Treatment of Human Cell Lines with 5-Azacytidine May Result in Profound Alterations in Clonogenicity and Growth Rate

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ABSTRACT Liquid medium cultures of three human cell lines (B-lymphoma, myeloma, and squamous lung carcinoma) with population-doubling times (PDT) and cloning efficiencies (CE) in the range of 32–43 h and 0.01–5.6%, respectively, were exposed to 5-azacytidine (5-azaC) for 3 d. The doses used (1–3 μM) were found to be nontoxic as measured by cell growth in liquid and semisolid agar medium and to be nonmutagenic as measured by the rate of generation of ouabain- and 6-thioguanine-resistant cell variants. After 5-azaC treatment, cell samples were subsequently harvested every day and assayed for their CE in semisolid agar medium. For each cell line, 30 to 42 individual clones were harvested at the day of maximal CE and expanded in liquid culture medium. PDT and CE were determined for each subclone about every 6 wk for 12 mo. The majority of the subclones had unaltered PDT and CE compared to the original lines. However, several clones had profoundly changed proliferative activity with PDT on ~12–14 h and/or CE 5 to >50%. Some of the clones with altered growth properties reverted to PDT and/or CE values of untreated clones. However, a few clones of each line had stable alterations with PDT on 12–14 h and CE 5 to >50%; these clones were all significantly hypomethylated. It is concluded that the human gene repertoire does contain genes that appropriately activated can result in growth properties with very short PDT and high CE (and comparable to animal cell lines), and that this activation may be obtained by 5-azaC treatment. It is conceivable that the procedure here described to alter growth properties of human cell lines may be applied to experimental situations, where alterations of cell growth properties are desired.

Many reports in recent years on regulation of both cellular (1–6) and viral genes (7–11) have provided experimental support to the hypothesis (12–15) that 5-methylcytosine (m^5Cyt)[1] may be important in the regulation of gene expression. Moreover, a number of experiments have shown that exposure of cells to 5-azacytidine (5-azaC) may result in hypomethylation of DNA and cause expression of otherwise silent genes of both cellular and viral origin (for review, cf. references 16, 17) to the extent that it mimics mutation induced genetic alterations, e.g., reversion frequency of thymidine kinase negative cells to thymidine kinase positive cells (18).

The growth properties of malignant tumor cell lines are at least in part reflected in the growth rate and CE. However, it has hitherto not been possible by exogenous factors to alter profoundly these biological features, despite numerous attempts that mainly have been based on alterations in the growth medium conditions such as addition of growth factors like insulin and transferrin. Moreover, human tumor cell lines generally have population-doubling times (PDT) >20–24 h and lower cloning efficiencies (CE) than comparable animal lines, and it has been an unresolved question, whether the gene repertoire in human cells upon appropriate activation can result in growth properties comparable to rodent cells. Finally, it is for many purposes desirable to have cell lines with high proliferative activity.

[1] *Abbreviations used in this paper:* 5-azaC, 5-azacytidine; CE, cloning efficiency; m^5Cyt, 5-methylcytosine; Oua^r, ouabain resistant; PDT, population-doubling time; RPMI/FCS, RPMI-1640 medium supplemented with 0.3% L-glutamine and 10–15% fetal calf serum; $6\text{-}TG^r$, 6-thioguanine resistant.

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We report here that treatment with 5-azaC resulted in profound changes of growth rates and CE of three human tumor cell lines (lymphoma, myeloma, squamous cell lung carcinoma). In particular, cell lines with very short PDT (12–14 h) and/or high CE (5 to >50%) values were obtained and were found to be significantly hypomethylated. This indicates that m^5Cyt is a significant regulatory factor in relation to genes that are involved in the determination of PDT and CE. Our experiments also suggest a general method to alter profoundly the growth properties of human cells.

MATERIALS AND METHODS

Cell Lines and Treatment with 5-azaC: The three human cell lines, RH-L4 (B-lymphoma [19]), RH-SLC-L11 (squamous lung carcinoma line [20]), and SKO-007 (myeloma [21]) were maintained routinely in RPMI-1640 medium supplemented with 0.3% L-glutamine and 10–15% fetal calf serum (RPMI/FCS). 5-AzaC was added to the culture medium, and the cells grown in this medium for 3 d. Subsequently, cells were harvested each day from the liquid cultures and cloned in semisolid agar medium (Bacto-Agar, Difco Laboratories, Inc., Detroit, MI) in RPMI/FCS. The procedure is outlined in Fig. 1.

