Biochemical and Biophysical Research Communications 372 (2008) 662–667

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Cell sorting analysis of cell cycle-dependent X-ray sensitivity in end joining-deficient human cells

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ARTICLE INFO

Article history:
Received 30 April 2008
Available online 4 June 2008

Keywords:
Flow cytometry
Cell sorter
Cell cycle
DNA double-strand breaks
Nonhomologous end joining
X-ray sensitivity
DT40
Nalm-6

ABSTRACT

Non-homologous end joining (NHEJ) plays a major role in the repair of ionizing radiation-induced DNA double-strand breaks (DSBs), especially during the G1-phase of the cell cycle. Using a flow cytometric cell sorter, we fractionated G1- and S/G2-phase cells based on size to assess the DSB-repair activity in NHEJ factor-deficient DT40 and Nalm-6 cell lines. Colony formation assays revealed that the X-ray sensitivities of the G1-enriched populations correctly reflected the DSB-repair activities of both the DT40 and Nalm-6 cell lines. Furthermore, as assessed by γ-H2AX foci formation, the sorted cells exhibited less DNA damage than chemically synchronized cells. Given that it does not use fluorescent labeling or chemical agents, this method of cell sorting is simpler and less toxic than other methods, making it applicable to a variety of cell lines, including those that cannot be synchronized by standard chemical treatments.

Of the lesions induced in DNA by ionizing radiation (IR), double-strand breaks (DSBs) are among the most serious because they can result in the loss or rearrangement of genetic information, leading to cell death or carcinogenesis. DSBs are repaired via two major pathways: homologous recombination (HR) and nonhomologous end joining (NHEJ) [1]. HR, which functions during late S-G2-phase [2], primarily uses the undamaged sister chromatid as a template allowing for accurate lesion repair [3]. In contrast, NHEJ is an error-prone method that involves the joining of DNA ends with little or no reliance on sequence homology. NHEJ is initiated by the recruitment of DNA-dependent protein kinase (DNA-PK), which consists of two DNA end-binding subunits (Ku70 and Ku80) and a catalytic subunit (DNA-PKcs). DNA-PKcs phosphorylates the Artemis nuclease, which facilitates the repair of a subset of IR-induced DSBs via NHEJ. In the final step, the DSB is sealed by DNA ligase IV with help from its binding partners XRCC4 and XLF (also called Cernunnos) [4]. NHEJ plays a major role in the repair of IR-induced DSBs, especially during the G1-phase of the cell cycle when sister chromatids are not available [2]. Thus, one of the hallmarks of NHEJ factor-deficient cells is pronounced IR sensitivity during G1 [5].

Gene targeting is a powerful tool for the study of gene function by reverse genetic approaches. The Nalm-6 cell line, which was established from the peripheral blood of a 19-year-old male with acute lymphoblastic leukemia, allows for high-efficiency gene targeting by HR [6]. Furthermore, Nalm-6 cells display a stable diploid karyotype with a single reciprocal translocation, and they express wild-type p53 [7]. Thus, the Nalm-6 cell line has been used in many gene-knockout studies in human cells. Although Nalm-6 cells have been applied to establish multiple NHEJ factor-deficient cell lines [8,9], a major disadvantage is that no simple method exists for cell cycle synchronization (Noritaka Adachi, unpublished data). This has prevented researchers from determining whether NHEJ-deficient Nalm-6 cells show increased IR sensitivity during the G1-phase of the cell cycle.

The fractionation of a cell cycle-synchronized population, which is critical for assessing IR sensitivity in cells irradiated at the G1-phase, is usually achieved in one of two ways [10]. In the first approach, cells are arrested at a particular point in the cell cycle by agents that inhibit specific cell cycle events. These agents include chemicals that prevent DNA synthesis (G1/S arrest), such as hydroxyurea, aphidicolin, or high doses of thymidine, and chemicals that inhibit mitotic spindle formation (metaphase arrest), such

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doi:10.1016/j.bbrc.2008.05.147

as nocodazole or colcemid [10]. In addition, contact inhibition of cell growth and the deprivation of nutrients or growth factors essential for cell growth have been used to arrest cells at G1-phase. Although these methods can be performed using equipment commonly found in cell biology laboratories, the agents used tend to be toxic; thus, a fraction of the cells may be killed or rendered susceptible to the induction of cell death. In the second approach, selection is applied at a particular point in the cell cycle based on changes in the physical properties of the cells [10]. For example, centrifugal elutriation makes use of the fact that cell size increases linearly as cells progress through the cell cycle [11]. This change in size is exploited to fractionate cells according to their position in the cell cycle. Although groups of cells at various stages of the cell cycle can be collected without the use of chemicals from a single batch of exponentially growing cells, chemical-induced synchronization is more widely used because it requires no specially designed centrifuge or elutriation rotor.

In the present study, we used a cell sorter to fractionate G1- and S/G2-phase cells from exponentially growing cells based on a difference in the cells' forward scatter characteristics (FSC), which reflects a difference in cell size, without the need for fluorescent labeling or the use of chemical agents that might perturb cell cycle progression. Using this method, we successfully obtained G1- and S/G2-enriched cell populations from adherent and suspension cell lines. The sorted G1-phase cells exhibited less DNA damage than chemically synchronized cells, and the sorted cells were successfully used to study the cell cycle-dependent IR sensitivity of NHEJ-deficient Nalm-6 cells. This method is simple and rapid, making it applicable to a variety of cell lines, including those that cannot be synchronized by standard chemical treatments.

