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Differentiation of myoblast cell lines and biological methylation: 3-Deazaadenosine stimulates formation of multinucleated myofibers

(induction of creatine kinase/3-deazaadenosylhomocysteine/3-deazaaristeromycin/*S*-adenosylhomocysteine hydrolase)

SIGFRIDO SCARPA*†, ROBERTO STROM*, ARGANTE BOZZI*, ROBERT R. AKSAMIT‡, PETER S. BACKLUND, JR.‡, JOSEPH CHEN‡, AND GIULIO L. CANTONI‡§

*Department of Biopathology and Institute of Applied Biochemistry, University of Rome, "La Sapienza," Rome, Italy; †Cell Biology Laboratory, Istituto Superiore di Sanità, Rome, Italy; and ‡Laboratory of General and Comparative Biochemistry, National Institute of Mental Health, Bethesda, MD 20205

Contributed by Giulio L. Cantoni, February 9, 1984

ABSTRACT **Treatment of myoblast cell lines with 3-deazaadenosine stimulates differentiation into myofibers. Myoblast clone L5/3B5, which does not form myofibers after 6 days in fusion medium, was stimulated to form myofibers after 5 days of culture in fusion medium containing 50 μM 3-deazaadenosine. Myoblast clone L5/3C4, which normally begins to form myofibers after 4 days in fusion medium, was stimulated by 50 μM 3-deazaadenosine to form myofibers after 3 days in culture and the extent of fusion was also increased. In the presence of 100 μM homocysteine thiolactone, the concentration of 3-deazaadenosine that stimulated maximal fusion was reduced by a factor of 10, from 50 μM to 5 μM 3-deazaadenosine. Stimulation of myofiber formation by 3-deazaadenosine suggests a requirement for one or more methylation reactions in myoblast differentiation and the potentiation by homocysteine thiolactone indicates that myofiber formation is specifically stimulated by an intracellular accumulation of 3-deazaadenosylhomocysteine.**

The differentiation of myoblasts into myofibers in tissue culture has been widely studied as a model of terminal differentiation. A myoblast cell line, L5/A10, subcloned from Yaffe's original L5 line (1, 2), is particularly suitable for study since it can be induced to differentiate into multinucleated fibers by appropriate manipulations of the culture medium. When cultured in F14 medium supplemented with 10% fetal calf serum (growth medium) the cells will grow to confluency without undergoing differentiation, and the cells can be carried for many passages in growth medium as mononucleated myoblasts. However, if L5/A10 myoblasts are transferred from growth medium to F14 medium supplemented with only 1% fetal calf serum (fusion medium), they will, after a few cell divisions, undergo differentiation and fuse into multinucleated myofibers containing muscle-specific proteins (unpublished data). As the number of passages in growth medium increases, line L5/A10, like other myoblast cell lines, exhibits a tendency toward a lower extent of fusion. When myoblast populations with reduced fusion potential are recloned it is possible, as shown below, to isolate clones that exhibit a range of different fusion capacities. Such clones, when exposed to the culture conditions described above, can be grouped into three classes: (*i*) *fusing* clones that resemble the parent strain L5/A10, (*ii*) *nonfusing* clones that continue to divide and by day 6 exhibit <10% fusion, and (*iii*) clones with intermediate fusion capacities.

Several recent reports have suggested that the methylation of cytosine to form 5-methylcytosine, the only modified base in the genomes of higher eukaryotes (3), plays a role in the regulation of gene expression (4, 5). This suggestion is supported by numerous examples that demonstrate an inverse relationship between the extent of methylation of specific genes and their transcriptional activity (6). The studies of Christman *et al.* (7) and of Jones and Taylor (8) have shown that compounds that inhibit cytosine methylation can induce differentiation of cultured mouse embryo cells into myofibers.

