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5-azacytidine-induced decrease in the frequency of Barr body in human fibroblasts

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Summary

5-azacytidine-treated human fibroblasts exhibit a significant decrease in the frequencies of Barr body$^+$ cells as compared to nontreated cultures. This presumably indicates that 5-azacytidine can induce a change in the degree of condensation of the Barr body. It is suggested that the state of chromatin condensation of the Barr body may be related to the reactivation process by 5-azacytidine of gene loci in the inactive *X*.

1. Introduction

It is now established that one of the *X* chromosomes in a diploid cell of the human female becomes genetically inactive during early embryogenesis and forms a nuclear structure at interphase, called the Barr body. This chromatin body could be unambiguously identified by fluorescence techniques (Mukherjee, Moser & Nitowsky, 1972; Kondra & Ray, 1978).

In mammalian cells about 5% of the cytosine residues in DNA occurs as 5-methylcytosine. It is also known that the cytidine analogue, 5-azacytidine (5-azaC), can induce hypomethylation of DNA, and that it can be used for reactivation of gene(s) on the inactive human *X* chromosome in cell hybrids (Mohandas, Sparkes & Shapiro, 1981; Lester, Korn & De Mars, 1982; Hors-Cayla, Hevertz & Frezal, 1983). The reversal of DNA methylation by 5-azaC also alters the *X* chromosome replication pattern (Schaffer & Priest, 1984) and 5-azaC can induce undercondensation of human lymphocyte chromosomes (Schmid, Haaf & Grunert, 1984). Moreover, it has been shown that 5-azaC treatment of mammalian cells can suppress the condensation of distinct chromosomal regions at metaphase (Viegas-Pequignot & Dutrillaux, 1976, 1981) and that it can prevent chromatin condensation only when incorporated into DNA during S phase (Viegas-Pequignot & Dutrillaux, 1976).

This study was performed to determine whether the degree of chromatin condensation of the Barr body in a human cell can be altered by 5-azaC. If 5-azaC can induce a change in the structure of the Barr bodies in a proporation of cells to the extend that they can no longer be morphologically distinguished from other chromocenters in the nuclei, then lower frequencies of the Barr body$^+$ cells would be found in treated cultures as compared to nontreated cells.

2. Materials and Methods

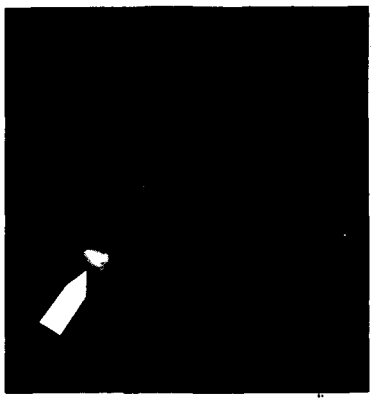
Two diploid cell strains were utilized: (1) fibroblasts from a normal (46, *XX*) female fetus and (2) IMR-90 (46, *XX*). Monolayer cultures were grown directly on microscope slides in a series of large Leighton tubes containing McCoy's 5A medium with 15% foetal bovine serum and antibiotics. After 24 h incubation at 37 °C, the foetal cells in each tube were exposed for either 24 or 48 h to a medium containing 2 μM 5-azaC (SIGMA). The cell strain, IMR-90, was exposed for 24 or 48 h to five different dosages of 5-azaC (1, 2, 4, 8, and 10 μM). The culture medium containing 5-azaC was then removed from each Leighton tube and all cells were rinsed thoroughly with HBSS. Fresh medium without 5-azaC was then added to each tube and the cells were allowed to grow for an additional 48 h before being fixed in 1:3 fixative for 30 min and air dried on slides. Control cultures were treated identically except for omission of 5-azaC. Since it has been shown that the frequency of Barr body$^+$ nuclei in cultured human fibroblasts is related to cell density (Klinger *et al.* 1968; Mukherjee & San Sebastian, 1978), the average cell densities for a particular set of treated and control cultures were kept more or less constant by seeding the same number of cells in each culture tube. The cell viability and growth were not adversely affected in the experimental cultures as determined by trypan blue test and by determination of cell numbers at various time intervals.

Fluorescent staining of cells with acridine orange (A0) was done according to a previously published

method (Kondra & Ray, 1978). All slides were screened blindly by a single individual under a Leitz fluorescent microscope. The light source had an HB0 200W mercury vapor lamp, a BG 12 exciter filter and a 510 nm barrier filter. The proportion of nuclei with brightly fluorescent Barr bodies was then determined for each cell preparation. The data were derived from duplicate series of each treated and control culture. Statistical analyses were done using a 2×2 contingency χ^2 test.

