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Advances in Brief

Reversal of Loss of Imprinting in Tumor Cells by 5-Aza-2′-deoxycytidine[1]

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Abstract

To determine whether loss of imprinting in cancer might be reversed by altering DNA methylation, we treated tumor cells with 5-aza-2′-deoxycytidine, a specific inhibitor of cytosine DNA methyltransferase. Treated cells showed several significant and reproducible changes. (*a*) Equal expression of maternal and paternal alleles of insulin-like growth factor 2 switched to predominant expression of a single parental allele. (*b*) H19 expression was reactivated. (*c*) Biallelic H19 expression switched to monoallelic expression. (*d*) Biallelic methylation of H19 switched to preferential allelic methylation. These results imply that abnormally imprinted cells are susceptible to epigenetic modification and that the effect of 5-aza-2′-deoxycytidine on tumor cells with loss of imprinting is not random but specific to one allele.

Introduction

Genomic imprinting is an epigenetic modification of a specific parental chromosome in the gamete or zygote that leads to monoallelic or differential expression of the two alleles of a gene in somatic cells of the offspring. We and others have shown previously that the human *IGF2*[3] and *H19* genes are normally imprinted; *i.e.*, they show preferential expression of a specific parental allele (1–5). We and others also have found that some tumors undergo LOI in cancer, with biallelic expression of *IGF2* (1, 5), epigenetic silencing of *H19* (6, 7), and/or abnormal expression of the paternal *H19* allele (1), and this observation has been extended to a wide variety of childhood and adult malignancies (8–10). Normal imprinting appears to be maintained in part by allele-specific, tissue-independent methylation of *H19*, because LOI is associated with abnormal methylation of the normally unmethylated maternal *H19* allele (6, 7). To test the hypotheses that LOI is reversible and that DNA methylation plays a role in LOI, we have attempted to modify allele-specific gene expression in tumor cells with LOI by using a specific inhibitor of DNA methylation, 5-azaCdR (11). These experiments suggest that LOI is partly reversible and amenable to changes in DNA methylation.

Materials and Methods

Cell Culture and Treatment with 5-azaCdR. JEG-3 human choriocarcinoma and COLO-205 human colon adenocarcinoma cell lines were obtained from American Type Culture Collection. Cells were cultured in RPMI 1640, 10% FCS, and 5% CO_2 with antibiotics. Cells (10^4–10^5) in 6-cm dishes were treated with 0.3–10 μM 5-azaCdR for 24 h. Cultures were washed in HBSS and grown to confluence. Concentrations of 0.3–3.0 μM caused minimal cytotoxicity (0–30%).

Analysis of Gene Expression. For quantitative analysis of gene expression, total RNA was isolated as described (12), and 10 μg were electrophoresed in 1.2% agarose gels containing formaldehyde. The RNA was then transferred to Hybond-N+ membranes (Amersham) and hybridized as described with *H19* and *IGF2* probes (6). Hybridization with *GAPDH* was used as a control for RNA loading. For analysis of allele-specific expression, 0.5 μg of RNA was treated with DNase I (Boehringer Mannheim), heated to 95°C for 7 min, and RT-PCR was performed as described for both *IGF2* and *H19* (1); however, nested PCR using primers HP1 and HP2 (13) was performed for *H19* analysis. PCR products were digested with either *Apa*I (for *IGF2* analysis) or *Alu*I (for H19 analysis) as described (14) and electrophoresed on 4% Nusieve-agarose (3:1) gels. Each sample was analyzed in duplicate in the presence and absence of RT to exclude DNA contamination. Gels were blotted and hybridized with a ^{32}P-labeled internal specific oligonucleotide probe (1) and quantified with a PhosphorImager (Molecular Dynamics).

Analysis of DNA Methylation. For quantitative analysis of DNA methylation, genomic DNA was prepared as described (1), and Southern analysis of methylation of the *H19* promoter CpG island was performed as described (15). Filters were hybridized with the 1.8-kb *Pst*I fragment of H19, which detects imprint-specific methylation (6). For analysis of allele-specific methylation, 1 μg of DNA was digested with *Bam*HI or with *Bam*HI plus *Hpa*II. After digestion, enzymes were inactivated by heating to 100°C for 10 min. Fifty ng of digested DNA were added to the PCR reaction mix in buffer F (Invitrogen), containing primers HP1 (5′-TACAACC-ACTGCACTACCTG-3′) and H3 (5′-TGGAATGCTTGAAGGCTGCT-3′; Ref. 13). One initial cycle (94°C, 4 min) was followed by 28 cycles of 94°C, 1 min; 60°C, 1 min, and 72°C, 1 min. PCR products (227 bp) were digested with *Alu*I, electrophoresed on 3% Nusieve/1% agarose gels, transferred to Hybond-N+ membranes (Amersham), hybridized with an internal oligonucleotide probe HP2 (5′-TGGCCATGAAGATG-GAGTCG-3′), and then quantified with a PhosphorImager (Molecular Dynamics).