The methylation of cytosine residues in DNA is catalyzed by an enzyme system(s) that utilizes *S*-adenosylmethionine (AdoMet) as the methyl donor (9). *S*-Adenosylhomocysteine (AdoHcy), one of the products of the methyl transfer reaction, is a potent inhibitor of cytosine methyltransferase(s) as well as nearly all other methyltransferases (10, 11). In eukaryotes, AdoHcy is metabolized through a single metabolic pathway by AdoHcy hydrolase (12). The reaction catalyzed by this enzyme is readily reversible and the equilibrium of the reaction is strongly in the direction of synthesis (12). Physiologically, however, the reaction proceeds in the direction of hydrolysis because one of the reaction products, adenosine, is rapidly converted to inosine by adenosine deaminase. It has been suggested by Cantoni and Chiang (13) that modulation of the ratio of intracellular levels of AdoMet and AdoHcy may play a role in the regulation of biological methylations. Experimentally, the intracellular AdoMet/AdoHcy ratio can be altered by administration of analogs of adenosine that are inhibitors of AdoHcy hydrolase. In this laboratory we have found that two analogs, 3-deazaadenosine and 3-deazaaristeromycin, are particularly useful for *in vivo* studies of methylation reactions. 3-Deazaadenosine is both a potent inhibitor of, and a good substrate for, AdoHcy hydrolase. Administration of 3-deazaadenosine to cells results in the accumulation of AdoHcy and the synthesis of 3-deazaAdoHcy, a congener of AdoHcy, that, like the natural compound, is an inhibitor of methyl transferases (14). 3-Deazaaristeromycin is a potent inhibitor of, but not a substrate for, AdoHcy hydrolase (15).

Administration of 3-deazaadenosine to several different cellular systems results in a variety of biological effects that have been attributed to the inhibition of methyl transfer reactions caused by the accumulation of AdoHcy, the formation of 3-deazaAdoHcy, or both (16–21). It must be pointed out, however, that it has not yet been possible to correlate the inhibition of a specific methylation reaction with a given biological response.

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Abbreviation: AdoHcy, *S*-adenosylhomocysteine.

§To whom reprint requests should be addressed at the National Institute of Mental Health, Building 36, Room 3A17, 9000 Rockville Pike, Bethesda, MD 20205.

It was of interest, therefore, in light of the studies of Christman *et al.* (7) and of Jones and Taylor (8) to explore whether administration of 3-deazaadenosine or 3-deazaaristeromycin might have an effect on the differentiation of poorly fusing myoblast clones. We report here that addition of 3-deazaadenosine 24 hr after transfer of myoblasts to fusion medium both accelerated the rate of fusion and increased the number of multinucleated myofibers, whereas 3-deazaaristeromycin had no such effect.

MATERIALS AND METHODS

Chemicals. 3-Deazaadenosine and 3-deazaaristeromycin were obtained from the Southern Research Institute (Birmingham, AL) and L-homocysteine thiolactone was obtained from Calbiochem.

Cultivation and Cytological Examination of Cell Cultures. The standard medium used for cell culture was F14 (22), a modification of Ham's F12. Fetal calf serum was added to final concentrations (vol/vol) of either 10% (growth medium) or 1% (fusion medium). For all growth and propagation experiments, 75-cm^2 plastic flasks were plated with 1×10^6 cells in 30 ml of growth medium, and the medium was changed every other day. Fusion assays were performed in 25-cm^2 plastic flasks, pretreated with 0.2% (wt/vol) gelatin, and 5×10^5 cells were plated in 5 ml of fusion medium (day 0). The next day and every other day thereafter the medium was changed. At scheduled intervals, the cell layer was rinsed for 10 min with phosphate-buffered saline, fixed with 50% methanol, and rinsed with absolute methanol. Cells were stained with Wright Giemsa stain. Cells that contained three or more nuclei were classified as multinucleated. The degree of fusion was expressed as the percent of total nuclei that were found in multinucleated cells. Enough nuclei were counted so that the standard error of the mean for the percent fusion was ≤4%.

Cells. The L5/A10 myoblast cell line was isolated by subcloning the L5 line established by Yaffe (1, 2) from primary cultures of newborn rat thigh muscle. Clones with different fusion capacities could be reisolated from L5/A10 cultures and two clones, named L5/3C4 and L5/3B5, were selected for this study. L5/3C4 is characterized by the capacity to undergo fusion to an extent of 58% within 6 days after transfer to fusion medium at passage 4. This value declined with passage, until by passage 13 it was reduced to 18%. The other clone, L5/3B5, was essentially unable to undergo differentiation into multinucleated fibers when transferred to fusion medium. Six days after transfer to fusion medium, these myoblasts developed, at most, a few small fibers containing an average of only three or four nuclei.

