Cellular Immunology 287 (2014) 106–111

Contents lists available at ScienceDirect

Cellular Immunology

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

HLA-G1 increases the radiosensitivity of human tumoral cells

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ARTICLE INFO

Article history:
Received 30 September 2013
Accepted 7 January 2014
Available online 15 January 2014

Keywords:
HLA-G1
Radiosensitivity
Ionizing radiation
Melanoma cells
Erythroleukemia cells

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.
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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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http://dx.doi.org/10.1016/j.cellimm.2014.01.005

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

more radiosensible than M8-pcDNA cells at all the doses tested. Similar results were obtained for K562 cells, exhibiting the K562-HLA-G1 cell line higher radiosensitivity than K562-pRc/RSV cells after irradiation with 0, 2, 5 and 8 Gy (Fig. 1B). The number of surviving cells at 10 Gy for K562 cells was extremely low and was not considered for the construction of the corresponding clonogenic survival curves (Fig. 1B).

The percentage of cell survival of M8 and K562 HLA-G1 expressing cells with respect to their corresponding HLA-G1 negative variants was determined after gamma irradiation. As shown in Table 1, the survival of HLA-G1 positive cells was significantly reduced with respect to HLA-G1 negative cells for all the tested doses.

Table 1
Percentage of cell survival of HLA-G1 expressing cells with respect to HLA-G1 negative cells after gamma irradiation with the indicated doses.

Cell line	2 Gy	5 Gy	8 Gy	10 Gy
M8	58.06 ± 14.50** ($n = 4$)	54.27 ± 10.61*** ($n = 5$)	60.08 ± 10.82** ($n = 3$)	63.41 ± 16.03* ($n = 3$)
K562	74.75 ± 12.27** ($n = 4$)	62.50 ± 11.48** ($n = 4$)	63.30 ± 12.37** ($n = 4$)	ND

*$p < 0.05$; **$p < 0.005$; ***$p < 0.0001$ with respect to HLA-G1 negative cells (100%) (Student's t-test).

3.2. HLA-G1 expressing cells generate more AVO after gamma irradiation

Numerous studies demonstrated the dual role of autophagy, regulating cell survival and cell death [12,13]. Additionally, the association between autophagy and cellular radiosensitivity has also been reported [14–17]. In order to study whether autophagy was involved in the regulation of radiosensitivity under our experimental conditions, we evaluated the formation of AVO, indicative of the induction of autophagy, using acridine orange staining [18]. HLA-G1 negative and HLA-G1 expressing cells from M8 and K562 cell lines were gamma-irradiated with 5 Gy and after 24 and 48 h of irradiation the cells were stained as described in the Materials and Methods section. We could observe that irradiation induced a larger increase in the development of AVO in HLA-G1 expressing cells, in comparison with their corresponding HLA-G1 negative variants for both cell lines (Fig. 2A and B).

3.3. M8 and K562 cells differed in their apoptosis response

Afterwards, we evaluated if the apoptotic mechanism could contribute to the observed differences in cell survival after irradiation between HLA-G1 negative and HLA-G1 expressing cells. For this end, we quantified apoptosis levels by flow cytometry with Anexin V-FITC/PI after 3, 24 and 48 h of irradiation with 5 Gy. At 3 h post-irradiation there were no differences in apoptosis levels between N-IR and IR cells, and between HLA-G1 positive and HLA-G1 negative cells, for M8 as well as for K562 cell lines.

After 24 and 48 h post-irradiation apoptosis began to be evident in M8 cells, (~20% and 65% of increase with respect to N-IR cells, respectively) although no significant differences could be observed between M8-pcDNA and M8-HLA-G1 cells (Fig. 3A).

For the K562 erythroleukemic cell line we obtained a different result: 24 and 48 h post-irradiation, K562-HLA-G1 cells reached significantly higher apoptosis values than K562-pRc/RSV cells (187% *vs.* 156% after 24 h, and 185% *vs.* 129% after 48 h of irradiation) (Fig. 3B).

3.4. M8-HLA-G1 cells exhibit a higher arrest in G2/M phase than M8-pcDNA cells

We also analysed if the occurrence of an arrest in some of the cell cycle phases could be contributing to the observed radiosensitivity phenomenon. For M8 cells, we could observe that HLA-G1 negative and HLA-G1 positive cells were both arrested in the G2/M phase 24 h post-irradiation. However, for HLA-G1 positive cells the percentage of arrest was slightly higher than for negative cells. This difference was overcome after 48 h of irradiation (Table 2).

