Interference with DNA Methyltransferase Activity and Genome Methylation during F9 Teratocarcinoma Stem Cell Differentiation Induced by Polyamine Depletion*

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When ornithine decarboxylase, the initial and highly regulated enzyme in polyamine biosynthesis, is irreversibly inactivated by α-difluoromethylornithine, F9 teratocarcinoma stem cells are depleted of putrescine and spermidine and as a result differentiate into a cell type which phenotypically resembles the parietal endoderm cells of the early mouse embryo. Simultaneously the level of decarboxylated S-adenosylmethionine (dcAdoMet), the aminopropyl group donor in spermidine and spermine synthesis, increases dramatically, as the aminopropyl group acceptor molecules (putrescine and spermidine) become limiting. When this excessive accumulation of dcAdoMet is prevented by specific inhibition of the AdoMet decarboxylase activity, the differentiative effect is counteracted, despite the fact that the extent of polyamine depletion remains almost identical. Therefore, it may be concluded that dcAdoMet plays an important role in the induction of differentiation. Moreover, this key metabolite acts as a competitive inhibitor of DNA methyltransferase and is therefore capable of interfering with the maintenance methylation of newly replicated DNA. During the course of F9 cell differentiation, the highly methylated genome is gradually demethylated, and its pattern of gene expression is changed. Our present findings, that the DNA remains highly methylated and that the differentiation process is counteracted when the build-up of dcAdoMet is prevented, provide strong evidence for a causative relation between the level of dcAdoMet and the state of DNA methylation as well as cell differentiation.

Cells require optimal levels of the polyamines, putrescine, spermidine, and spermine, for their growth and differentiation and are therefore equipped with many intricate mechanisms for the control of these levels (1, 2). The polyamine biosynthetic pathway consists of two highly regulated enzymes, ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC), and two constitutively expressed enzymes, spermidine synthase and spermine synthase (Fig. 1). Any cell that becomes polyamine-deficient is severely limited in its ability to grow and proliferate. There is also evidence suggesting that polyamines play a role in apoptotic cell death (3, 4).

More specifically, the polyamines have been shown to regulate ion channel gating (5–7) and polyamine derivatives are constituents of venoms produced by many invertebrates (e.g. funnel-web spiders), where they act as potent neurotoxins (8). Polyamines also regulate programmed translational frameshifting, which is essential for the expression of ODC antizyme, a protein involved in the regulation of polyamine levels both in yeast (9) and in mammalian (10) cells.

Of particular interest, with regard to growth regulation, is the finding that when ODC genes are transfected into cells and overexpressed, the cells go through malignant transformation (11). The fact that inhibition of the ODC activity counteracts malignant transformation suggests that the ODC gene is a proto-oncogene. In this context it also should be noted that the ODC gene is regulated by the Fos (12) and Myc (13) proteins, and by Wilms’ tumor suppressor WT1 (14). In view of the fact that constitutive overproduction of ODC has been observed in many types of cancer cells, the ODC gene appears to be of central importance in the regulation of cell growth, with deregulation (15) or mutation (16) leading to malignant transformation.

In an effort to elucidate the poorly understood role of polyamines in cell differentiation and development, we have focused our attention on F9 teratocarcinoma stem cells, which can be induced to differentiate in a controlled manner in culture, and which provide a model system for studying early events in mammalian development. All-trans-retinoic acid (RA) is a well-known inducer of F9 cell differentiation (17). Less attention has been paid to the fact that F9 cells also differentiate into a parietal endoderm-like phenotype when inhibited in their ODC activity by treatment with the highly specific suicide inhibitor α-difluoromethylornithine (DFMO) (18, 19). That the differentiative effect is indeed due to polyamine depletion is shown by the fact that supplementation of putrescine (the product in the ODC-catalyzed reaction) within the first 2–4 days of DFMO treatment prevents the expression of the