Creatine Kinase and DNA Determinations. Cultures were rinsed with phosphate-buffered saline and frozen at −80°C. After thawing, the cells were scraped into 1 ml of 0.05 M Tris buffer at pH 7.2. The cell suspension was sonicated for 30 sec at 4°C and creatine kinase activity was determined in 50-μl aliquots by use of the creatine kinase *N*-acetyl-L-cysteine-activated Boehringer Mannheim kit. DNA was determined on 150-μl aliquots by the fluorimetric method of Labarca and Paigen (23).

Analysis of Cell Metabolites. The medium from cells growing in 6-cm dishes was removed and replaced with 2 ml of serum-free F14 medium containing 10 μCi of [^{35}S]methionine per ml (1 Ci = 37 GBq) and either no inhibitor, 50 μM 3-deazaadenosine, or 50 μM 3-deazaaristeromycin. The final concentration of methionine in the medium was 0.2 mM. After incubation at 37°C for 2 hr, the radioactive medium was removed and 2 ml of 5% sulfosalicylic acid at 0°C was added to each dish. The sulfosalicylic acid suspension was removed and each dish was rinsed twice with 1 ml each of 5% sulfosalicylic acid at 0°C. The sulfosalicylic acid suspension and the two rinses from each dish were combined and centrifuged at 3000 × *g* for 20 min at 4°C. The amount of AdoHcy, 3-deazaAdoHcy, and AdoMet in the acid-soluble supernatant was determined by the chromatographic method of Hoffman (24). The number of cells in each culture was determined in replicate dishes.

RESULTS

The nonfusing myoblast clone L5/3B5 forms very few myofibers when cultured in fusion medium for 6 days (Fig. 1). However, addition of 50 μM 3-deazaadenosine on day 1, 24 hr after transfer to fusion medium, resulted in myofiber formation beginning between day 4 and day 5 (Fig. 1). After treatment with 3-deazaadenosine, as many as 50% of the nuclei appeared in multinucleated fibers. The fibers were similar to those that develop in the parent strain with respect to nuclear density, but the length of the fibers was somewhat shorter. The induction of differentiation caused by 3-deazaadenosine treatment can be seen both at the cytological level as the appearance of multinucleated fibers and at the biochemical level as increased levels of muscle-specific creatine kinase (Fig. 1).

Differentiation of a fusing clone of myoblasts, L5/3C4, was increased by addition of 3-deazaadenosine on day 1 (Fig. 2). Fusion was accelerated and a greater extent of fusion occurred relative to the cell cultures that did not receive 3-deazaadenosine. Cell fusion reached 80% by day 4 for cultures treated with 3-deazaadenosine compared to a value of only 22% in the control cultures (Fig. 2*A*). Cell creatine kinase activity also increased upon treatment with 3-deazaadenosine (Fig. 2*B*). For both the fusing (L5/3C4) and nonfusing (L5/3B5) clones, the transfer of cells to fusion medium was necessary for the increased fusion capacity with 3-deazaadenosine, since fusion did not occur in the presence of 3-deazaadenosine in growth medium.

In previous studies that have utilized many different types of cells, it has been shown that both the biological effects of 3-deazaadenosine and the intracellular levels of 3-deaza-AdoHcy were greatly increased by addition of homocysteine thiolactone (18, 19). Potentiation of the biological effects of 3-deazaadenosine by the addition of homocysteine thiolactone can also be demonstrated in the myoblast system. In the

