Mechanism of in vivo sister-chromatid exchange induction by 5-azacytidine


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The aim of the present study was to explore the in vivo mechanism of sister-chromatid exchange (SCE) induction by 5-azacytidine (5-azaC) in murine bone marrow cells. Experiments were performed to examine SCE induction in response to different doses of 5-azaC as well as several exposures. Additionally, we examined the persistence of SCE induction and the effect of bromodeoxyuridine (BrdU) incorporation. Sister-chromatid differentiation was obtained by injecting mice intraperitoneally with BrdU absorbed to activated charcoal. Before BrdU injection, different doses of 5-azaC were administered intraperitoneally either singly or in multiples. Colchicine in an aqueous solution was administered subcutaneously 22 h after BrdU injection. Two hours later, animals were sacrificed by cervical dislocation and both femurs were dissected. Bone marrow cells were processed to obtain chromosome preparations, which were stained by the fluorescence plus Giemsa method. Results indicate that 5-azaC caused SCE, albeit to a limited extent. In order to discern whether the limitation was due to cytotoxicity or to partial 5-azaC incorporation, we administered multiple sub-toxic doses of 5-azaC. This treatment increased 5-azaC incorporation and reduced cytotoxicity, but did not raise SCE frequency, indicating that the limitation was not due to either of the two factors mentioned above. SCE frequency induced by 5-azaC persisted for at least eight cell divisions, confirming that this agent had caused inhibition of DNA methyltransferase and subsequently the reduction of DNA re-methylation, which in turn induced the expression of a number of SCE-prone sites. Finally, SCE induction in response to 5-azaC was completely dependent on BrdU incorporation. The data allow us to conclude that 5-azaC causes SCE to a limited extent; limited SCE induction was not due to the direct effect of incorporation or cytotoxicity of 5-azaC, but rather the generation of a number of SCE-prone sites, the expression of which persists for at least eight cell divisions and is dependent on BrdU incorporation.

Introduction

Sister-chromatid exchange (SCE) represents DNA double-strand exchange between chromatids of the same chromosome. According to the more supported hypothesis, SCE induction represents recombination repair (1) of DNA lesions that persist before duplication (2,3). However, a paradox exists due to evidence obtained from a three-way differential staining protocol, that agents could cause increased SCE frequencies that transcend cell division (4–7), although they do not seem to be caused at the same locus (8). The persistence of lesions involved in SCE for several cell divisions implies that lesions are not eliminated and SCE represents a mechanism to tolerate them. Evidence suggests that gamma rays induce a statistically significant increase in SCE frequency up to six cell divisions after exposure (9).

There is also evidence that DNA de-methylation causes a persistent and constant increase in SCE frequencies for more than eight cell divisions (10). This was interpreted as a result of an epigenetic alteration in DNA represented by de-methylation. A hypothesis that unifies the persistence of SCE induction caused by de-methylation and by alklylation of nucleophilic sites in DNA bases is that the latter is able to cause cytosine de-methylation in DNA by extensive repair of alkylated sites. In support of this possibility, a de-methylating effect of alkylating agents has been previously reported (11).

The inhibitory effect of 5-azacytidine (5-azaC) on DNA re-methylation is well established (12), as are the consequences of DNA de-methylation on several cell processes, such as mismatch repair (13), transduction control and genome stability (14). However, the mechanism that relates de-methylation with SCE induction is not clearly understood (15). Understanding this phenomenon is important because it would establish a link between DNA de-methylation, an epigenetic effect, and the persistent occurrence of homologous recombination.

Evidence with respect to the effect of 5-azaC on SCE induction in vitro include the following: (i) 5-azaC is able to induce significant increase in SCE (16), (ii) SCE frequency induced by this agent remains high and constant for several cell divisions (10) and (iii) SCE induction by 5-azaC showed a synergistic effect with various mutagens (17).

The aims of the present study were to determine the in vivo dose–response curve of SCE induction by 5-azaC in murine bone marrow cells, the persistence of SCE frequency induced after exposure to 5-azaC, the effect of several exposures to 5-azaC during the same cell division and the role of bromodeoxyuridine (BrdU) incorporation on SCE elicited by 5-azaC. All this was carried out in order to approach the mechanism of SCE induction by 5-azaC in the same biological system.

Materials and methods

Animals

BALB/c male mice (2- to 3-month old) weighing 30 g were used in all experiments. Animals were bred and maintained in plastic cages with sawdust bedding under controlled conditions of temperature (22°C) and dark–light periods (light 7 am–7 pm). They were fed Purina chow for small rodents and given water ad libitum. Animals were treated and housed in accordance with the Guide for the Care and Use of Laboratory Animals (18).

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Chromosomal aberrations

Genotoxic and cytotoxic effect of multiple dose exposure to 5-azaC (40 μmole/kg per dose)

<table>
<thead>
<tr>
<th>Number of doses</th>
<th>SCE (mean ± SD)</th>
<th>MI (mean ± SD)</th>
<th>AGT (mean ± SD)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 (NaCl)</td>
<td>3.5 ± 0.15</td>
<td>46.7 ± 3.4</td>
<td>12.6 ± 0.3</td>
<td>5</td>
</tr>
<tr>
<td>3 (5-azaC)</td>
<td>5.1 ± 0.46*</td>
<td>33.0 ± 1.25*</td>
<td>15.8 ± 2.0*</td>
<td>4</td>
</tr>
<tr>
<td>4 (NaCl)</td>
<td>3.5 ± 0.06</td>
<td>49.8 ± 1.03</td>
<td>12.35 ± 0.3</td>
<td>5</td>
</tr>
<tr>
<td>4 (5-azaC)</td>
<td>NA</td>
<td>13.2 ± 1.3*</td>
<td>20.44 ± 0.8*</td>
<td>2</td>
</tr>
</tbody>
</table>

n, number of animals; NA, non-analysable.