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§ The abbreviations used are: ODC, ornithine decarboxylase; AdoMetDC, S-adenosylmethionine decarboxylase; RA, all-trans-retinoic acid; DFMO, α-difluoromethylornithine; dcAdoMet, decarboxylated S-adenosylmethionine; MTase, methyltransferase; MGBG, methylglyoxal-bis(guanylylhydrazone); AbeAdo, S-[(Z)-4-amino-2-butenyl]methylaminol-5′-deoxyadenosine; iPA, tissue plasminogen activator; AdoHey, S-adenosylhomocysteine; m°C, 5-methylcytosine; m°DC, 5-methyl-2′-deoxycytidine; 4C, 2′-deoxycytidine; HPLC, high performance liquid chromatography.
Inhibitors—DFMO (MDL 72527), an enzyme-activated irreversible inhibitor of ODC (30), and AbeAdo (MDL 73811), an irreversible inhibitor of AdoMetDC (31), were kind gifts from Marion Merrell Dow Research Institute (Cincinnati, OH, and Strasbourg, France). MGBG was purchased from Aldrich-Chemie, and 5-azaadecytidine from Sigma.

Cell Line—The F9 teratocarcinoma stem cell line used was initially isolated by Bernstein et al. (32) from embryoid bodies of the transplantable pluripotent teratocarcinoma OTT 6050-970, a subline of OTT 6050 (33), which originated from the grafting of a 6-day male mouse embryo to the testis of a strain 129/Sv mouse. OTT 6050-970 produces well differentiated tumors containing derivatives of all three germ layers when maintained intraperitoneally in strain 129/Sv mice by serial transfer of ascitic fluid. F9 cells also produced well differentiated tumors at the time of their isolation, but subsequently lost most of their potentialities (32). Since F9 cells do not form the differentiated tissues diagnostic of teratocarcinomas when propagated in the mouse as a solid subcutaneous tumor, they have been designated nullpotent.

Cell Culture—During routine passages, monodisperse F9 cells were seeded to yield a density of 1.8 × 10^6 cells/ml of Dulbecco’s modified Eagle’s medium supplemented with 20% fetal calf serum. The cells were seeded into gelatinized tissue culture flasks and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The cells were routinely subcultured every other day.

For the experiments, monodisperse F9 cells were seeded into a growth medium containing either (a) 5 mM DFMO, (b) 5 μM MGBG, (c) 5 μM DFMO + 5 μM MGBG, (d) 5 mM DFMO + 25 μM AbeAdo, (e) 2.5 μM 5-azaadecytidine, or (f) 1 μM RA. The cells were grown in 75- or 225-cm² Costar tissue culture flasks using 19 and 56 ml of growth medium, respectively. The medium, including inducer and/or inhibitor(s), was exchanged every other day. After various times in the absence or presence of the drug(s), the cells were washed in phosphate-buffered saline (pH 7.2), trypsinized (0.25% trypsin, and 0.02% EDTA in phosphate-buffered saline) and counted. Cell aliquots were pelleted by centrifugation (500 × g, 5 min, 4°C) and stored at −80°C. Cell morphology was documented and photographed at 2, 4, and 12 days of culture in the absence or presence of the various agents. Growth medium components were purchased from Life Technologies, Inc., Europe.

Northern Blot Analysis—Cytoplasmic RNA was isolated from cells essentially as described by Gough (34). RNA from to 3 × 10^6 cells was fractionated by electrophoresis in 1% agarose gels containing 0.66 M formaldehyde in 10 mM sodium phosphate buffer (pH 6.5), and transferred by vacuum blotting to a nylon membrane (Hybond N, Amersham Corp.) using 25 mM sodium phosphate buffer (pH 6.5). The membranes were prehybridized at 60°C for at least 3 h in 0.25 M sodium phosphate buffer (pH 7.2) containing 50% deionized formamide, 0.25 M NaCl, 1 mM EDTA, 7% sodium dodecyl sulfate (SDS), and 100 μg/ml yeast tRNA, using a Hybridization oven equipped with cylindrical bottles.