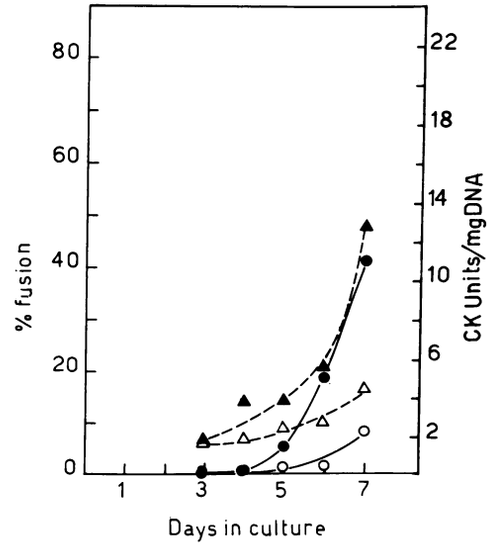

FIG. 1. Differentiation of L5/3B5 myoblasts in the absence (open symbols) or the presence (closed symbols) of 50 μM 3-deazaadenosine added 24 hr after transfer of the cells from growth medium to fusion medium. ——, Percent fusion; – – –, creatine kinase (CK) activity.

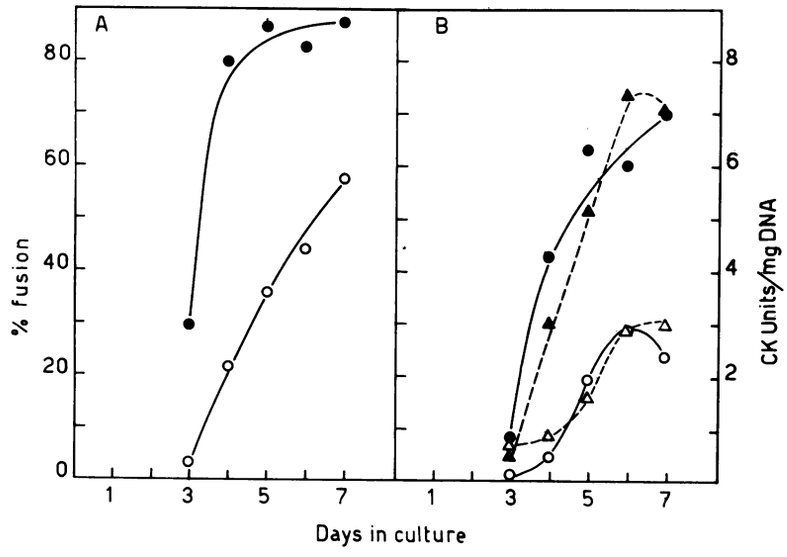

FIG. 2. Differentiation of L5/3C4 myoblasts at passage 4 (*A*) or at passage 10 (*B*) in the presence (closed symbols) or the absence (open symbols) of 50 μM 3-deazaadenosine added 24 hr after transfer of the cells from growth medium to fusion medium. ——, Percent fusion; --, creatine kinase (CK) activity.

absence of 3-deazaadenosine the addition of homocysteine thiolactone (50–100 μM) to culture medium has no effect either on the formation of multinucleated fibers (Fig. 3) or on the appearance of creatine kinase (not shown). In other experiments, we have determined that the optimal concentration of 3-deazaadenosine required to induce differentiation is 50 μM, and that little, if any, differentiation occurs at 3-deazaadenosine concentrations <15 μM. When both homocysteine thiolactone and 3-deazaadenosine were added to myoblast cultures in fusion medium, the concentration of 3-deazaadenosine required to induce differentiation could be reduced by a factor of 10 (Fig. 3). Treatment of L5/3C4 myoblasts with 50 μM 3-deazaadenosine increased the level of fusion from 18% to 43% at day 6. When cells were treated with 100 μM homocysteine thiolactone and *only* 5 μM 3-deazaadenosine, 75% of the nuclei were found grouped in multinucleated fibers. Potentiation by homocysteine thiolactone could be seen both as an increase in the extent of fusion and as an accelerated rate of fusion (Fig. 4).

