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Research paper

Radiation-resistant B-1 cells: A possible initiating cells of neoplastic transformation

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ABSTRACT

The role of B-1 cells in the hyperproliferative hematologic disease has been described. Several reports bring evidences that B-1 cells are the main cell population in the chronic lymphatic leukemia. It is also described that these cells have an important involvement in the lupus erythematous systemic. The murine model used to investigate both disease models is NZB/NZW. Data from literature point that mutation in micro-RNA 15a and 16 are the responsible for the B-1 hyperplasia in these mice. Interestingly, it was demonstrated that NZB/NZW B-1 cells are radioresistant, contrariwise to observe in other mouse lineage derived B-1 cells and B-2 cells. However, some reports bring evidences that a small percentage of B-1 cells in healthy mice are also able to survive to irradiation. Herein, we aim to investigate the malignant potential of ionizing-radiation resistant B-1 cells *in vitro*. Our main goal is to establish a model that mimics the neoplastic transformation originate to a damage exposure of DNA, and not only related to intrinsic mutations. Data shown here demonstrated that radiation-resistant B-1 cells were able to survive long periods in culture. Further, these cells show proliferation index increase in relation to non-irradiated B-1 cells. In addition, radiation resistant B-1 cells showed hyperploid, morphologic alterations, increased induction of apoptosis after anti-IgM stimulation. Based on these results, we could suggest that radiation resistant B-1 cells showed some modifications in that could be related to induction of malignant potential.

1. Introduction

In 1983, for the first time, murine B-1 cells were described as a splenic $CD5^+$ B cell population (Hayakawa et al., 1983). Later on, these cells were also identified in the peritoneal and pleural cavity (Hayakawa et al., 1985). B-1 cells were characterized as $CD19^+CD23^-CD43^+IgM^{high}IgD^{low}CD11b^+$ cells (reviewed by Lopes and Mariano, 2009). The expression of CD5 classified B-1 cells into two subtypes: B-1a cells which express CD5, and B-1b which are $CD5^-$ (Hayakawa et al., 1983). Moreover, B-1 and B-2 cells are different in theirs origin. While B-2 cells develop from progenitors from adult bone marrow, and before birth, from fetal liver precursors (Abbas, 2008); it is established that B-1 cells emerge at the beginning of the hematopoietic system from precursors derived from splancnopleura paraortic (Montecino-Rodriguez et al., 2006) and later from fetal liver (Herzenberg and Kantor, 1993). There is a small population of B-1 precursors cells in the adult bone marrow, but the functionality and maintenance of B-1 cell population derived from them is not completely elucidated (Montecino-Rodriguez et al., 2006).

B-1 cell population is larger in NZB/NZW mice, a murine model to study systemic lupus erythematosus and chronic lymphocytic leukemia (CLL), than normal mice (Kasar et al., 2012). This murine CLL is similar to human CLL, meaning that the course of the disease depends on the age and it is associated with the expansion of B $CD5^+$ cell clones (Caligaris-Cappio, 1996; Phillips et al., 1992). It has been demonstrated that one-year-old NZB/NZW mice have hyperplasia in long life B-1 cells, where it is possible to observe the presence of polyploidy (Kasar et al., 2012). Furthermore, it was observed larger quantity of mature lymphocytes in peritoneal NZB/NZW mice with a high nucleus:cytoplasm proportion, as observed in patients with CLL (Phillips et al., 1992). Interestingly, these cells are able to proliferate after intravenous transference into irradiated NZB/NZW mice (Phillips et al., 1992; Raveche, 1990). NZB/NZW B-1 cells are able to survive for a long time, showing self-renewal capacity and increase malignant potential (Seldin et al., 1987). It is important to mention that NZB/NZW B-1 cells are radioresistant to high doses of ionizing

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radiation. e Brito et al. (2007) observed that the selective radiation of the peritoneal cavity was not able to deplete B-1 cells from NZB/NZW mice, while another authors observed that this treatment depleted efficiently B-1 cells from the peritoneal cavity of healthy mice (C57/BL6 and BALB/c) (e Brito et al., 2007; Staquicini et al., 2008).

Despite of B-1 cells from healthy mice are radiosensitive, it has been described that a small percentage of these cells are able to survive *in vitro* after exposure to high doses of ionizing radiation (8 Gy) (Staquicini et al., 2008). The mechanisms involved in the acquisition of radioresistance by B-1 cells from healthy mice were not studied yet. The elucidation of mechanisms involved in the resistance to apoptosis and cellular cycle control are fundamental to the understanding of early events related to transformation and tumor progression. Herein, we purpose to investigate the characteristics of radiation resistant B-1 cells, which could lead these cells to a neoplastic transformation.

2. Materials and methods

2.1. Mice

C57BL/6 mice was obtained from CEDEME/UNIFESP (Centro de Desenvolvimento de Modelos Experimentais da Universidade Federal de São Paulo). All animals were maintained under specific pathogen free conditions. All procedures described herein were approved by the Ethical Committee of UNIFESP (2014/8832030914).