3. Results

Fig. 1 and 2 show typical fluorescent Barr bodies in control and 5-azaC-treated fibroblast cultures. Table 1 presents our data on the frequency of Barr body$^+$ nuclei in fetal fibroblasts following 2 μM 5-azaC treatment. For each 24 or 48 h exposure, the decrease in the % Barr body$^+$ cells was statistically significant ($P < 0{\cdot}001$). Tables 2 and 3 show the frequencies of Barr body$^+$ nuclei in another cell strain, IMR-90, exposed to 5-azaC for 24 or 48 h at various dose levels (1–10 μM). Again the % Barr body$^+$ nuclei in treated cells was lowered significantly ($P < 0{\cdot}001$) for each of the five dose levels and the decrease was dose-dependent, i.e. the higher the dose, the higher was the % Barr body$^-$ cells. It was noted that the longer exposure time (48 h) seemed to induce a greater drop in the % Barr body$^+$ nuclei compared to the shorter exposure time (24 h) (compare Tables 2 and 3).



Human fibroblasts (IMR-90) showing typical Barr bodies (arrow) following 48 h growth in Leighton tubes.
Fig. 1. Control culture.

Fig. 2. 10 μM 5-azaC-treated culture.

4. Discussion

A fluorescent staining method using acridine orange permitted accurate identification of the Barr body on the basis of its typical size, shape and fluorescence intensity. The Barr body showed bright yellow fluorescence and the nucleoli showed red fluorescence. The rest of the nuclear chromocenters and nuclear membrane had a relatively dull fluorescence. All slides were screened blindly by a single individual. For this reason, we were quite confident about the unambiguous identification of the Barr body.

It is now well-established that, at interphase, the higher degree of condensation of the inactive *X* (Barr body) as compared to that of the active *X* is the most important factor in unambiguous detection of the Barr body by the fluorescence and/or conventional staining methods. We have also found that the frequency of Barr body$^+$ nuclei in a population of cells decreased at S phase of the cell cycle when the Barr body presumably becomes undercondensed/decondensed to allow DNA replication (Mukherjee & Czirbik, 1984). It has also been clearly shown that 5-azaC can cause undercondensation of human chromosomes (Viegas-Pequignot & Dutrillaux, 1976; Schmid *et al.* 1984). A significant drop in the % Barr body$^+$ nuclei at each experimental step, therefore, most probably indicates that 5-azaC induces undercondensation of the Barr bodies. This chemical might break up a large chromocenter, like the Barr body, into two or more smaller chromatin bodies by undercondensation of specific chromatin regions so that it can no longer be distinguished from morphologically similar structures in a nucleus. Since the longer exposure time (48 h) caused a greater decrease in the frequencies of Barr body$^+$ cells (compare Tables 1–3), it seems that the treatment time is an important factor in the degree of presumptive undercondensation of the Barr body. Also, it was quite apparent from the data (Tables 2, 3) that the extent of probable undercondensation of the Barr body was dose-dependent, i.e. the higher the dosage of 5-azaC, the higher is the % Barr body$^-$ nuclei. It is important to note that similar results were also obatined with human lymphocytes where it was shown that the number of chromosome regions undercondensed in metaphase increases with increasing 5-azaC dose and treatment time (Schmid *et al.* 1984).

To our knowledge, this is the first report on the effect of 5-azaC in diploid human cells at interphase. The dose levels of 5-azaC (1–10 μM) used in this study are not found to be toxic to cells and are the same used for testing the genetic derepression of the inactive *X* in human diploid fibroblasts (Wolf & Migeon, 1982). The significant decrease in the Barr body frequency in treated cells is highly unlikely, therefore, to arise from

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Table 1. *Frequencies of fluorescent Barr body⁺ nuclei in a human foetal fibroblast culture following 2 μM 5-azacytidine[a] treatment*

	24 hr exposure		
	Average cell density[b]	% Barr body⁺ cells	Total cell count
Control	2·3	58·7	491
Experimental	2·6	41·3	470
	$\chi^2 = 28{\cdot}99$	$P < 0{\cdot}001$	
	48 hr exposure		
Control	4·2	69·7	435
Experimental	4·4	41·9	582
	$\chi^2 = 76{\cdot}51$	$P < 0{\cdot}001$	

[a] Dosage used in previous studies for reactivating HPRT alocus on the human inactive *X* in mouse-human hybrids (Mohandas *et al.* 1981).
[b] Represents the average no. of cells found in 50 nonoverlapping fields of 0·01 mm² of each culture surface.

