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HLA-G1 increases the radiosensitivity of human tumoral cells

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ABSTRACT

Different molecules regulate the response of tumoral tissues to ionizing radiation. The objective of this work was to determine if HLA-G1 expression modulates the radiosensitivity of human tumoral cell lines. To this end, human melanoma M8 and human erythroleukemia K562 cell lines, with their correspondent HLA-G1 negative and positive variants, were gamma irradiated and the survival frequency was determined by clonogenic assay. The survival fraction of HLA-G1 expressing cells was around 60% of HLA-G1 negative cells. The generation of acidic vesicular organelles was higher in HLA-G1 positive cells. Apoptosis levels showed statistically significant differences only in K562 cells, whereas the variation in G2/M cycle progression was only significant in M8 cells. In addition, irradiation diminished cell-surface HLA-G1 and increased soluble HLA-G1 levels. Soluble HLA-G1 has no influence on cell survival in any cell line. In summary, we could demonstrate that HLA-G1 confers higher radiosensitivity to HLA-G1 expressing cells.

1. Introduction

Tumor cells show a very broad range of radiosensitivities: lymphoid tumors are in general highly sensitive to radiation, whereas melanomas and gliomas are one of the most radioresistant tumors [1]. The differential radiosensitivity may depend on many factors, being the efficiency to recognize and/or repair the DNA lesion, and the cell cycle control mechanisms, the most important [2]. Activation of cell cycle checkpoints is a common cellular response to DNA-damaging agents such as ionizing radiation. The tumor suppressor gene p53 is one of the key proteins in these checkpoint pathways, coordinating DNA repair with cell cycle progression and apoptosis [3]. Furthermore, regulating autophagy and apoptosis p53 contributes to cellular radiosensitivity [4]. For a given cell line, radiosensitivity also varies along the cell cycle: G2/M is the most radiosensitive phase, followed by G1, and being the latter part of the S phase the least sensitive to radiation [3].

Human leukocyte antigen-G (HLA-G) is a non-classical HLA class I molecule involved in fetus protection from the maternal immune system, transplant tolerance, and viral and tumoral immune escape. It is normally absent on healthy tissues except for trophoblast, thymus, cornea, pancreas, nail matrix and erythroid precursors [5]. HLA-G can be expressed as seven isoforms: four membrane-bound (HLA-G1 to -G4) and three soluble (HLA-G5 to -G7) [6]. Soluble HLA-G1 (sHLA-G1) could be generated by proteolytic cleavage of surface HLA-G1 [7].

In a previous work we could demonstrate that ionizing radiation down-regulates the surface expression of HLA-G1 in human melanoma cells [8]. Similar results were reported for basal cell carcinoma of the skin after radiotherapy [9]. However, the involvement of this molecule in tumoral radiosensitivity has not been demonstrated yet.

The aim of this work was to evaluate if the expression of HLA-G1 intervenes in the survival response to ionizing radiation of human tumoral cells cultured *in vitro*. For that purpose, we compared the survival frequency after gamma irradiation of HLA-G1 positive and HLA-G1 negative cell lines from melanoma and erythroleukemia. In order to establish the possible mechanisms by which HLA-G1 was exerting its radiosensitizing action, we evaluated the presence of acidic vesicular organelles (AVO), apoptosis, cell cycle evolution, surface HLA-G1 expression and sHLA-G1, together with its biological activity.

The main finding of our work was that HLA-G1 confers a significant reduction in cell survival after gamma irradiation, postulating HLA-G1 as a possible tumoral radiosensitivity marker. The mechanism implicated in this radiosensitivity phenomenon seems to be dependent on the histological origin of the neoplastic tissue, and remains to be determined.

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2. Materials and methods

2.1. Cell lines and cell culture

The parental cell lines of human melanoma M8 cells and human erythroleukemia K562 cells are HLA-G negative. In the present study we used HLA-G1 positive and HLA-G1 negative, stable transfectant cells from both cell lines. These transfectant cell lines were obtained previously [10,11] by incorporation of the vectors containing the cDNA of the HLA-G1 molecule (HLA-G1 positive cells), or the vector alone (HLA-G1 negative cells). The vectors used were the pcDNA3-1/hygromycin expression vector for M8 cells and the pRc/RSV eukaryotic expression vector for K562 cells. The cell lines were named M8-HLA-G1 and K562-HLA-G1 for HLA-G1 expressing cells, and M8-pcDNA and K562-pRc/RSV for HLA-G1 negative cells. The cells were maintained in RPMI 1640 medium supplemented with 2 mM glutamine, 10% fetal calf serum, penicillin/streptomycin (100 U/ml – 50 μg/ml) and 50 μg/ml hygromycin B (for M8-pcDNA and M8-HLA-G1 cells) or 1 μg/ml geneticin (for K562-pRc/RSV and K562-HLA-G1 cells), and cultured in a 37 °C, 5% CO_2, humidified incubator. The cell lines used in this study were routinely tested for freedom of mycoplasma contamination.

2.2. Gamma irradiation

The cells were gamma irradiated with a Gammacell 220 equipment (Nordion International Inc., Kanata, Ontario, Canada) at room temperature, with a final dose of 2, 5, 8 and 10 Gy at a dose rate of 0.48 Gy/min for M8-pcDNA and M8-HLA-G1 cell lines, and with a final dose of 2, 5, and 8 Gy at the same dose rate for K562-pRc/RSV and K562-HLA-G1 cell lines.