Assays for Toxicity and Mutagenicity of 5-AzaC: The dose of 5-azaC was for each cell line chosen as the highest concentration that did not show any sign of toxic or mutagenic effects on the cells. Toxicity was measured by the effect on growth properties as estimated from conventional growth curves and from CE in semisolid agar medium. In the latter test, 10^3 cells were seeded into wells of 24-well plates (Nunc, Roskilde, Denmark) in 1.0 ml 0.3% agar-RPMI/FCS semisolid medium. The number of clones (>50 cells) was counted under a microscope 2–3 wk later and the CE determined as the percentage of clones among the total number of cells seeded. The experiments demonstrated (Fig. 2) that a dose of 3 μM 5-azaC was applicable for the RH-L4 and the RH-SLC-L11 line, and a dose of 1.0 μM for the SK0-007 cell line.

The mutagenic effect of 5-azaC was analyzed by measurement of the rate of induction of ouabain-resistant (Oua^r) variants and of 6-thioguanine-resistant ($6\text{-}TG^r$) variants. Cells were grown for 3 d in 5-azaC in large cultures, washed three times in RPMI-1640 medium and reseeded into wells of 24-well plates in 1.0 ml RPMI/FCS with 10 μM ouabain with a cell concentration of 5×10^6/well, 10^6/well, 5×10^5/well, or 5×10^4/well. 24 wells were seeded for each cell concentration and medium exchanged every 2–3 d. In wells with cell growth, the ouabain concentration was increased to 100 μM, and the number of wells with growth counted 2 wk later. The mutation frequency was calculated from the number of wells with growth in the cell concentration that did not result in growth in all wells; e.g., growth in all wells seeded with 10^6 cells indicates mutation frequency $>10^{-6}$, and growth in 5 wells of 24 wells seeded with 10^5 cells/well indicates a mutation frequency at $\sim 2 \times 10^{-6}$. Table I shows that the doses selected for the three lines (3 μM for RH-L4 and RH-SCL-L11, 1 μM for SKO-007) did not result in a measurable increase in the number of Qua^r or $6\text{-}TG^r$ variants.

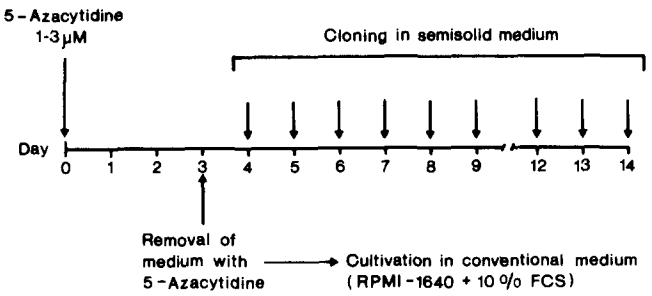

FIGURE 1 Experimental procedure for 5-azaC treatment of the human B-lymphoma cell line (RH-L4), the human SKO-007 myeloma cell line, and the human squamous lung carcinoma cell line (RH-SLC-L11). 5-AzaC (3 μM for RH-L4; 1 μM for SKO-007; 3 μM for RH-SLC-L11) was added to the culture medium at day 0 and removed 3 d later. The cells were then grown in RPMI-1640 medium with 15% FCS and without 5-azaC. Cells were harvested each day from the liquid cultures and cloned in 0.3% semisolid agar medium (Bacto-agar, Difco Laboratories, Inc.) in RPMI-1640 with 15% FCS. Clones were scored 14–30 d later. 5-AzaC-treated RH-SLC-L11 were cloned every day for 30 d and the RH-L4 and SKO-007 cell lines for 16–28 d.