Statistically significant with respect to current control, Student’s t-test P > 0.05.
behaviour indicates that both events were caused by the same process.

**Role of BrdU on SCE induction by 5-azaC**

Table V presents the effect of BrdU dosage on SCE induction by 30 μmoles of 5-azaC. SCE induction only increased slightly, up to 0.5 SCEs per cell, when we administered a BrdU dose of 0.75 mg/g of body weight. This increase did not prove to be statistically significant with respect to control, which implies that 5-azaC did not induce SCE per se. However, a BrdU dose of 1.1 mg/g of body weight caused a clear increase of 1.6 SCEs per cell using the same dose of 5-azaC. Subsequent increases in BrdU dose resulted in SCE increases. Figure 3A shows the dose–response curves of BrdU induction of SCE in the presence or absence of 5-azaC. Slopes of curves from 5-azaC-treated and untreated control groups suggest that these curves would converge at lower 5-azaC doses; at the point of convergence, the SCEs should be induced only by BrdU. This result indicates that SCE induction in response to 5-azaC was completely dependent on BrdU incorporation. Figure 3B shows the curve of SCE increase caused by different doses of BrdU in cells treated with 5-azaC. The curve fit with a Bolzman sygmoidal curve (r = 1.0) having a plateau around 2.7 SCEs per cell indicates that there was a limited number of SCE-prone sites. This curve supplies evidence that SCE induction by 5-azaC was completely dependent on BrdU incorporation.

**Discussion**

Our results indicate that 5-azaC also caused SCE in vivo, but with a lower frequency than that obtained in vitro (10). Importantly, the induction of SCE in our in vivo study showed a clear limit. This could be explained by sensitization that resulted from the level of BrdU incorporation, given that basal SCE frequencies were substantially higher in previous in vitro studies (10,24).

Use of a single acute dose and the proven increased cellular toxicity by higher doses of 5-azaC would suggest that limited SCE induction by an acute dose of 5-azaC is due to limited DNA incorporation or that the cytotoxic effect of higher 5-azaC doses did not allow a dose-dependent SCE increase. However, our results with multiple low doses of 5-azaC, causing a condition of reduced toxicity that allowed higher DNA incorporation, did not result in SCE increase. This suggests that these factors do not cause limited SCE induction but, possibly, the occurrence of a restricted number of prone SCE-occurring sites.

Based on previous evidence that 5-azaC inhibits methylation by sequestering molecules of DNA methylase (25), results of the present study could be explained by reduction of DNA re-methylation caused by 5-azaC, which results in the expression of limited SCE-prone sites.

Our results concerning the dependence of SCE induced by 5-azaC on BrdU incorporation, which were not previously reported, denote that both de-methylation and BrdU incorporation concurred in these SCE-prone sites.

The persistent SCE increase caused by 5-azaC for several divisions, which agrees with previously reported data (10), indicates that the epigenetic modification caused by de-methylation caused the appearance of SCE-eliciting sites in DNA which are revealed by BrdU. These sites are conceptually equivalent to fragile sites as evidenced by their small number and requirement for exposure to an agent to be manifested. In this context, our results are relevant to early observations that common fragile sites have been observed as hot spots not only for chromosomal lesions, such as gaps, but also for SCE formation (26). A remarkably high frequency of SCE was observed at gaps on common fragile sites after treatment with 5-azaC and BrdU (27). SCE has also been associated with fragile sites after treatment with aphidicolin (27), 4′, 6-diamidino-2-phenylindole (28) and Mitomycin C (29).
Both DNA de-methylation and BrdU incorporation relax chromatin structure (30,31); the question is whether this determines susceptibility to the induction of lesions that could produce SCE or gaps arising from incomplete SCE. An additional explanation is that such a relaxation makes DNA more susceptible to damage induced by endogenous and exogenous mutagens. In fact, this hypothesis is also supported by permanent susceptibility or epigenetic susceptibility to mutagens induced by 5-azaC (10,17). Likewise, the scientific literature supports the sensitization to exogenous mutagens caused by both 5-azaC (17) and BrdU (2,3).

The hypothesis that SCE-prone sites caused by DNA de-methylation and subsequent BrdU incorporation are equivalent to fragile sites must be further explored.

Although the synergy of cytotoxic effects of BrdU and 5-azaC is not related with the aim of the present study, we consider it pertinent to comment on the subject. Our results indicate (Table V) that BrdU caused a slightly and not statistically significant cytotoxic effect, this determined as an increase in the AGT and as a reduction in MI; however, the pretreatment with 5-azaC caused an important and significant effect in both parameters. The importance of this resides in the possibility of using 5-azaC followed by BrdU as an anti-neoplastic treatment.

Results of the present in vivo study allow us to conclude that 5-azaC cause SCE to a limited extent and that limited SCE induction was not produced by the incorporation or cytotoxicity of 5-azaC, but rather by the epigenetic production of a number of SCE-prone sites, whose expression persists for at least eight cell divisions and is dependent on BrdU incorporation.

Acknowledgements

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References

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