For one of the differentiation markers (tissue plasminogen activator (tPA)), hybridization was performed at 60°C for 18 h, in the sense RNA probe. This probe was synthesized with T7 RNA polymerase, using a Hybaid hybridization oven equipped with cylindrical bottles.

For the other differentiation marker (collagen type IV α1), hybridization was performed as described below for the DNA MTase cDNA. An 843-base pair BamHI fragment from the mouse α1 type IV collagen-cDNA (pCV-1-PE16) (36) was used as a probe.

To analyze the cellular DNA MTase mRNA content a DNA probe was synthesized using a 933-base pair mouse DNA MTase cDNA BglII/ EcoRI fragment (37) and the Megaprime random primer labeling system (Amersham). Nylon membranes were prehybridized at 50°C for at least 3 h in 5 × Denhardt’s solution containing 6 × SSC (20 × SSC = 3 M NaCl, 0.3 M trisodium citrate (pH 7.0), 0.5% SDS, and 0.1 mg/ml herring sperm DNA. Hybridization was performed at the same temperature for 18 h, using a solution containing 6 × SSC, 0.5% SDS, 0.1 mg/ml herring sperm DNA, and 1–10 × 10^6 cpm/ml of random-labeled DNA probe. The membranes were washed at 55°C for 3 × 20 min in a solution containing 0.2 × SSC and 0.1% SDS.

Quantification of Nucleosides and Polyamines—Homogenates obtained by sonication of F9 cell pellets (1–5 × 10^6 cells) in 100 μl of ice-cold 0.2 M perchloric acid, were kept on ice for 15 min and centrifuged at 1,000 × g for 10 min. The amounts of AdoMet, decAdoMet, and spermidine were measured using a standard HPLC method.
S-adenosylhomocysteine (AdoHcy), putrescine, spermidine, and spermine contained in the supernatant were determined by reversed-phase high performance liquid chromatography (HPLC) (38). The method is based on the separation of the ion pairs formed with 1-octanesulfonic acid on a reversed-phase column (Kromasil KR 100–5C18; Eka Nobel, 15 cm × 4.6 mm inside diameter). The eluate first passed a UV detector at 254 nm to detect AdoMet and deAdoMet. It was then mixed with o-phthalaldehyde reagent and passed through a fluorescence detector to detect the polyamines. For these analyses we used a Varian Vista 5500 liquid chromatography system equipped with a model 9900 AutoSampler, a model 2050 UV variable wavelength detector, a model 2010 HPLC pump, and a Fluorichrom fluorescence detector. A Dynamax HPLC Method Manager and MacIntegrator (Rainin Instrument Company) were used together with a Macintosh SE/30 for method editing and HPLC control and for data collection and analysis (peak identification and quantification).

Quantification of the m5C Content in Genomic DNA—DNA was extracted from F9 cells using a simple salting out procedure (39). Digestion and hydrolysis of DNA was performed using 5 μg of genomic DNA in 200 μl of a solution containing 30 mM sodium acetate (pH 5.3), 0.1 mM ZnCl2, 5 units of nuclease P1 (Sigma), and 0.54 unit of bacterial alkaline phosphatase (Fluka) at 37 °C for 150 min (40). Proteins were precipitated with 96% ethanol. The supernatant was evaporated, and the pellet was dissolved in sterile double-distilled H2O. A quantitative analysis of 5-methyl-2′-deoxycytidine (m5dC) and 2′-deoxyctydine (dC) was performed using reversed-phase HPLC and UV detection at 254 nm. The nucleosides were separated on a Supelcosil LC 18-S (15 cm × 4.6 mm inside diameter) column using a two-buffer system: (A) 0.05 mM KH2PO4 (pH 4.0):methanol (97.5:2.5), and (B) 0.05 mM KH2PO4 (pH 4.0) methanol (80:20). The percentage of methylated cytosines (m5C) in genomic DNA was calculated by entering the measured concentrations of the nucleosides in the formula: m5dC × 100/(dC + m5dC).