For K562-pRc/RSV and K562-HLA-G1 cells, gamma irradiation also caused G2/M arrest at 24 and 48 h post-irradiation, but without statistically significant differences between HLA-G1 negative and HLA-G1 positive cells (Table 2).

3.5. Gamma irradiation decreases cells surface HLA-G1 and increases sHLA-G1 levels

In a previous work from our laboratory, we could demonstrate that ionizing radiation caused the reduction of surface HLA-G1 in FON cells (a melanoma cell line which naturally expresses the HLA-G1 molecule) with the concomitant increase in sHLA-G1 levels in the culture medium after 24 h of irradiation [8]. To examine if gamma irradiation induces the same effect in HLA-G1 transfected cells, the surface expression of HLA-G1 in M8-HLA-G1 and K562-HLA-G1 cells was determined by flow cytometry after 24 h of irradiation with 5 Gy. We could observe a significant decrease in surface HLA-G1 levels (16% for M8-HLA-G1 and 30% for K562-HLA-G1 cells) (Fig. 4A).

The concentration of sHLA-G1 in the culture medium of N-IR and IR M8-HLA-G1 and K562-HLA-G1 cells was measured 24 h post-irradiation by ELISA assay. We obtained an increase of 97% for M8-HLA-G1 cells and of 145% for K562-HLA-G1 cells (Fig. 4B).

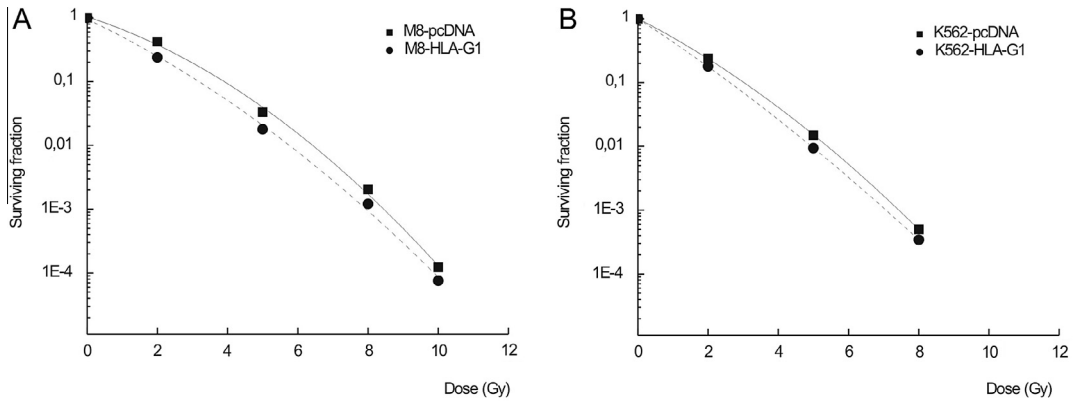

Fig. 1. Clonogenic survival curves for M8-pcDNA and M8-HLA-G1 cells (A) and for K562-HLA-G1 and K562-pRc/RSV cells (B). (Black circles, HLA-G1 positive cells; black squares, HLA-G1 negative cells).

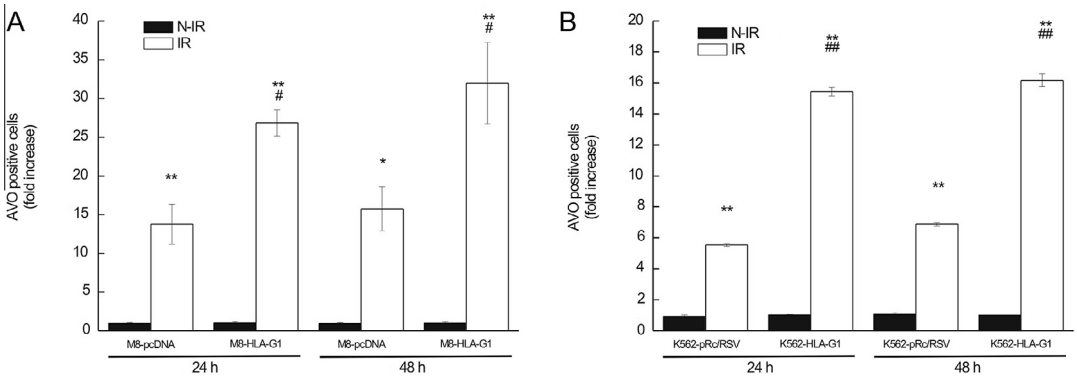

Fig. 2. The development of acidic vesicular organelles (AVO) was determined using acridine orange staining followed by FACS analysis for both M8 (A) and K562 (B) cells. $^{*}p < 0.005$; $^{**}p < 0.0001$ with respect to N-IR cells (ANOVA). $^{\#}p < 0.001$; $^{\#\#}p < 0.0001$ with respect to HLA-G1 negative cells (ANOVA). Results are the average of three independent experiments.