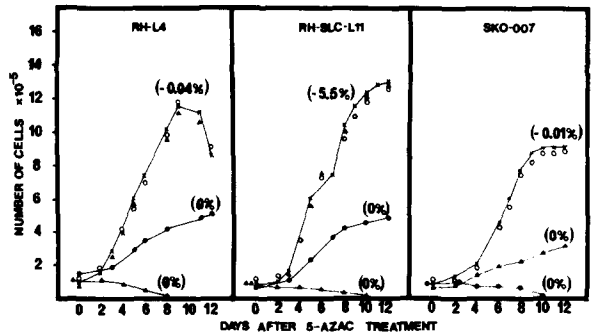

FIGURE 2 Growth curves and cloning efficiency of RH-L4, RH-SLC-L11, and SKO-007 cells after treatment with 5-azaC in various doses (×, no treatment; O, 1.0 μM; Δ, 3.0 μM; ●, 6.0 μM; ▲, 10.0 μM). The cloning efficiencies are indicated in parenthesis. The 5-azaC treatment was for all doses done for 3 d as described in detail in Materials and Methods.

TABLE I
Data

Cell line	Treatment*	No. of drug resistant cells/10^6 cells: 10 μM Oua	1 μM 6-TG
RH-L4	None	0.2	0.3
	0.1 μM 5-azaC	0.1	0.5
	1.0 μM 5-azaC	0.2	0.5
	3.0 μM 5-azaC	0.2	0.5
	6.0 μM 5-azaC	0.2	0.3
	3.0 μg/ml MNNG	19	12
RH-SLC-L11	None	0.1	0.2
	0.1 μM 5-azaC	0.1	0.1
	1.0 μM 5-azaC	0.1	0.1
	3.0 μM 5-azaC	0.1	0.1
	6.0 μM 5-azaC	0.2	0.3
	3.0 μg/ml MNNG	11	9
SKO-007	None	0.7	1.0
	0.1 μM 5-azaC	0.5	1.0
	1.0 μM 5-azaC	0.7	1.0
	3.0 μM 5-azaC	0.7	1.0
	6.0 μM 5-azaC	1.0	1.7
	3.0 μg/ml MNNG	9	14

Frequency of Oua^r and $6\text{-}TG^r$ cells in 3 human cell lines after treatment with 5-azacytidine (5-azaC) or with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (*MNNG*).

* The cells were treated for 3 d with 5-azaC, washed, and reseeded in wells (as indicated in Materials and Methods) with either Oua or 6-TG. Treatment with *MNNG* was performed as described by Boon and Kellerman (22). The cells were exposed to *MNNG* at 3 μg/ml for 60 min, washed, and placed in culture.

DNA-Methylation: The amounts of m^5Cyt in newly replicated DNA was estimated by labeling the cells with ^{14}C-deoxycytidine (120 nCi/ml; sp.act. 460 Ci/mol; Amersham) and subsequently hydrolysis of the bases in 96% formic acid for 60 min at 175°C, separation of the bases by thin-layer chromatography (23), and the percentage of enzymatic methylation calculated as 100% $m^5Cyt(cpm)/Cyt(cpm) + m^5Cyt(cpm)$.

RESULTS

Effects of 5-AzaC on CE

The CE of RH-L4 cells varied significantly after 5-azaC treatment (Fig. 3), and was associated with a remarkable

change in morphology of the agar colonies (Fig. 4). The corresponding data for the effects of 5-azaC on a human myeloma line and a squamous cell lung carcinoma line are seen in Table II.

30–42 clones were harvested individually from agar cultures of the untreated parental cell lines and 10–20 clones from 5-azaC–treated cultures. All clones were subsequently expanded in liquid culture medium. The cloning efficiency and growth rate were determined for each clone (Table III) after expansion to ~10^6–3 × 10^6 cells. A high growth rate was not necessarily associated with high CE and vice versa. The values for CE and growth rate of the clones harvested from untreated cultures did not vary significantly. Expression of cell surface HLA class I and class II antigens was not changed significantly either quantitatively or qualitatively in the various clones as analyzed on a fluorescence activated cell sorter after staining with fluorescein isothiocyanate–conjugated monoclonal antibodies against monomorphic determinants of HLA class I and class II antigens (data not shown).

