Formation and Expansion of Leukemia-Specific Chromosome Aberrations in Hematopoietic Cells of X-ray Irradiated Mice

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Abstract. C3H/He mice develop acute myeloid leukemia (AML) after whole-body irradiation, and typical chromosome 2 deletions are found in the leukemic cells. To investigate a process of the formation and the expansion of the AML-specific deletions, we have examined its frequency in primitive hematopoietic cells that could be the target of the leukemogenesis. Male C3H/He mice were exposed to 3Gy x-rays and sacrificed after certain periods of time. Bone marrow cells were collected from the femora and a single-cell suspension from each animal was divided into two parts. One part of the cell suspension was cultured in methylcellulose medium, and metaphase spreads were prepared from each growing colony. The other part was sorted to obtain Lin− and Lin Sca1+ cells, and those cells were scored with FISH for the AML-specific deletions. Karyotyping of the cultured cells detected signs of the delayed chromosomal instability, but an aberration involving chromosome 2 has not been found so far. FISH to the sorted cells, however, revealed the AML-specific deletions could be produced in the primitive hematopoietic cells as an early event of radiation exposure.

1. Introduction

While a lot of studies have shown the evidence of carcinogenic effect of ionizing radiation, its mechanism is still unresolved [1]. A classical theory assumes that DNA damage caused by radiation is directly responsible for the genetic changes found in tumor cells. In the last decade, however, another hypothesis has been introduced based on the findings of radiation-induced genomic instability [2-4]. It emphasizes radiation destabilizes the genome of the exposed cells and the elevated level of mutations accumulates changes in cancer-related genes. This hypothesis was first suggested for solid tumors, but recently it was also applied to explain the leukemogenic process; delayed chromosomal instability followed by radiation exposure is assumed to have a key role in the formation of the leukemia-specific chromosome aberrations [5-7].

Some strains of mice, such as RFM, CBA and C3H/He, develop acute myeloid leukemia (AML) after whole-body irradiation [8-10]. It serves as an animal model of radiation-induced leukemia since the symptoms are similar to those in human AML and the incidence increases in dose-dependent manner. The murine AML is characterized by a partial deletion of chromosome 2 in one homologue that is observed in more than 90% of the affected cases [11, 12]. The “classical theory” implies this AML-specific deletion is formed just after irradiation while the “instability hypothesis” claims the delayed chromosomal rearrangements eventually produces the deletion. Although it is difficult to know what is actually happening in vivo, a time-course analysis of the chromosomal events could be informative to judge which scenario is more likely. In this study, we have examined the aberration patterns and the frequencies of the AML-specific deletions in murine hematopoietic cells after certain periods of time post-irradiation.

Some studies have already reported the karyotyping results of the bone marrow cells in mice up to two years after irradiation [13-15]. Those studies used the whole population of bone marrow cells, but leukemia is thought to originate from the primitive hematopoietic cells that comprise a tiny part of the population. Unless the kinetics of the primitive cells is similar to that of the differentiated cells, it is impossible to detect early changes in the target cells by looking at the whole cell population. Therefore, we have adopted two approaches to investigate chromosomal events in the primitive cells. In vitro culture was set up from the bone marrow cells and each colony of the culture was examined for chromosome aberrations with an emphasis on asymmetrical or non-clonal aberrations. A part of the cells from the same animal was sorted to obtain the primitive cells themselves, and they were scored with FISH for the AML-specific deletions.
2. Materials and methods

2.1. Cell preparation

Ten-week old male C3H/He mice were exposed to 3Gy of x-rays and sacrificed at 1 or 90 days after irradiation. Another group of mice was also prepared for 300 days post-irradiation, but any of them have not reached the time to be sacrificed. Bone marrow cells were aseptically taken from the femora and suspended in Iscove’s MDM (StemCell Technologies) supplemented with 2% fetal bovine serum. After pipetted and sieved with Cell Strainer (Falcon), the cell suspension was divided into two aliquots for each mouse. One part was used to establish in vitro culture and the other was served for the cell sorting.

2.2. In vitro culture and metaphase preparation

Two dishes of methylcellulose medium GF M3534 (StemCell Technologies) containing $1.5 \times 10^4$ bone marrow cells in 1.1ml volume were prepared for each mouse, and were incubated at 37°C, 5% CO$_2$. On 10–11 days of incubation, each growing colony was collected into a microtube containing Iscove’s MDM supplemented with 20% fetal bovine serum and 0.02µg/ml colcemid, and was further incubated at 37°C for one and half hour. Cells were then treated with 0.075M KCl for 20 minutes, fixed with methanol/acetic acid (3:1 in volume), and dropped onto the slides. The slides were stained with DAPI and actinomycin D, and visualized with an epifluorescence microscope Optiphot-2 (Nikon) equipped with a chilled CCD camera C5810-1 (Hamamatsu Photonics). Acquired metaphase pictures were converted into inverted grayscale images and karyotyped.

2.3. Cell sorting

Approximately $1 \times 10^7$ bone marrow cells were suspended in PBS with 0.5% bovine serum albumin, and were sorted using a MACS (magnetic cell sorting) system. Lineage Cell Depletion Kit (Miltenyi Biotec) was first applied to separate Lin$^+$ and Lin$^-$ cells. The Lin$^-$ cells were further processed with Anti-Sca1 MicroBeads (Miltenyi Biotec) to obtain Lin–Sca1$^+$ cells.