DNA MTase Activity Assay—The activity of DNA MTase was determined essentially as described by Li et al. (41). F9 cells were lysed by sonication on ice in 5 volumes of 20 mM Tris-HCl (pH 7.4) containing 0.4 mM NaCl, 25% glycerol, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin (Sigma). Then 1 volume of a 50% (v/v) DEAE-Sephacel slurry, equilibrated with 20 mM Tris-HCl (pH 7.4), was added. After vortexing the mixture for 10 min, the DEAE-Sephacel was removed by centrifugation at 1,600 × g for 5 min. This purification step was done twice. The clarified lysate was stored at −70 °C until used. Lysate equal to 20 μg of total protein, quantified with a Coomassie Brilliant Blue G-250 assay (42), was added to a solution of 20 mM Tris-HCl (pH 7.4) containing 5% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, 5 μg/ml of [methyl-3H]-S-adenosyl-l-methionine (12 Ci/mmol; Amersham), 4 μg of poly(dI-dC)poly(dI-dC) (Pharmacia Biotech Inc.), and 200 μg/ml bovine serum albumin Fraction V. The final volume was 250 μl. The reaction mixture was incubated at 37 °C for 2 h and was then extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous solution was separated by reversed-phase HPLC, stained with o-phthalaldehyde, and quantified using a fluorescence detector. Means ± S.D. (n ≥ 3).

RESULTS

Polyamine Content as Affected by Inhibition of ODC and AdoMetDC—When F9 cells were inhibited in their ODC activity by treatment with 5 mM DFMO, their putrescine and spermidine content was depleted within 2 days of treatment (Fig. 2, A and B). During the remainder of the treatment period, putrescine and spermidine were barely detectable. During the first 2 days of DFMO treatment, the spermine content decreased by more than 50%, but subsequently there was a gradual increase (Fig. 2C). When 5 μM MGBG (43) or 25 μM AbeAdo (31) was provided, in addition to 5 mM DFMO, in order to block the AdoMetDC activity (Fig. 1), the patterns of changes in
polyamine content did not differ significantly from those of F9 cells treated with 5 mM DFMO alone.

AdoMet, dcAdoMet, and AdoHcy Content as Affected by Inhibition of ODC and AdoMetDC—As compared to untreated control cells, DFMO-treated F9 cells exhibited a lower AdoMet content (Fig. 3A). This effect may be due to the fact that DFMO-mediated polyamine depletion has been shown to cause a compensatory increase in AdoMetDC activity (20, 21), which increases the consumption of the substrate (AdoMet).

In untreated F9 cells the dcAdoMet content was only 2–4% of the AdoMet content (Fig. 3, A and B). By reducing the number of acceptor (putrescine and spermidine) molecules for dcAdoMet-derived aminopropyl groups, however, DFMO treatment caused a dramatic increase (almost 30-fold) in the dcAdoMet content of the F9 cells (Fig. 3B). As a result, the dcAdoMet content reached the level of AdoMet by day 2, and exceeded it from day 3 on (Fig. 3, A and B). This increase in dcAdoMet content was largely prevented when the F9 cells were treated with 5 μM MGBG, in addition to 5 mM DFMO.

In untreated F9 cells the AdoHcy content was only 2–4% of the AdoMet content (Fig. 3, A and C). By reducing the number of acceptor (putrescine and spermidine) molecules for AdoHcy synthesis, however, DFMO treatment caused a dramatic increase (almost 30-fold) in the AdoHcy content of the F9 cells (Fig. 3C). As a result, the AdoHcy content reached the level of AdoMet by day 2, and exceeded it from day 3 on (Fig. 3, A and C). This increase in AdoHcy content was largely prevented when the F9 cells were treated with 5 μM MGBG, in addition to 5 mM DFMO.