2.3. Survival assays

For M8-pcDNA and M8-HLA-G1 cell lines, the survival fraction at different doses was determined by clonogenic assay (Franken et al., 2006). Briefly, after irradiation the cells were detached with 0.1% EDTA–0.25% trypsin at 37 °C and an adequate number of cells was seeded in 100 mm tissue culture dishes in order to obtain approximately 50–70 colonies/dish. The cells were cultured for 15 days at 37 °C. Cell colonies were stained with May-Grunwald stain (Merck Chemicals, Darmstadt, Germany). Colonies containing more than 50 cells were considered positive and were counted. The ratio between the number of colonies of HLA-G1 positive cells respect to the number of HLA-G1 negative cells was determined and expressed as percentage.

Since K562 cells grow in suspension, we used a different method for evaluation of the survival fraction of K562-pRc/RSV and K562-HLA-G1 cells. A number of 1×10^4 cells were seeded in 60 mm tissue culture dishes for control and for 2, 5 and 8 Gy irradiated cells. After 10 days of culture at 37 °C the number of surviving cells was counted in a Neubauer chamber. The survival fraction was determined as the ratio between the number of HLA-G1 positive cells and the number of HLA-G1 negative cells, and expressed as percentage.

2.4. Acidic vesicular organelles (AVO) staining

In order to quantify the development of AVO after 24 and 48 h of irradiation with 5 Gy, HLA-G1 positive and negative M8 and K562 cells were stained with acridine orange at a final concentration of 1 μg/ml during 15 min at 37 °C, washed with PBS and analysed in a FACSCalibur cytometer (BD Biosciences, San Jose, CA, USA). Data analysis was carried out with BD Cell Quest Pro software (BD Biosciences).

2.5. Annexin V-FITC/propidium iodide (PI) staining

Apoptotic levels in HLA-G1 positive and negative M8 and K562 cells were determined after 0, 3, 24 and 48 h of irradiation with 5 Gy by flow cytometry using the Annexin V-FITC/propidium iodide (PI) kit (BD Biosciences) following manufacturer's instructions. Results were expressed as the ratio between the percentage of apoptotic cells in the irradiated condition (IR) and the percentage of apoptotic cells under non irradiated conditions (N-IR).

2.6. Cell cycle analysis

Samples of HLA-G1 positive and HLA-G1 negative cells irradiated with 5 Gy were collected at 0, 3, 24, 48 and 192 h post-irradiation for M8 cells and at 0, 3, 24 and 48 h post-irradiation for K562 cells, and assessed for cell-cycle distribution by flow cytometry as described in [8]. Briefly, 1×10^6 cells were fixed in ethanol 70% (v/v) during 24 h, then washed and resuspended in PBS buffer containing 100 μg/ml RNAse and 40 μg/ml PI, incubated for 15 min in the dark and analysed in a FACSCalibur cytometer.

2.7. Evaluation of HLA-G1 surface expression

Plasma membrane HLA-G1 expression in M8-HLA-G1 and in K562-HLA-G1 cells irradiated with 5 Gy was analyzed by flow cytometry 24 h after irradiation. The cells were labeled as described in [8] using for isotype controls mouse IgG1 pure (BD Biosciences) and anti-HLA-G (MEM-G/9) monoclonal antibody (Exbio, Prague, Czech Republic) as a primary specific antibody. Goat anti-mouse immunoglobulin coupled to R-phycoerythrin (RPE) (Dako, Glostrup, Denmark) was used as secondary antibody. The specific fluorescence index (SFI) in IR and N-IR cells was calculated as the ratio of the mean fluorescence values obtained with the specific antibody and the control isotype antibody. The level of HLA-G1 at the surface of IR cells was express as the ratio: SFI IR/SFI N-IR.

2.8. Evaluation of sHLA-G1 levels and its biological activity

The presence of sHLA-G1 in the culture medium of N-IR and IR cells was analyzed 24 h after irradiation with 5 Gy using a specific ELISA kit from Exbio, according to the manufacturer's recommendations.

For the determination of the biological activity of sHLA-G1, 24 h after irradiation with 5 Gy, the medium of HLA-G1 positive and negative M8 and K562 cells was centrifuged, filtered through 450 μm filters and transferred to 60 mm tissue culture dishes seeded with 5×10^4 of N-IR M8-HLA-G1 and K562-HLA-G1 cells, respectively. After 10 days, the ratio between the number of cells cultured with the medium of IR cells and the number of cells grown with the medium of N-IR cells was determined for both cell lines.

2.9. Statistical analysis

The results were reported as mean ± standard deviation. Statistically significant differences were determined using Student's *t*-test (two-tailed) or one-way ANOVA test followed by Tukey's test, according to the case. A $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. HLA-G1 is involved in tumoral radiosensitivity

We compared the survival fraction after 0, 2, 5, 8 and 10 Gy gamma irradiation for M8-pcDNA and M8-HLA-G1 cell lines by clonogenic survival analysis. As shown in Fig. 1A, M8-HLA-G1 cells are