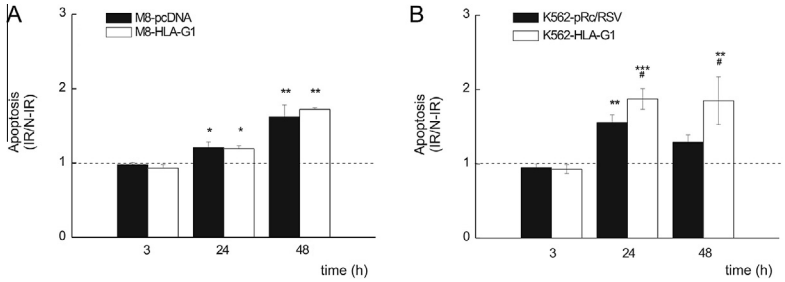

Fig. 3. Annexin V-FITC/PI flow cytometry evaluation of apoptosis in M8 (A) and K562 (B) cells after the indicated times post-irradiation. Results are expressed as apoptotic cells in the IR condition/apoptotic cells in N-IR condition. $^{*}p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.0001$ with respect to N-IR cells (1.00) (ANOVA). $^{\#}p < 0.05$ with respect to K562-pRc/RSV cells (ANOVA). Results are the average of three independent experiments.

Table 2
Cell cycle distribution of M8 and K562 cells after the indicated times of irradiation with 5 Gy.

	G0/G1	S	G2/M
M8-pcDNA (h)			
0	58.94 ± 3.9	16.53 ± 3.2	23.96 ± 1.4
3	52.63 ± 9.8	20.12 ± 4.0	27.37 ± 5.5
24	5.68 ± 0.2*	2.97 ± 1.6**	91.4 ± 1.7*
48	10.37 ± 1.7*	3.61 ± 0.9**	86.07 ± 2.5*
192	44.31 ± 5.3*	25.48 ± 4.4	28.78 ± 0.4**
M8-HLA-G1 (h)			
0	58.79 ± 4.7	15.60 ± 3.8	25.57 ± 0.7
3	57.67 ± 12.9	18.92 ± 4.9	23.41 ± 6.6
24	3.55 ± 0.4*	1.84 ± 1.1**	94.65 ± 1.0*,#
48	11.73 ± 0.6*	5.13 ± 0.6**	83.16 ± 1.2*
192	47.12 ± 1.9	22.02 ± 0.6	30.32 ± 1.4**
K562-pRc/RSV (h)			
0	39.57 ± 2.9	29.93 ± 2.6	30.95 ± 4.6
3	38.23 ± 4.2	28.77 ± 1.5	32.58 ± 3.1
24	37.42 ± 1.3	17.80 ± 0.3***	45.08 ± 1.5***
48	35.88 ± 1.4	24.02 ± 0.9*	40.48 ± 1.1*
K562-HLA-G1 (h)			
0	40.75 ± 2.2	30.34 ± 3.0	29.44 ± 3.8
3	37.98 ± 5.1	27.82 ± 2.3	33.64 ± 4.7
24	33.61 ± 2.3**	18.42 ± 2.1**	48.29 ± 0.6***
48	35.90 ± 1.8*	25.27 ± 1.0	39.08 ± 0.8*

$^{*}p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.0005$ with respect to N-IR cells (ANOVA). $^{\#}p < 0.05$ with respect to M8-pcDNA values (ANOVA). Results are the average of three independent experiments.

3.6. sHLA-G1 is not involved in the regulation of cellular radiosensitivity

Finally, we evaluated if the molecule sHLA-G1, released after gamma irradiation, has some influence on the regulation of the radiosensitivity of the studied HLA-G1 positive cell lines. For this purpose, the culture medium of N-IR and 5 Gy IR, M8-HLA-G1 and K562-HLA-G1 cells was transferred 24 h post-irradiation to N-IR M8-HLA-G1 and K562-HLA-G1 cells, respectively, and the final number of cells in each condition was determined after 10 days of incubation. We could not observe any statistical differences in the final cell number from both cell lines, indicating that sHLA-G1 has no effect on the cell growth of HLA-G1 expressing cells.