F9 Cell Growth as Affected by Inhibition of ODC and AdoMetDC—When the polyamine content of F9 cells was reduced by treatment with 5 mM DFMO, their growth rate decreased, and after day 3 there was no further increase in cell number (Fig. 4). The fact that provision of 5 μM MGBG, in addition to 5 mM DFMO, did not significantly change the polyamine content of the cells as compared to DFMO-treated cells (Fig. 2) is consistent with the finding that F9 cell growth was reduced to the same extent in both cases (Fig. 4). The result was similar when 25 μM AbeAdo was used instead of 5 mM MGBG as inhibitor of dcAdoMet formation (not shown). RA, another inducer of F9 cell differentiation (17), exerted a less inhibitory effect on growth than did DFMO, at least at the concentration used (1 μM).

Induction of F9 Cell Differentiation by Inhibition of ODC—Untreated F9 cells grew as tightly packed colonies, characteristic of teratocarcinoma stem cells (Fig. 5, A and B). Within 4 days, the F9 cell cultures had reached confluency and certain areas were overcrowded and cells began to detach from the substrate (Fig. 5B). In the presence of 5 mM DFMO, the F9 cells were not only arrested in their growth (Fig. 4), but gradually changed their phenotype. Thus, the morphology changed from small cells with a round shape and a large nucleus:cytoplasm ratio, to large cells with a flat shape and a small nucleus:cytoplasm ratio (Figs. 5, A–D, and 6). Concomitantly, there was a change from rapidly proliferating cells, with a cell cycle time of 7–8 h, to cells with no further proliferative capacity (18). The terminally differentiated cells resembled parietal endoderm cells, both in terms of morphology and in terms of their increased expression of tPA mRNA (Fig. 7) and collagen type IV α1 mRNA (not shown) (46). Parietal and visceral endoderm form from primitive endoderm and, together with trophectoderm,
they represent terminal lineages whose role is to produce extraembryonic structures which support the developing fetus in the uterus (47, 48). The primitive endoderm as well as the more differentiated parietal endoderm synthesize and secrete tPA and collagen type IV α-1, whereas visceral endoderm does not (46). The induction of tPA mRNA expression occurred after 2–3 days of DFMO treatment, and the cellular tPA mRNA content increased during subsequent DFMO treatment (Fig. 7). The increase in collagen type IV α-1 mRNA was greater in DFMO-treated than in RA-treated F9 cells (not shown).

Since the DFMO-mediated decrease in putrescine and spermidine content resulted in overproduction of dcAdoMet (Fig. 3B), a metabolite that can act as a competitive inhibitor of DNA MTase when present in high enough concentrations (26), and since genome-wide demethylation has been observed during RA-induced F9 cell differentiation (29), it was essential to evaluate the demethylating and differentiative potentials of this nucleoside.

Prevention of the DFMO-induced Accumulation of dcAdoMet—To prevent the DFMO-induced accumulation of dcAdoMet, the F9 cells were inhibited in their AdoMetDC activity, the enzyme catalyzing the synthesis of dcAdoMet, by provision of 5 μM MGBG (or 25 μM AbeAdo). This treatment almost completely blocked the accumulation of dcAdoMet seen in the presence of 5 mM DFMO alone (Fig. 3B).

At variance with data obtained for L1210 leukemia (49), MGBG did not potentiate the antiproliferative effect of DFMO in F9 cell cultures (Fig. 4). Nevertheless, MGBG exerted a slight antiproliferative effect when used as a single agent, at the same (5 μM) concentration (Fig. 5, E and F). Despite this antiproliferative effect, MGBG-treated cultures became overcrowded as early as day 4, and dead cells began to accumulate from then on (Fig. 5, E and F).

Although prevention of dcAdoMet accumulation (by inclusion of 5 μM MGBG or 25 μM AbeAdo in the culture medium) had no effect on the DFMO-mediated growth arrest, there was a pronounced effect on the differentiative process. Thus, F9 cells treated with a combination of 5 mM DFMO and 5 μM MGBG (or 25 μM AbeAdo) did not differentiate to the same extent as did F9 cells treated with DFMO alone (Fig. 5). Eventually, however, there was a slight change in morphology (Fig. 6B). This antidifferentiative effect of MGBG was apparent in analyses of both morphological criteria (Figs. 5 and 6) and expression patterns (Fig. 7). Thus, MGBG counteracted the DFMO-induced accumulation of transcripts encoding tPA and collagen type IV α-1, two differentiation markers representative of parietal endoderm (46).