Results

The purpose of the experiments described here was to determine whether any of the features of LOI in tumor cells was reversible with 5-azaCdR, a specific inhibitor of DNA methylation. We chose as a model system two tumor cell lines, JEG-3 choriocarcinoma and COLO-205 colorectal carcinoma cells, by screening cell lines for those that fulfilled two criteria: (*a*) They were heterozygous for transcribed polymorphisms in *IGF2* and *H19*, and thus informative for imprinting studies; and (*b*) They showed LOI. Cells were treated with 5-azaCdR for 24 h (approximately 1 cell division) and at concentrations experimentally determined both in our laboratory (16) and others (17) to maximize hypomethylation and minimize cytotoxicity.

We first examined the effect of 5-azaCdR on LOI of *IGF2*. Tumor cells with LOI show equal biallelic expression of *IGF2*, in contrast to cells with normal imprinting, which exhibit preferential expression of a specific parental allele (1, 5, 8–10). As expected, untreated tumor cells, as well as control tumor cells treated with PBS, showed approximately equal expression of the two alleles of *IGF2* (Fig. 1*A*), indicating LOI. In contrast, treatment with increasing doses of 5-azaCdR led to unequal expression of the two *IGF2* alleles, with preferential expression of the allele lacking the *Apa*I polymorphic site (Fig. 1*A*). Although preferential expression of one allele was partial and not absolute, three repetitions of these experiments always gave the same results, with the predominantly expressed allele always represented by the upper band. Thus, the switch to predominant expression of one

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[3] The abbreviations used are: IGF2, insulin-like growth factor II; LOI, loss of imprinting; 5-azaCdR, 5-aza-2′-deoxycytidine; RT, reverse transcriptase.

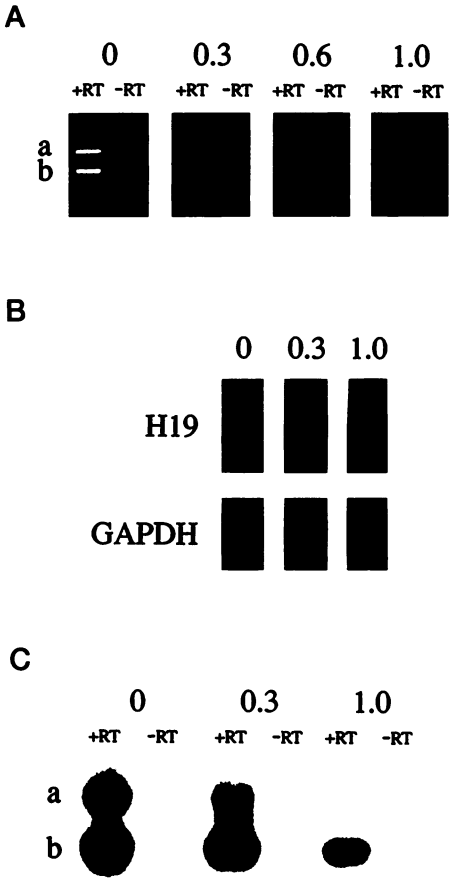

Fig. 1. Effects of 5-azaCdR on imprinting of tumor cells with LOI. *A*, switch to preferential allelic expression of IGF2. RT-PCR was performed on total RNA extracted from JEG-3 cells after a single 24-h treatment with 0, 0.3, 0.6, or 1.0 μM 5-azaCdR. Alternating lanes represent simultaneous experiments with (+) and without (−) *RT*. PCR products were digested with *ApaI*. The *a* and *b* alleles are 236 and 173 bp, respectively. *B*, induction of H19 expression. Expression of H19 in JEG-3 cells after treatment with 5-azaCdR was measured by Northern blot analysis. Blots were rehybridized with GAPDH as a control for loading. *C*, switch to monoallelic expression of H19. RT-PCR was performed as in *A*. PCR products were digested with *AluI* to detect allele-specific expression. The *a* and *b* alleles are 227 and 100 bp, respectively. Note that the RT-PCR assay is not quantitative, and the total amounts of RNA in the three sets were not normalized.

allele was not a random effect of 5-azaCdR, but was specific to one allele. Consistent switching by 5-azaCdR from equal biallelic expression to preferential expression of a single parental allele was also observed with a second cancer cell line. Treatment of COLO-205 colorectal carcinoma cells also showed a switch from biallelic to predominately monoallelic expression of *IGF2*, and the same allele was preferentially expressed in repeated experiments (data not shown). Thus, 5-azaCdR partially abrogated the pattern of equal biallelic expression of *IGF2* in tumor cells with LOI.