irreversible random break-down of the Barr body chromatin, but most likely is caused by undercondensation of the Barr body. However, stable derepression of a specific gene, such as the HPRT locus on the inactive *X* of diploid human fibroblasts, has not been achieved by 1–10 μM 5-azaC treatment (Wolf & Migeon, 1982). It is possible that a greater degree of decondensation of the Barr body is required for reactivation of specific gene loci on the inactive *X* and that the dosage of 5-azaC must be adjusted accordingly. It is interesting to note, however, that 1–10 μM 5-azaC can induce reactivation of the HPRT locus on the inactive *X* in mouse-human hybrids (Mohandas *et al.* 1981; Lester *et al.* 1982). These differential results may actually stem from the differential chromatin condensation of the inactive *X* (Barr body) in its normal habitat in a diploid cell and in the foreign environment of the hybrid cell. The presumptive greater chromatin condensation of the inactive *X* at interphase in a diploid fibroblast might make it less accessible to 5-azaC for demethylation of DNA than is the case with mouse-human hybrids in which less condensation presumptively occurs. Indeed, a recent study found only 7% Barr body⁺ cells in a mouse-human hybrid cell line (37-26R-D) containing a structurally

Table 2. *Frequencies of fluorescent Barr body⁺ nuclei in IMR-90 cells following 5-azacytidine treatment at various dose levels (1–10 μM)[a]*

	24 hr exposure			
	Dosage	Average cell density[b]	% Barr body⁺ cells	Total cell count
Control	1 μM	1·9	53·3	500
Experimental		2·1	41·6	500
	$\chi^2 = 13{\cdot}95$	$P < 0{\cdot}0$ 01		
Control	2 μM	1·9	53·4	500
Experimental		2·4	34·0	500
	$\chi^2 = 38{\cdot}23$	$P < 0{\cdot}0$ 01		
Control	4 μM	1·9	53·4	500
Experimental		2·0	28·0	500
	$\chi^2 = 66{\cdot}82$	$P < 0{\cdot}0$ 01		
Control	8 μM	1·9	53·4	500
Experimental		1·8	25·8	500
	$\chi^2 = 79{\cdot}62$	$P < 0{\cdot}0$ 01		
Control	10 μM	1·9	53·4	500
Experimental		2·3	11·4	500
	$\chi^2 = 201{\cdot}34$	$P < 0{\cdot}0$ 01		

[a] Dosage used in an effort to reactivate HPRT locus on the inactive human *X* in diploid fibroblasts (Wolf & Migeon, 1982).
[b] Represents the average no. of cells found in 50 nonoverlapping fields of 0·01 mm² of each culture surface.

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Table 3. *Frequencies of fluorescent Barr body⁺ nuclei in IMR-90 cells following 5-azacytidine treatment at various dose levels (1–10 μM)*[a]

	48 hr exposure			
	Dosage	Average cell density[b]	% Barr body⁺ cells	Total cell count
Control	1 μM	2·2	56·2	500
Experimental		2·5	34·8	500
	$\chi^2 = 46{\cdot}16$	$P < 0{\cdot}0$		
		01		
Control	2 μM	2·2	56·2	500
Experimental		2·0	28·2	500
	$\chi^2 = 80{\cdot}34$	$P < 0{\cdot}0$		
		01		
Control	4 μM	2·2	56·2	500
Experimental		1·9	22·3	500
	$\chi^2 = 119{\cdot}96$	$P < 0{\cdot}0$		
		01		
Control	8 μM	2·2	56·2	500
Experimental		2·4	20·1	500
	$\chi^2 = 137{\cdot}22$	$P < 0{\cdot}0$		
		01		
Control	10 μM	2·2	56·2	500
Experimental		1·8	10·3	500
	$\chi^2 = 236{\cdot}1$	$P < 0{\cdot}0$		
		01		

[a] Dosage used in an effort to reactivate HPRT locus on the inactive human *X* in diploid fibroblasts (Wolf & Migeon, 1982).
[b] Represents the average no. of cells found in 50 nonoverlapping fields of 0·01 mm² of each culture surface.

normal genetically inactive human *X* and 4% of a hybrid cell line (37-26R-D-1b) with a 5-azaC-reactivated HPRT locus on the inactive human *X* (Worsham, Van Dyke & Weiss 1985). These frequencies of Barr body⁺ nuclei in hybrids are far lower than those of cultured diploid fibroblast.

Recently, a correlation has been suggested between the 5-azaC-induced temporal change to earlier DNA replication, induced hypomethylation of DNA and gene activation in human diploid cells (Schaffer & Priest, 1984). Studies of the 5-azaC-reactivated inactive *X* in mouse-human hybrids (Schmidt, Wolf & Migeon, 1985) has also provided evidence suggesting a relationship between DNA methylation and DNA replication. Most recently, complete reversal of human *X* inactivation has been observed in mouse-human chorionic villi hybrids where the late replicating *X* (allocyclic) becomes early replicating (isocyclic) (Migeon *et al.* 1985). Based on this information and on the results of our present study, we might suggest that components such as chromatin undercondensation/decondensation of specific regions of the Barr body, hypomethylation of inactive *X* DNA, and temporal changes in the DNA replication pattern of the inactive *X* are all related to the reactivation process of specific gene loci on the inactive *X* by 5-azaC.

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