Cytosine Methylation as Affected by Inhibition of ODC and AdoMetDC—In agreement with other studies (27–29), the nuclear DNA of F9 cells was found to be highly methylated. During the course of treatment with 5 mM DFMO, however, the F9 cell genome underwent a marked loss of methyl groups, first apparent by day 3 (Fig. 8). It is conceivable that this loss is due to DNA replication in the absence of maintenance DNA methylation. A plausible mechanism is that maintenance DNA methylation is prevented by the high concentration of dcAdoMet, which is known to compete with AdoMet for the active site of DNA MTase, thus inhibiting its CpG methylation capacity (26). In agreement with this hypothesis we found that
Enzyme activity was determined by measuring the incorporation of 1.8 mCi of the F9 cell genome. (Phase HPLC and quantified by UV detection at 254 nm. Means ± S.D. (n ≥ 3).

The cells were plated at a density of 1.8 × 10⁴ cells/ml and were cultivated in the absence (○) or presence of 1 μM RA (●), 5 mM DFMO (▲), or a combination of 5 mM DFMO and 5 μM MGBG (■). After treatment of the DNA with nuclease P1 and alkaline phosphatase, the nucleosides were separated by reversed-phase HPLC and quantified by UV detection at 254 nm. Means ± S.D. (n ≥ 3).

When the DFMO-mediated accumulation of dcAdoMet was prevented by simultaneous treatment with MGBG, the F9 cell genome remained highly methylated (Fig. 8). The fact that the decrease in methylated cytosines from day 2 to day 3 is greater than the increase in cell number during the same time period, indicates that interference with maintenance methylation is not solely responsible for this decrease. It is conceivable that the demethylating activity, which has been demonstrated in F9 cells (50, 51), partly contributes to the demethylation observed during DFMO-induced F9 cell differentiation.

The gradual demethylation of DNA seen in F9 cells during DFMO-induced differentiation was comparable to that observed during RA-induced differentiation (Fig. 8). It has been previously shown that the number of methylated CpG sites is markedly reduced on induction with RA, and that this loss of methyl groups affects a large number of CpG sites spread out over the entire F9 cell genome (27–29). However, the molecular mechanism behind the RA-induced DNA demethylation and differentiation of F9 cells remains elusive.

In agreement with the results of Young and Tilghman (29), we found that DNA demethylation brought about by treatment with 5-azacytidine, a demethylation agent (52), did not result in differentiation of the F9 cells. Yet, the methylcytosine content of the genome was lower in 5-azacytidine-treated (about 2% on day 6) (not shown) than in DFMO-treated or RA-treated F9 cells (cf. Fig. 8). Even though these findings suggest that genome demethylation alone is not sufficient to induce the parietal endoderm phenotype, we cannot exclude the possibility that 5-azacytidine, in addition to inhibiting DNA methylation, may have blocked other events necessary for the differentiation of the F9 cells.

The gradual demethylation of the F9 cell genome during DFMO treatment was at least partly attributable to a decrease in DNA MTase activity (Fig. 9). Rather than preceding DNA demethylation, this decrease was found to follow DNA demethylation. It should be emphasized, however, that the DNA MTase activity assay provides an indication of the enzyme activity under optimal conditions, which may not be representative for the in vivo situation with its elevated dcAdoMet content. This fact should also be taken into account when evaluating how the DNA MTase activity is affected in cells treated with both DFMO and MGBG.