We next examined the effect of 5-aza-CdR treatment on overall expression of *H19*. As we and others had found earlier, LOI of *IGF2* is linked in Wilms' tumors to down-regulation of *H19* (6, 7). Untreated JEG-3 cells, like Wilms' tumors, displayed little to no expression of *H19* (Fig. 1*B*). After treatment with 0.3 μM or 1.0 μM 5-azaCdR, tumor cells showed a substantial increase in *H19* expression (Fig. 1*B*), 30- or 50-fold, respectively, as determined by phosphorimager analysis. These changes were also reproducible in the second cancer cell line, as COLO-205 cells showed virtually no expression of H19 before treatment. After treatment with 1.0 μM 5-azaCdR, cells showed a 20-fold increase in *H19* expression (data not shown). Thus, 5-azaCdR abrogated the epigenetic silencing of *H19* in tumor cells with LOI.

We had also observed earlier that LOI of *IGF2* in Wilms' tumor is associated with biallelic expression of *H19* (1), despite the relatively low overall quantitative expression of *H19* in the tumors. Biallelic expression of *H19* is also a characteristic of JEG-3 cells (13). Therefore, we examined allele-specific expression of *H19*. We used an RT-PCR assay that reflects allele-specific expression (1) but should not be used to quantify total mRNA levels. 5-azaCdR treatment caused a change from biallelic expression to preferential expression of a single *H19* allele. This change was even more dramatic than that seen for *IGF2*, with monoallelic expression of the allele containing the *AluI* polymorphic site (Fig. 1*C*).

We and others showed earlier that LOI is associated with abnormal methylation of the normally unmethylated maternal *H19* promoter, to a degree similar to normal methylation of the paternal allele (6, 7, 15). To determine whether treatment with 5-azaCdR altered methylation of this imprint-specific region, cells were digested with *PstI* and *SmaI*, as described (15). Normal cells show approximately 50% methylation, because the paternal allele is methylated and the maternal allele is unmethylated (6, 7, 15). Cells with LOI show increased methylation, reflecting abnormal methylation of the maternal allele (6, 7, 15). As expected, control cells treated with PBS showed increased methylation of the *H19* CpG island (Fig. 2*A*). However, after treatment with 5-azaCdR, the *H19* CpG island showed a pattern of methylation approaching 50% (Fig. 2*A*). As with the changes in *IGF2* imprinting after 5-azaCdR treatment, these changes in DNA methylation were also reproducible in the second cancer cell line, COLO-205 (data not shown).

Finally, we asked whether the effects of 5-azaCdR were specific to a single allele, as would be expected if these changes were related to changes in genomic imprinting, and not a random effect of 5-azaCdR

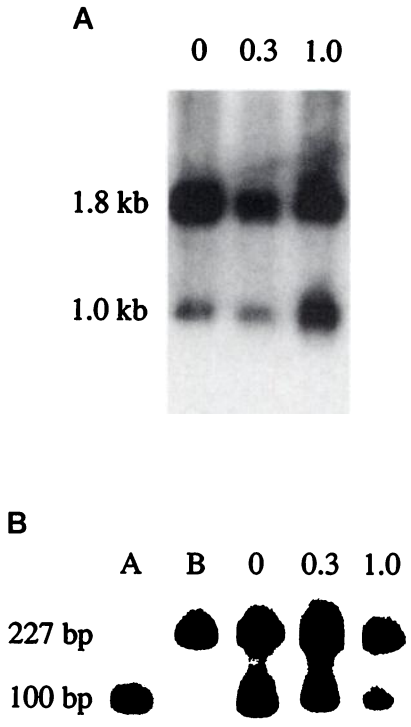

Fig. 2. Effects of 5-azaCdR on H19 promoter methylation. *A*, decreased methylation of H19 after 5-azaCdR treatment. DNA was extracted from cells treated with 0, 0.3, or 1.0 μM 5-azaCdR and digested with *PstI* and *SmaI* to assay DNA methylation as described (24). The 1.8-kb fragment represents methylated DNA, and the 1.0-kb fragment represents unmethylated DNA. *B*, allele specificity of altered methylation. *Bam*HI and *Hpa*II digested DNA was PCR amplified and digested with *AluI* to detect allele-specific methylation. The *A* and *B* alleles (shown) are 100 and 227 bp, respectively.

