothesis by showing recovery of membrane fusion with 0.1 mM of either spermidine

or spermine added externally (Fig. 2). However, complete recovery was not achieved with higher concentrations of added polyamines. These results might be due to unknown side effects of MGBG. MGBG is a potential specific inhibitor of the key polyamine enzyme SAMDC. However, it also induces alterations in mitochondrial morphology and function in various cell types (Pleshkewych *et al.*, 1980; Porter *et al.*, 1979). Therefore, the partial recovery of membrane fusion might be related to mitochondrial damage, an idea which should be studied further in detail.

Due to their polycationic nature polyamines interact with nucleic acids. Therefore, they are involved in gene expression. In mouse lymphocytes, exogenously added putrescine or spermine induced an increase in expression of genes coding for tubulin and actin (Kaminska *et al.*, 1990 and 1992). Actin synthesis became most active at the time of myoblast fusion. However, in this study polyamine depletion almost completely blocked actin synthesis without any significant growth inhibition, and without any noticeable morphological alteration. Polyamines promote polymerization of actin monomers to filaments and association of filaments into bundles (Grant *et al.*, 1983). In nonmuscle cells actin filaments are associated with the plasma membrane through a specific actin attachment protein (Burridge *et al.*, 1982; Mercier *et al.*, 1989; Tilney *et al.*, 1981). In skeletal muscle cells the association of actin with the plasma membrane is not well understood but, actin could play an important role in membrane reorganization, i.e., membrane fusion if actin filaments are associated with the plasma membrane. Therefore, polyamine depletion by MGBG might inhibit membrane fusion through inhibition of the synthesis or polymerization of actin.

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