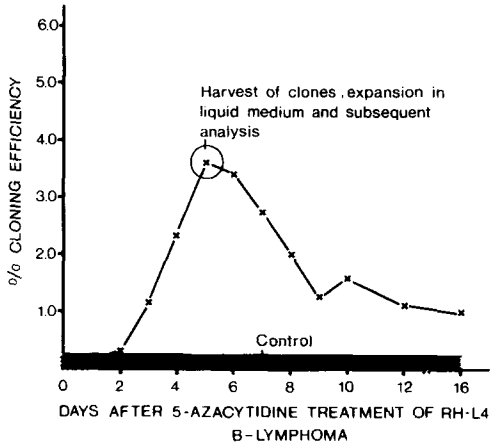

FIGURE 3 Cloning efficiency of the human RH-L4 B-lymphoma cell line 0–16 d after the cells had been cultivated for 3 d in RPMI-1640 medium with 15% FCS, 0.3% L-glutamine, and 3 μM 5-azaC. The cells were cloned in 0.3% agar in 24-well plates (Nunc, Roskilde, Denmark) with 10^3–5 × 10^4 cells per well. The number of clones were counted 14–24 d later and cloning efficiency calculated as the number of colonies per 100 cells seeded. Control cultures were RH-L4 cells in RPMI-1640 medium with 15% FCS, and were continuously adjusted to the same density as 5-azaC-treated cells. The experiments were repeated five times. The curve shows a typical result. Colonies were harvested individually from the agar cultures at day 5 and each colony expanded in liquid RPMI-1640 medium with 15% FCS.

CE and Growth Rate of 5-AzaC–treated Subclones

Clones with high and low CE and/or growth rates were selected from the RH-L4 line for further analyses and main-

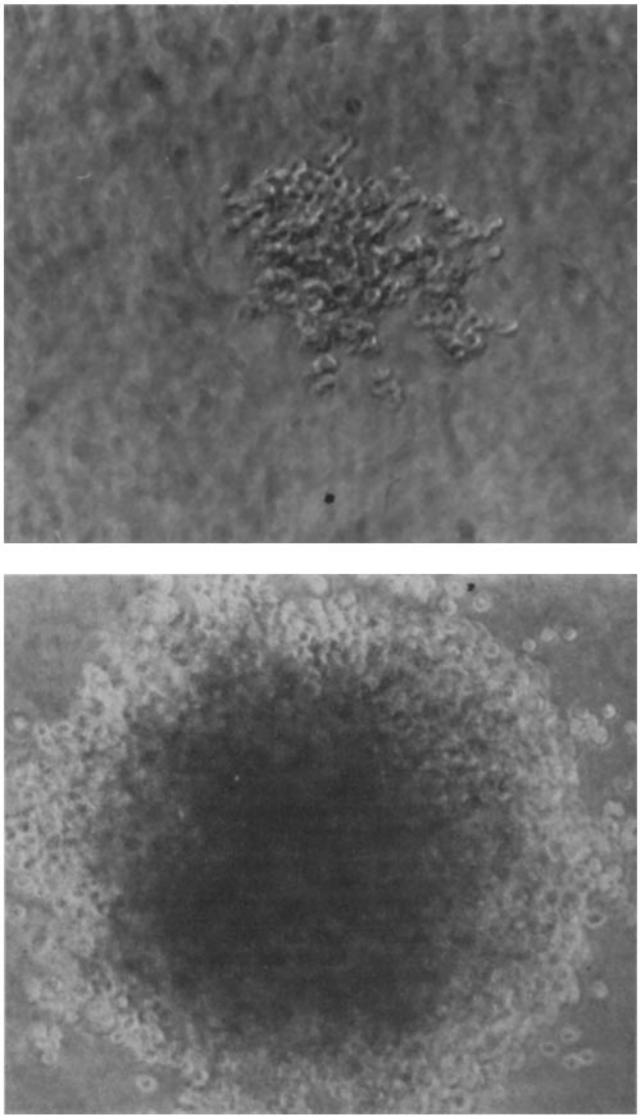

FIGURE 4 Typical RH-L4 B-lymphoma cell colony in semisolid agar medium 14 d after seeding of cells taken from (*top*) conventional cultures and (*bottom*) cultures treated for 3 d with 3 μM 5-azaC and subsequently cloned.