on both methylated alleles. We PCR-amplified a region of *H19* containing an *Hpa*II site that shows imprint-specific methylation (18), as well as an *Alu*I polymorphism that distinguishes the two alleles of the gene. By digesting genomic DNA with *Hpa*II before PCR, we could determine which alleles were methylated before and after treatment of cells with 5-azaCdR. This analysis showed, as expected, that both alleles were methylated in the absence of 5-azaCdR (Fig. 2*B*). However, after treatment with 5-azaCdR, the allele lacking the *Alu*I polymorphic site was predominantly amplified (Fig. 2*B*). Thus, the non-*Alu*I-containing allele remained methylated, whereas the *Alu*I-containing allele became unmethylated after 5-azaCdR treatment. Thus, one allele was preferentially affected by 5-azaCdR. Furthermore, the change in *IGF2* methylation was not affected by 5-azaCdR (data not shown), again indicating a specific effect of 5-azaCdR on these cells.

In summary, 5-azaCdR restored a pattern of normal genomic imprinting to tumor cells with LOI by each of the following criteria. (*a*) Equal biallelic expression of *IGF2* switched to preferential expression of the same allele in repeated experiments. (*b*) Epigenetic silencing of *H19* was reversed (*c*) Biallelic expression of *H19* switched to monoallelic expression of the same allele in repeated experiments. (*d*) Hypermethylation of the *H19* promoter switched to demethylation of one allele but not the other, the same allele that was transcribed. These effects on tumor cells with LOI were not random but were specific in each instance to one allele, and they were reproducible both within and between the cell lines.

Discussion

We have shown that 5-azaCdR switched *IGF2* from approximately equal expression of maternal and paternal alleles to predominant expression of a specific parental allele in repeated experiments, and caused a switch from biallelic to monoallelic expression of *H19*, as well as quantitative activation of *H19* expression. These effects were not a random response to 5-azaCdR, or else tumor cells would have continued to show equal expression of both alleles of *IGF2* and *H19*. Consistent with the nonrandom effect of 5-azaCdR in these experiments, the loss of DNA methylation of the *H19* promoter was specific to one allele. Thus, 5-azaCdR treatment confers a normal pattern of genomic imprinting on tumor cells with LOI. This pattern was complete for *H19* and partial for *IGF2*, because in the latter case both alleles were expressed, although the same allele was preferentially expressed in repeated experiments.

These results have three important implications:

(*a*) They indicate that abnormal imprinting in tumor cells can be modified pharmacologically. These effects are different than those seen in normal cells. Thus, Eversole-Cire *et al.* (19) showed previously that 5-azaCdR treatment of mouse cells with maternal disomy for chromosome 7 causes epigenetic activation of the normally silent maternal *IGF2* gene on that chromosome. Similarly, Hu *et al.* (20) recently found that treatment of normal human brain cells with 5-azaCdR switched *IGF2* from a pattern of preferential expression of one allele to equal biallelic expression. In contrast, in the experiments described here using tumor cells with LOI, the pattern of allele-specific expression of *IGF2* switched from equal biallelic expression to predominant expression of a single allele. Thus, the mechanism for LOI in cancer may be different than that of establishment of imprinting in normal cells.

(*b*) These experiments show that some imprint-specific information must still be present in tumor cells with LOI. Because normal cells from the patients from whom these cell lines were derived are not available, we cannot know at this point whether 5-azaCdR caused restoration of normal imprinting to these cells, or, alternatively, allele switching of imprinting, with activation of the paternal *H19* allele and relative epigenetic silencing of the paternal *IGF2* allele. We are currently addressing this question by examining tumor cell explants from which paired normal tissue and parental DNA are available. However, regardless of which allele was affected by 5-azaCdR, the fact that one allele was affected differently than the other, and reproducibly in repeated experiments, indicates that some mark still exists on one or both chromosomes that distinguishes their parental origin and renders then differentially susceptible to the effects of 5-azaCdR.

(*c*) This work may provide a novel experimental strategy toward understanding the molecular basis of abnormal imprinting in cancer, since these experiments generate isogenic cell lines that differ in their imprinting of *IGF2* and *H19*. These cell lines can then be tested for alterations at additional target sites, such as $p57^{KIP2}$, which we have also found to be imprinted (21), or for alterations in *trans*-acting factors that may have been activated by 5-azaCdR to mediate these effects.

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