Materials and methods

Cell culture. HeLa human cervical carcinoma cells were cultured in Dulbecco's modified Eagle's medium (Nissui Seiyaku, Tokyo, Japan) containing 10% fetal calf serum (FCS; Hyclone, Logan, UT, USA) at 37 °C. The wild-type, Ku70-deficient, and 53BP1-deficient DT40 cells used in this study were described previously [12]. The DT40 cells were cultured in RPMI1640 medium (Nissui Seiyaku) supplemented with 10% FCS, 1% chicken serum (Invitrogen, Carlsbad, CA, USA), and 10 μM β-mercaptoethanol at 39.5 °C. The wild-type and DNA ligase IV-deficient Nalm-6 cells used in this study are described elsewhere [8]. The Nalm-6 cells were cultured in RPMI1640 medium supplemented with 10% FCS and 50 μM β-mercaptoethanol at 37 °C.

Cell sorting and flow cytometric analysis. Dispersed HeLa cells, which were detached from their culture dishes using trypsin, DT40 cells, or Nalm-6 cells, were suspended in their respective culture medium and applied to a JSAN cell sorter (Bay Bioscience, Hyogo, Japan). Using their FSC values, the cells were sorted into groups based on the different stages of the cell cycle at room temperature over a 60- or 90-min period for the DT40 and Nalm-6 cell lines, respectively. Asynchronous cells were separated from the exponentially growing cells by gating and sorting. All measurements were made using an argon laser tuned at 488 nm. The sorted cells were washed once with the appropriate culture medium, suspended in the same medium, and then subjected to cell cycle analysis, immunofluorescent staining or colony formation assays. For the cell cycle analyses, cells were cultured for 5 min with 10 μM bromodeoxyuridine (BrdU) (Sigma–Aldrich, St. Louis, MO, USA), then harvested, fixed, and stained with a fluorescein isothiocyanate-conjugated anti-BrdU antibody as described previously [12]. Flow cytometric analysis of the HeLa cells was performed using a JSAN sorter, while a FACSCalibur flow cytometer (Becton–Dickinson, Plymouth, UK) was used for the DT40 and Nalm-6 cell lines.

Cell cycle synchronization and γ-H2AX staining in the DT40 cells. The method used to achieve G1 synchronization in the DT40 cells was described previously [12]. Briefly, the cells were cultured in medium containing 1.0 μg/ml nocodazole (Sigma–Aldrich) for 8 h, washed three times with PBS containing 5% calf serum, and then cultured in medium containing 0.8 mM mimosine (Sigma–Aldrich) for 8 h. The synchronized cells were then washed three times as described above and cultured in medium to allow them to reenter the cell cycle. γ-H2AX staining was performed as described previously [12].

Colony formation assay and X-irradiation. Colony formation assays were performed as described elsewhere using DT40 [12] and Nalm-6 [13] cells with the following modification: the cells were irradiated, and then an appropriate number of cells were plated in agarose-containing medium. X-irradiation was performed at 150 kVp and 20 mA with a dose rate of 1.9 Gy/min using a Hitachi Medico X-ray irradiator (Tokyo, Japan) for the DT40 cells, and at 50 kVp and 2.5 mA with a dose rate of 0.25 Gy/min using a Softex X-ray irradiator (Kanagawa, Japan) for the Nalm-6 cells.

Results and discussion

Sorting of G1-phase cells from exponentially growing HeLa cells

Because cell size increases linearly as cells proceed through the cell cycle [11], we tested whether the sorting of cells with lower FSC values could improve the proportion of G1-phase cells. HeLa cells were trypsinized, and the dispersed cell suspension was applied to a cell sorter without fluorescent staining or fixing. The sorted cells were then pulse-labeled with BrdU, and the cell cycle profile was assessed by flow cytometric analysis of the DNA content and BrdU uptake. As shown in Fig. 1A, the side scatter characteristics (SSC) value, which represents internal structure, increased as the FSC value increased in the cells. Since approximately 53% of the exponentially growing HeLa cells were in G1-phase (Fig. 1B, left panel), we assumed that if we gated the cells with lower FSC and SSC values by less than half of 53%, S-phase cell contamination would be negligible. As expected, by gating and sorting 10% of the cells with lower FSC and SSC values, we were able to obtain a cell population in which 90% of the cells were in G1-phase (Fig. 1B, right panel). These data indicate that this sorting method can be used for the enrichment of G1-phase HeLa cells.

X-ray sensitivity in sorted NHEJ-deficient DT40 cells

We next applied this sorting method to a suspension cell line. The chicken B cell line DT40 is highly useful for gene targeting by HR [14], and many NHEJ factor-deficient lines have been established [15]. DT40 cells can be synchronized in G1-phase by treatment with nocodazole followed by mimosine. Previously, we suggested that in DT40 cells, 53BP1 plays a role in the repair of DNA DSBs by NHEJ via a pathway that is distinct from the Ku70/Ku80/DNA-PKcs-dependent pathway [12]. Colony formation assays using chemically synchronized G1-phase cells revealed that 53BP1-deficient DT40 cells are less sensitive to X-rays than Ku70-deficient cells when the cells are irradiated during G1. This indicates that the 53BP1-dependent pathway contributes less than the Ku70/Ku80/DNA-PKcs-dependent pathway to the repair of X-ray-induced DNA damage in G1-phase cells. To determine whether G1-enriched cells obtained by cell sorting have similar X-ray sensitivities to chemically synchronized G1-enriched cells, we applied our cell sorter-based fractionation method to DT40 cells. Compared to the HeLa cells, the DT40 cells had relatively constant SSC values (Fig. 2A), and the proportion of G1-phase cells was only 24% of the total (Fig. 2B, left panel). Accordingly, we gated the cells with lower FSC