TABLE II

Effects of 5-Azacytidine on CE of Three Human Tumor Cell Lines

	Cell lines		
	RH-L4	SKO-007	RH-SLC-L11
PDT before 5-azaC*	35 h	43 h	32 h
CE before 5-azaC‡	0.04 ± 0.01%	0.01 ± 0.01%	5.6 ± 0.4%
Maximum CE after 5-azaC treatment	3.7 ± 0.3%	2.2 ± 0.3%	12.8 ± 1.2%
Day of maximum CE after removal of 5-azaC from culture medium	5	17	12

* PDT and CE were established as described in Materials and Methods.
‡ The values are mean ± SD of three to eight experiments. The variation in PDT was below 15% for all three cell lines.

TABLE III

PDT and CE of Subclones of the RH-L4, SKO-007, and RH-SLC-L11 Cell Lines after Treatment with 5-AzaC

Cell line	Subclone* No.	5-AzaC treatment	PDT in hours 25–35 d after harvest of colonies‡ (range)	CE 28–31 d after harvest of colonies§ (range)
				%
RH-L4	40–71	–	34 (29–41)	0.04 (0.02–0.06)
	1	+	14	24.4
	3	+	26	>50
	13	+	12	7.0
	14	+	14	9.0
	17	+	18	5.6
	18	+	31	12.6
	2, 5, 6, 7, 8, 9, 10, 11, 12, 15, 16, 19, 20	+	32–41	0.01–0.08
SKO-007	20–50	–	43 (37–54)	0.01% (<0.01–0.0)
	5	+	18	1.6
	6	+	44	16.5
	8	+	15	11.3
	1, 2, 3, 4, 7, 9, 10	+	42–49	<0.01
RH-SCL-L11	25–67	–	32 (24–36)	5.5% (3.7–7.1)
	2	+	34	>50
	3	+	14	11.0
	6	+	16	5.8
	9	+	41	6.1
	11	+	24	21.8
	1, 4, 5, 7, 8, 10, 11	+	30–41	5.1–6.2

* Colonies were harvested individually from the agar under a microscope and expanded in liquid RPMI-1640 medium with 10–15% FCS.

‡ Population doubling time and cloning efficiency were determined as described in Materials and Methods. Moreover, flow cytofluometric measurements (data not shown) on propidium iodine stained nuclei revealed that the shortening of PDT in all cases was a result of a decrease in G1-phase duration and an increase in growth fraction.

tained in conventional RPMI-1640 medium with 10% FCS. The CE and generation doubling times were determined every 6 wk for a 12-mo period (Table IV). Most of the clones reverted to values of untreated cultures, but some clones have been stable in respect to their CE and growth rates now for more than 12 mo. The total amount of DNA in the clones did not differ significantly from the original cell lines. However, 5-azaC treatment resulted in significant hypomethylation of most of the subclones, whereas the level of m^5Cyt in untreated subclones was stable (Table V). Those 5-azaC-treated clones that reverted to growth properties of untreated clones regained all approximately the same amounts of m^5Cyt as untreated cultures (data not shown).

DISCUSSION

The data demonstrate that the growth rates and CEs of human malignant cell lines can be significantly altered by 5-azaC treatment in doses that seemed neither mutagenic nor toxic as measured by the effects on cell growth and on the generation of Oua^r and/or $6\text{-}TG^r$ variants; this is in line with findings in other cell systems (24). It seems also excluded that subclones with short PDT and/or high CE preexisted in the culture, because (*a*) such subclones within only a few weeks would be predominant in the culture, and the culture would consequently have a very fast growth rate and/or high CE, and because (*b*) all subclones isolated from the nontreated culture had growth properties comparable to the original culture.