2.4. FISH

The sorted cells were treated with 0.075M KCl for 20 minutes, fixed with methanol/acetic acid, and spread on the slides. FISH probes were prepared from two BAC clones that were mapped within the deleted region of chromosome 2 in most AMLs. An Sfpi1-positive clone was labeled with SpectrumOrange-dUTP (Vysis) and a Wt1-positive clone was labeled with SpectrumGreen-dUTP (Vysis). The slides were immersed in 70% fomamide/2×SSC, pH7 at 73°C for 5 minutes, and dehydrated with ethanol series. The probe solution was denatured at 75°C for 5 minutes, and applied onto the slides. After the incubation at 37°C overnight, the slides were washed with 0.4×SSC at 70°C for 2 minutes, rinsed with 2×SSC/0.1% NP-40, and mounted with DAPI II counterstain (Vysis). The slides were visualized with the microscope and the CCD camera mentioned above.

3. Results

A limited number of data has been compiled so far as a part of the ongoing project. Table I summarizes a preliminary result of the karyotyping for the colonies grown in vitro. Each treatment category represents data of a single animal, and the colonies examined for at least two metaphases were scored. For the cells taken at one day post-irradiation, the result was comparable to the non-irradiated, i.e. no clonal aberration and a small number of non-clonal aberrations. The type of the aberration was chromatid break in the control, but translocations were found in the exposed. At 90 days post-irradiation, aberrant cells came into cell cycle to produce clonal aberrations, and the frequency of non-clonal aberration was also high. The clonal aberrations were reciprocal translocations and a terminal deletion while non-clonal aberrations were mostly chromatid breaks (FIG. 1). No aberration involving chromosome 2 has been found in any category.
Table I. Preliminary result of the karyotyping for in vitro colonies.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days after irradiation</th>
<th>No. of colonies</th>
<th>Colonies with clonal aberration</th>
<th>No. of metaphases [A]</th>
<th>Cells with non-clonal aberration [B]</th>
<th>B/A (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>6</td>
<td>0</td>
<td>29</td>
<td>2</td>
<td>6.9</td>
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<tr>
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<td>1</td>
<td>12</td>
<td>0</td>
<td>61</td>
<td>4</td>
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<tr>
<td>3Gy</td>
<td>90</td>
<td>6</td>
<td>4</td>
<td>54</td>
<td>8</td>
<td>14.8</td>
</tr>
</tbody>
</table>

**FIG. 1.** Examples of clonal and non-clonal aberrations. Both are metaphases found in the same colony grown from the cells of 90 days post-irradiation. Panel (a) shows a reciprocal translocation while the metaphase of panel (b) carries additional chromatid breaks indicated with arrow heads.

**FIG. 2** shows a result of FISH. A nucleus of the normal diploid cell shows two red signals of the Sfpi1-positive probe and two green signals of the Wt1-positive probe (FIG. 3(a)). If a cell carries the AML-specific deletion, a segment of chromosome 2 is lost and only one signal should be detected for each probe (FIG. 3(b)). Such a cell was scored as "positive" for the AML-specific deletion, and the graph in FIG.2 compared its frequency between the control and one day post-irradiation. Since the two probes were discrete in the interphase nuclei, excluding the cells with uneven number of red and green signals decreased false-positive rate due to the overlapping signals. No positive case was actually observed in the control group, that endorses reliability of data. In contrast, the loss of the signals was observed in 2–3% of the cells in the exposed group irrespective of cell maturity. This indicates the AML-specific deletion could be produced as an early event of radiation exposure not only in the differentiated cells but also in the primitive hematopoietic cells.

**FIG. 2.** Percentage of the cells showing one signal for each probe. 100–200 cells were counted for each category.

**FIG. 3.** Examples of FISH image. See text for the details.
4. Discussion

Although the available data are not enough to make any conclusion, there found some implication regarding the aberration pattern of the primitive hematopoietic cells in the exposed mice. The colonies arising from the cells taken at one day post-irradiation showed no clonal aberration. As to 90 days post-irradiation, however, four out of six colonies originated from the aberrant cells. Those results imply intact cells are selectively recruited to the cell cycle shortly after irradiation, and cells with balanced aberrations subsequently emerge. Alternatively, initially damaged cells become extinct but the delayed chromosome aberrations might be accumulated during a recovery of the cell population. The apparently high frequency of the non-clonal aberrations at 90 days post-irradiation may support the latter idea, but genomic integrity of the cells could have been artificially perturbed in the culture since the non-clonal aberrations were also found in the control. If the delayed chromosomal rearrangement is responsible for the formation of the AML-specific deletion, non-clonal chromosome 2 aberrations should appear at certain point of time. Further compilation of data is required in this regard.

In a previous study, we have demonstrated the AML-specific deletion could be produced in the bone marrow cells as an early event of radiation exposure [16]. That finding has been challenged by a speculation that observable aberrations in the bone marrow cells do not necessarily reflect those in the target cells of radiation-leukemogenesis. FISH to the sorted cells in the present study not only confirmed the previous result, but also showed the AML-specific deletions were formed in the primitive cells. It is reported that the frequencies of the AML-specific deletions in the bone marrow cells were low until a year after irradiation [13-15]. The point is whether the primitive cells with the AML-specific deletions are retained in silence during that period or they experience cell deaths shortly after irradiation. The upcoming data are expected to provide an indication on this issue.

References