In the presence of homocysteine the cytotoxicity of 3-deazaadenosine was markedly increased. Fifty micromolar 3-deazaadenosine, a concentration that was well tolerated in the absence of homocysteine thiolactone, became quite toxic in its presence. The number of cells that remained attached to the surface of the culture dish decreased very significantly as a result of both cell death and loss of adhesion capacity

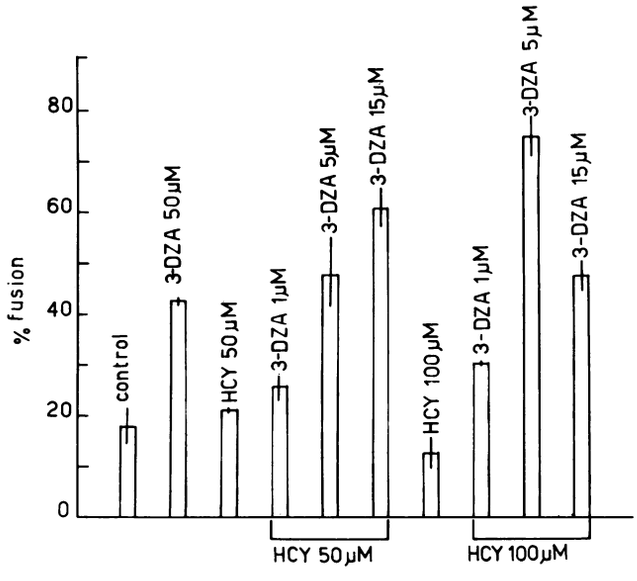

FIG. 3. Potentiation by homocysteine thiolactone (HCY) of the effect of 3-deazaadenosine (3-DZA) on differentiation of L5/3C4 myoblasts. 3-Deazaadenosine and homocysteine thiolactone were added to the cultures 24 hr after transfer from growth medium to fusion medium and the percent fusion was determined on day 6.

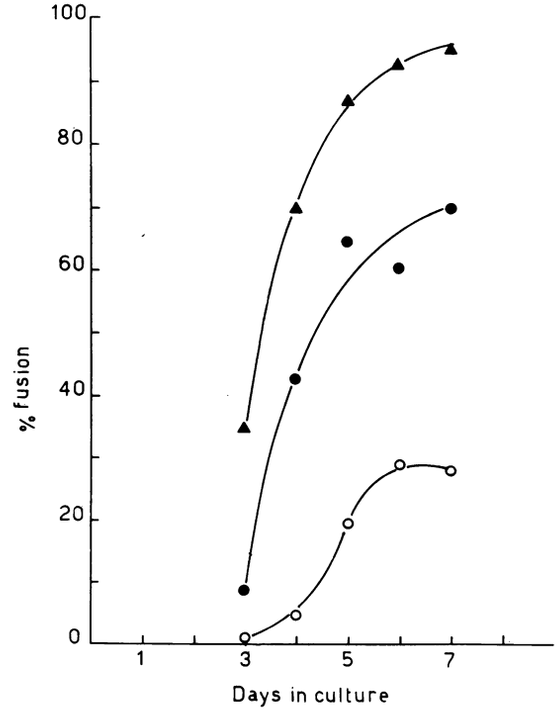

FIG. 4. Time course of L5/3C4 myofiber formation in the presence of 3-deazaadenosine and homocysteine thiolactone. No addition (○), 50 μM 3-deazaadenosine (●), or 5 μM 3-deazaadenosine and 100 μM homocysteine thiolactone (▲) was added 24 hr after transfer from growth medium to fusion medium.

Table 1. Metabolite changes and myofiber formation in the L5/3B5 myoblast cell line upon incubation with 3-deazaadenosine and 3-deazaaristeromycin

Adenosine analog	Intracellular metabolite, dpm per 10^6 cells			AdoMet/PurHcy*	Stimulation of myofiber formation
	AdoMet	AdoHcy	3-DeazaAdoHcy		
None	29,400 ± 1100	470 ± 20		63	−
3-Deazaadenosine, 50 μM	42,600 ± 1500	12,400 ± 2700	8800 ± 400	2.0	+
3-Deazaaristeromycin, 50 μM	49,000 ± 1100	52,300 ± 300		0.9	−

Cells were labeled on day 2 with 10 μCi of [^{35}S]methionine per ml for 2 hr and acid-soluble metabolites were separated by cation-exchange chromatography. Data are the mean ± SEM for duplicate cultures.
*Purine nucleosidylhomocysteine calculated by addition of the radioactivity in AdoHcy and 3-deazaAdoHcy.