To obtain a more reliable estimation of the DNA MTase activity under in vivo conditions, we determined the amount of DNA MTase protein, using a polyclonal antibody (41). During treatment with 5 mM DFMO there was a gradual decrease in methylated cytosines from day 2 to day 3 is greater than the increase in cell number during the same time period, indicates that interference with maintenance methylation is not solely responsible for this decrease. It is conceivable that the demethylating activity, which has been demonstrated in F9 cells (50, 51), partly contributes to the demethylation observed during DFMO-induced F9 cell differentiation.

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DNA MTase Activity and DNA MTase Protein Content as Affected by Inhibition of ODC and AdoMetDC—The gradual loss of methylation of the F9 cell genome during DFMO treatment was at least partly attributable to a decrease in DNA MTase activity (Fig. 9). Rather than preceding DNA demethylation, this decrease was found to follow DNA demethylation. It should be emphasized, however, that the DNA MTase activity assay provides an indication of the enzyme activity under optimal conditions, which may not be representative for the in vivo situation with its elevated dcAdoMet content. This fact should also be taken into account when evaluating how the DNA MTase activity is affected in cells treated with both DFMO and MGBG.

To obtain a more reliable estimation of the DNA MTase activity under in vivo conditions, we determined the amount of DNA MTase protein, using a polyclonal antibody (41). During treatment with 5 mM DFMO there was a gradual decrease in DNA MTase protein (Fig. 10). As for the decrease in DNA MTase activity, the decrease in DNA MTase protein was counteracted by simultaneous treatment with 5 μM MGBG.

DNA MTase mRNA Content as Affected by Inhibition of ODC and AdoMetDC—Treatment with 5 mM DFMO was found to reduce not only the amount and activity of DNA MTase, but also the amount of DNA MTase transcripts (Fig. 11). The size of the DNA MTase mRNA corresponds to that observed in other F9 cells (53) and in a majority of mouse tissues (54). The DFMO-inducible reduction in DNA MTase mRNA level did not...
take place when the build-up of dcAdoMet was prevented by treatment with 5 μM MGBG (Fig. 11).

**DISCUSSION**

Inactivation of ODC, a key enzyme in polyamine biosynthesis, by DFMO treatment, causes a permanent block in F9 cell proliferation and gradual changes in cell morphology and gene expression, resembling those occurring during the differentiation of inner cell mass to parietal endoderm (18). The latter events take place in the early mouse embryo, generating the extrabryonic endoderm of the parietal yolk sac, which surrounds the developing mouse embryo (47).

In view of the fact that addition of polyamines has been shown to reverse or prevent antiproliferative and differentiative effects of DFMO, it has been concluded that these effects are due to polyamine depletion (18, 19). However, the possibility remains that antiproliferative and differentiative effects of DFMO treatment are at least partly due to overproduction of dcAdoMet. This key metabolite accumulates to very high levels during the course of DFMO-mediated polyamine depletion, in F9 cells (the present study) as well as in other types of cells (23–25). The highest dcAdoMet levels recorded (1–1.5 nmol/10^6 F9 cells (the present study) as well as in other types of cells (23–25). The highest dcAdoMet levels recorded (1–1.5 nmol/10^6 F9 cells), have been observed after removal of the selective pressure exerted by an AdoMetDC inhibitor from cells that overexpress AdoMetDC due to a 10–16-fold amplification of the AdoMetDC gene (55). In cells other than these AdoMetDC overproducers, the accumulation of dcAdoMet is a direct consequence of the polyamine-deficient state, because putrescine and spermidine are the only acceptor molecules for the amino- propyl group of dcAdoMet, that is normally transferred in the enzymatic reactions catalyzed by spermidine synthase and spermine synthase, respectively.

When putrescine and spermidine are added, they do not only replenish the cellular polyamine pools, but they also serve as acceptor molecules for dcAdoMet-derived aminopropyl groups in the synthesis of spermidine and spermine, thus reducing or eliminating the excess of dcAdoMet. Therefore, the fact that polyamines can prevent or reverse antiproliferative and differentiative effects caused by DFMO treatment does not prove that polyamines are involved in these processes.