2.2. Cell sorting

B-1 cells were obtained from the peritoneal cavity washout of C57BL/6 mice. First, cells were incubated with anti-CD16/CD32 (BD-Pharmingen, California; USA) to block the Fc receptor, and after, cells were stained with anti-CD19 allophycocianin (APC, BD-Pharmingen, California; USA) and anti-CD23 phycoerythrin (PE, BD-Pharmingen, California; USA). B-1 cell population ($CD19^+CD23^-$) was enriched from cell sorting by FACSAria II cytometer (BD-Pharmingen, California; USA). Enrichment above 95% was considered to perform the following analysis. Supplementary Fig. 1 illustrated the gate strategy used to obtain B-1 cells.

2.3. B-1 cells radiation

Purified B-1 cells were exposed to 3,5 Gy gamma radiation using Gammacell® 3000 (Nordion International Inc., Ottawa; Canada). After that, cells were maintained in culture with OP9 (ATCC CRL-2749) cells. Non-irradiated cells were used as control.

2.4. B-1 cells in co-culture with OP9 cells

It has been demonstrated that B-1 cells viability *in vitro* is increased when these cells were cultivated in a feeder layer (Thies et al., 2013). Considering that OP9 is commonly used in hematopoietic precursors cultures, herein we co-culture B-1 cells with OP9 in order to allow the maintenance of B-1 cell culture for long periods. OP9 cells (ATCC CRL-2749) were cultivated in 6 or 96 wells plates during four days in specific A20 medium (alfa-MEM medium added with 20% of Fetal Bovine Serum (FBS) (Life Technologies, USA) at 37 °C with 5% CO_2. Afterward, control or irradiated B-1 cells were co-cultured with OP9 cells with 50% A20 medium and 50% A10 medium (A20/10medium) (alfa-MEM with 10% FBS, 1% L-glutamine 100 mM and 0,1% β-mercapto 100uM). B-1 cells were added at 5×10^5 cells/well in the 6 wells plate and $2{,}5 \times 10^5$ cells/well in the 96 wells plate. The co-cultures were incubated at 37 °C with 5% CO_2 and maintained for 3, 7 or 15 days.

2.5. Analysis of viability and proliferation

Firstly, control or irradiated B-1 cells were stained with CellTrace™ Violet Cell Proliferation Kit (Life Technologies). After this, these cells were added to OP9 culture, as described early. B-1 cells were labeled with anti-CD19 allophycocianin (APC, BD-Pharmingen, California; USA) and anti-CD23 fluorescein isothiocyanate (FITC, BD-Pharmingen, California; USA). The proliferation and viability of B-1 cells ($CD19^+CD23^-$ cells) were analyzed by using CellTrace™ Violet Cell Proliferation Kit (Life Technologies) and propidium iodide (PI) (Life Technologies), respectively, accordingly manufacturer's instructions. Cells were acquired by FACSCanto II cytometer (BD-Pharmingen, California; USA) and analyzed by FlowJo9.5 software (TreeStar, USA). The absolute number was calculated considering the initial cells input. Division index is the average number of cell divisions that a cell in the original population has undergone.

2.6. Analysis of the DNA quantity

The DNA quantification was analyzed by Vybrant® DyeCycle™ Violet Stain (VDC) (Life Technology, USA). To exclude OP9 cells from this analysis, these cells were previously labeled with CellTrace™ APC Cell Proliferation Kit (Life Technologies, USA). Control or irradiated B-1 cells were added to OP9 cell cultures, and after different time points they were collected and stained with Vybrant® DyeCycle™ Violet Stain, following the manufacturer's protocol. Then, cells were acquired by FACSCanto II cytometer (BD-Pharmingen, California; USA) and DNA quantity was analyzed by FlowJo9.5 software (TreeStar, USA). To determine the G0 peak, bone marrow derived macrophages in a stationary culture were used.

2.7. Detection of apoptosis by anti-IgM

Purified goat antibody anti-IgM (eBiocience, USA) was added to B-1/OP9 co-culture at 8 μg/mL for 20 h. Afterwards, control or irradiated B-1 cells were stained with PE Anexin V Apoptosis Detection Kit I (BD-Pharmingen, California; USA), following the manufacturer's protocol. The cells were acquired in FACSCanto II cytometer (BD-Pharmingen, California; USA) and apoptosis was analyzed by FlowJo9.5 software (TreeStar, USA).

2.8. Morphological analysis

After different times point of B-1/OP9 co-culture, control or irradiated B-1 cells were collected and analyzed morphologically by Zeiss Axio Imager A2 microscope (Carl Zeiss Mycroscopy). Firstly, 5×10^4 cells were suspended in 200 μL of PBS and then submitted to cytospin (FANEM model 2400–Guarulhos/BR). After this, the glass-coverslips with adhered cells were fixed and stained with *Panótipo Rápido kit* (Laborclin, BR).

2.9. Expression of Bcl-family

RNA was isolated from control or irradiated B-1 cells using the llustraRNAspin Mini (Ge Healthcare Life Sciences). The samples were quantified by NanoDrop 1000 spectrophotometer (Thermo Scientific) and maintained in freezer −80 °C. cDNA synthesis was obtained using the kit Superscript III First-Strand synthesis System (Invitrogen-Life Technology). Expression levels of Bcl-2 (CTGCACCTGACGCCCTTCACC/CACATGACCCCACCGAACTCAAAGA), Bcl-9 (AGTGCTCTCTCCAGGATATGATG/GGGCAAAGAGTGTGAAATGTTG) and Bax (TGAAGACAGGGGCCTTTTTG/AATTCGCCGGAGACACTCG)