(not shown). The increased cytotoxicity of 3-deazaadenosine in the presence of homocysteine thiolactone may account for the smaller extent of fusion by 15 μM 3-deazaadenosine as compared to 5 μM 3-deazaadenosine when 100 μM homocysteine thiolactone was present (Fig. 3).

As noted in the Introduction, treatment of cells with 3-deazaadenosine or 3-deazaaristeromycin results in the formation of 3-deazaAdoHcy with the former and in the accumulation of AdoHcy and in changes in the AdoMet/PurHcy (purine nucleosidylhomocysteine) ratio with both compounds. Table 1 shows that L5/3B5 myoblasts respond to administration of 3-deazaadenosine and 3-deazaaristeromycin in the expected manner. With 50 μM 3-deazaadenosine there was a 26-fold increase in AdoHcy and formation of a similar amount of 3-deazaAdoHcy. The AdoMet/AdoHcy ratio decreased from 63 in the control cells to 2.0. With 50 μM 3-deazaaristeromycin the intracellular accumulation of AdoHcy was more than four times larger than with 3-deazaadenosine, and the AdoMet/PurHcy ratio decreased further to 0.9. It is also seen that the level of 3-deazaAdoHcy accumulating in response to 3-deazaadenosine administration was smaller by a factor of ≈6 than the level of AdoHcy formed in response to 3-deazaaristeromycin. 3-Deazaaristeromycin, however, had no effect on the time course or the extent of myoblast differentiation in these cell lines. It may be concluded, therefore, that the potentiation of differentiation in the presence of 3-deazaadenosine and homocysteine thiolactone is not due to accumulation of AdoHcy, but, rather, is specifically related to formation of 3-deazaAdoHcy.

DISCUSSION

From the data reported above it is evident that treatment with 3-deazaadenosine stimulates the differentiation of myoblasts into myofibers and that the effects of 3-deazaadenosine are greatly potentiated by the addition of homocysteine thiolactone. In myoblast clones, the loss of fusion capacity could be overcome partially by treatment with 3-deazaadenosine and more completely by 3-deazaadenosine and homocysteine thiolactone.

It is important to point out that in most of the previous studies the biological effects of 3-deazaadenosine administration were inhibitory. By contrast, we report here that 3-deazaadenosine administration caused potentiation of differentiation rather than its inhibition. Chiang (25) has previously reported that 3-deazaadenosine increased the frequency of differentiation of 3T3 L1 fibroblasts to fat cells. More recently, Lucas *et al.* (26) presented evidence that inhibition of methylation reactions may induce HL-60 cell maturation.

It has been shown in other systems (18, 19) that addition of homocysteine thiolactone greatly increased the intracellular formation of 3-deazaAdoHcy, whose synthesis is otherwise limited by the availability of homocysteine, and these earlier data account for the potentiation brought about by combined treatment with 3-deazaadenosine and homocysteine thiolactone. 3-DeazaAdoHcy, a congener of AdoHcy, has biological and presumably biochemical effects that are different from, or more specific than, those produced by AdoHcy. Aksamit *et al.* (21) have reported that chemotaxis by the RAW264 cell line is inhibited specifically by 3-deazaadenosine but not by 3-deazaaristeromycin and have concluded that the biological effects of 3-deazaadenosine in the macrophage cell line system were related to the intracellular formation of 3-deazaAdoHcy.

As to the molecular mechanism(s) responsible for the effect of 3-deazaAdoHcy on the differentiation of myoblasts, only conjecture is possible at this time. The formation of 3-deazaAdoHcy could result in the inhibition of AdoMet-dependent transmethylation reactions such as those involved in the methylation of DNA or of other macromolecules of importance in cellular differentiation. As noted in the Introduction, there is a large body of evidence that points to an inverse relationship between DNA methylation and gene expression, and it has been reported that agents such as 5-azacytidine that inhibit DNA methylation may have therapeutic significance (27). Although it is tempting to ascribe the results described above to modulation of DNA methylation, a great deal of additional work will be required before this correlation is firmly established.

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