It is conceivable that dcAdoMet, when present at an abnormally high concentration, may compete with AdoMet in various methylation reactions, thus reducing, e.g. the degree of DNA methylation. In fact, when the concentration of dcAdoMet exceeded that of AdoMet by 5-fold in an *in vitro* assay, almost no DNA MTase activity remained (26). We cannot entirely exclude the possibility that the accumulation of AdoHcy, a competitive inhibitor of virtually all transmethylases (44), including DNA MTase (45), could at least partly contribute to the inhibition of DNA methylation. However, the relatively modest change in the AdoHcy/AdoMet ratio (from approximately 0.08 to 0.43) makes this possibility very unlikely. Only AdoHcy/AdoMet ratios >1 appear to exert a significant inhibitory effect on the utilization of AdoMet for methylation (56).

The view advanced above is consistent with the present finding, that DFMO-induced accumulation of dcAdoMet is paralleled by genome-wide DNA demethylation (occurring during the course of F9 cell differentiation). To test the possibility of interference with DNA methylation, using a more direct approach, the DFMO-induced excessive accumulation of dcAdoMet was prevented by simultaneous treatment with MGBG (43) or AbeAdo (31), potent inhibitors of dcAdoMet formation. This treatment counteracted the demethylation of the genome and interfered with the differentiative processes induced by DFMO alone, thus lending support for our view that dcAdoMet accumulation is an important factor in these events. From this also follows that it is not the lack of polyamines per se that acts as a differentiative signal in DFMO-mediated F9 cell differentiation.

These findings indicate that polyamine deficiency may induce terminal differentiation through an indirect, inhibitory effect on DNA methylation. The high level of dcAdoMet is likely to compete with AdoMet, the methyl group donor, at the active site of DNA MTase, thus reducing the methylation capacity of the enzyme. Consistent with a decreasing DNA methylation capacity, we also find that the amounts of DNA MTase mRNA and DNA MTase protein are gradually reduced during the course of DFMO-mediated F9 cell differentiation. The latter effects may be, at least partly, due to the fact that 1) the largest amounts of DNA MTase mRNA^2 and protein (57) are found during the S phase of the cell cycle and that 2) the percentage of cells in the S phase decreases markedly during the course of F9 cell differentiation (18). The fact that there is still a relatively large amount of DNA MTase protein present in the F9 cells on day 3, although the percentage of S phase cells is low (18), may be a consequence of a relatively slow turnover of the protein.

The observed decrease in DNA methylation is likely to be an integral part of the corresponding differentiation process *in vivo*, because a similar decrease has been observed in the developing mouse embryo. Thus, there is a striking global undermethylation of the DNA in the extraembryonic lineages derived from the primitive endoderm as compared to the mouse embryo itself (28, 48, 58). Moreover, the fact that retroviral genomes are suppressed by DNA methylation when introduced into early embryos and F9 cells, but not when introduced into their differentiated counterparts (59), suggests that the DNA methylation capacity decreases during the corresponding differentiation process *in vivo*. The genes which are directly responsible for parietal endoderm differentiation are still unidentified, but are likely to be found among those genes activated by demethylation.

The experiments described in this study focus on a key metabolite, dcAdoMet, which normally acts as an aminopropyl group donor in polyamine biosynthesis, but which is shown to act as an inhibitor of DNA methylation and as an inducer of teratocarcinoma stem cell differentiation. The extremely low steady-state level of dcAdoMet observed under physiological conditions is maintained by a balance between the rate of synthesis (*i.e.* the AdoMetDC activity), which is feedback-regulated by the polyamines (20–22), and the rate of utilization of the aminopropyl group for polyamine synthesis. This balance may be disrupted by AdoMetDC gene amplification (55) or inhibition of polyamine biosynthesis (this study), and may result in excessive dcAdoMet levels. In the case of teratocarcinomas, where the consequence of ODC inhibition is their differentiation into a cell type with no further proliferative capacity, our finding may have clinical utility in the development of a therapeutic approach to ovarian and testicular cancer.

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