The effects of 5-azaC can therefore be assumed to be a result of its incorporation into DNA in place of cytosine, resulting in expression of genes that have been silent due to m^5Cyt or due to the general inhibitory effect of 5-azaC on DNA methylase activity (16). This concurs with the hypomethylated state of most clones treated with 5-azaC, although hypomethylation patterns are only very indirect evidence of the importance of methylation in gene control, as the replacement of cytosine by 5-azaC during DNA replication can be assumed to be a random process. Moreover, it is possible that the gene(s) responsible for the altered growth rate/CE is different not only between the three histologically different types of malignant cell lines, but also in relation to the different subclones of a given cell line. However, this problem cannot be approached properly before the genes controlling growth activity and clonogenicity are known. The present experiments nevertheless strongly indicate that the genome of human malignant cells contains genes that appropriately activated result in profound alterations of proliferation patterns that may result in cell lines with very short PDT and/or high CE.

Intratumoral phenotypic diversity is known to be expressed in malignant cell populations in respect to a number of biological features, including growth rate and clonability (25–30) and onc-gene expression (31). The biological basis for this diversity is unknown, but involves probably both genetic and epigenetic factors. The effects of 5-azaC on the proliferative features of the three lines suggest that changes in methylation patterns have a central role in the generation of biological

TABLE IV

Data

Cell line	Subclone No.	Time after harvest of tumor cell colonies from agar (d) 60–65 PDT (h)	60–65 CE (%)	104–114 PDT (h)	104–114 CE (%)	172–186 PDT (h)	172–186 CE (%)	200–365 PDT (h)	200–365 CE (%)
RH-L4	1	35	0.08	32	0.05	39	0.06	37	0.05
	3	32	>50	30	>50	31	>50	32	>50
	4	14	0.4	14	8.3	12	7.6	12	8.3
	13	14	1.1	16	1.0	18	0.7	18	0.1
	14	14	9.0	12	9.0	11	8.5	14	9.0
	17	37	3.4	37	1.8	38	1.0	34	1.0
	18	34	2.2	36	0.9	33	0.06	33	0.05
SKO-007	5	43	<0.01	47	<0.01	48	<0.01	44	<0.01
	6	51	2.6	49	2.3	55	0.1	51	0.1
	8	16	10.2	45	9.9	47	7.6	47	8.6
RH-SCL-L11	2	36	9.6	42	7.2	36	6.1	35	5.8
	3	16	4.9	17	5.9	28	5.7	30	5.7
	6	12	6.8	13	7.2	15	8.1	16	7.2
	9	44	5.7	49	6.1	44	7.2	46	5.8
	11	49	19.3	48	18.7	47	16.2	43	17.3

Changes in PDT and CE in subclones of RH-L4, SKO-007, and RH-SCL-L11 as a function of time after treatment with 5-azaC. All subclones were maintained in liquid RPMI-1640 medium with 10–15% FCS. The experiment has been run thrice and the results in the table are from the second experiment. PDT and CE were determined as explained in Materials and Methods.

TABLE V

DNA Methylation in Subclones of RH-L4, SKO-007, and RH-SCL-L11 with Stable (>12 mo) Changes in PDT and CE

Cell line	Subclone No.	5-AzaC treatment	% m^5Cyt of total cytosine
	Original RH-L4 clone	−	3.9 ± 0.3
	31 subclones	−	3.7 ± 0.5
RH-L4	3	+	0.7 ± 0.2
	4	+	2.4 ± 0.1
	13	+	0.4 ± 0.3
	14	+	0.5 ± 0.3
	Original SKO-007 clone	−	4.6 ± 0.2
	30 subclones	−	4.7 ± 0.5
SKO-007	6	+	2.1 ± 0.1
	8	+	0.7 ± 0.3
	Original RH-SCL-L11 clone	−	3.0 ± 0.1
	42 subclones	−	3.0 ± 0.4
RH-SCL-L11	2	+	2.1 ± 0.1
	6	+	0.8 ± 0.2
	11	+	1.7 ± 0.1

diversity. Such diversity may on the other hand be a necessary feature of malignant behavior (26, 32) as well as it is conceivable that demethylation is an important event in oncogenesis (33). It was also recently demonstrated that demethylation of certain genes in the murine Lewis lung tumor system may lead to metastatic activity of otherwise nonmetastatic cells (34).

Finally, the various subclones here reported may provide the experimental basis for isolation of DNA sequences that can be useful for analysis of the genetic basis for alteration of growth rate and cloning efficiency, e.g., through studies of certain "candidate" genes like various oncogenes and genes encoding for various growth factors.

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