4. Discussion

Ionizing radiation is presently considered as a useful component of the antineoplastic treatment. However, some malignancies are relatively resistant to radiation treatment while others are more responsive. To improve the efficacy of radiotherapy, the research of tumoral markers of radiosensitivity is one of the main areas of radiobiology studies.

In the present work, we evaluated the possibility that the HLA-G1 molecule could be a possible radiosensitivity marker modulating the response to ionizing radiation of those tumor cells that express this antigen. For that purpose, we used two human tumoral cell lines from different histological origin: the M8 cell line, from melanoma, and the K562 erythroleukemia cell line. From both cell lines we have the HLA-G1 negative and HLA-G1 positive variants. The possibility to have the same cell line with and without HLA-G1 expression makes this system the most appropriate for this kind of study, discarding the possible differences attributable to the use of dissimilar cell lines.

We compared the survival frequency after gamma irradiation of M8-pcDNA and M8-HLA-G1 cells, and K562-pRc/RSV and K562-HLA-G1 cells and we could observe that in both HLA-G1 positive cell lines, the cell survival was significantly decreased with respect

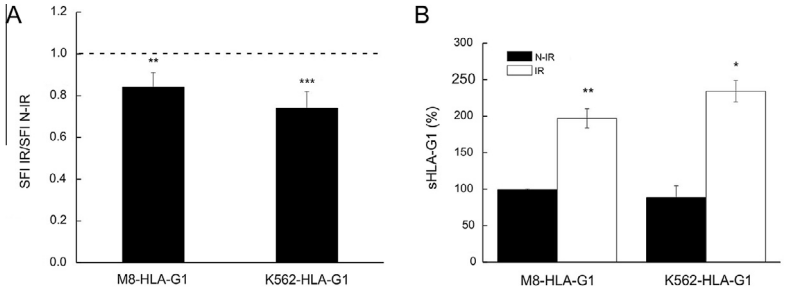

Fig. 4. (A) Flow cytometry evaluation of surface HLA-G1 in M8-HLA-G1 and K562-HLA-G1 cells after 24 h of irradiation with 5 Gy. Results are expressed as SFI IR/SFI N-IR. $^{**}p < 0.01$; $^{***}p < 0.0001$ with respect to N-IR cells (1.00) (Student's *t*-test). (B) Concentration of sHLA-G1 in the culture medium of N-IR and IR M8-HLA-G1 and K562-HLA-G1 cells after 24 h of irradiation with 5 Gy. Results are expressed as the percentage of sHLA-G1 in the culture medium. $^{*}p < 0.05$ and $^{**}p < 0.01$ with respect to N-IR cells (100%) (Student's *t*-test). Results shown in (A) and (B) are the average of three independent experiments.

to HLA-G1 negative cells. This effect was independent of the dose of radiation, since the percentage of decrease was very similar for all the doses tested (Fig. 1A and B; Table 1).

In order to understand the possible mechanisms involved, we evaluated the induction of autophagy, apoptosis levels, the progression trough the cell cycle and the effect of sHLA-G1 on cellular growth after irradiation with 5 Gy. We decided to perform all these determinations at 5 Gy as it is an intermediate dose and because at this dose the difference in cells survival between HLA-G1 positive and negative cells was slightly bigger than at 2 and 8 Gy.

Up regulation of autophagy has been observed in many types of cancers and it has been demonstrated to promote both cell survival and cell death [12,13]. To analyze the possible occurrence of this mechanism, we evaluated the generation of AVO 24 and 48 h post irradiation as an indication of autophagy development [18]. Numerous studies demonstrated an association between autophagy and cellular radiosensitivity: Kuwahara et al. [14] reported that X-ray irradiation of HepG2 and SAS cancer cell lines significantly induced autophagy in the parental cells, whereas this phenomenon was insignificant in their corresponding radioresistant cell variants suggesting that autophagic cell death is involved in cellular radiosensitivity. Similar results have been reported for glioma cell lines [19–22]. Indeed, induction of autophagy with mTOR inhibitors, such as rapamycin, increases the radiosensitivity of glioma stem cells and pancreatic carcinoma cells [15–17]. In accordance with these studies, we could demonstrate that gamma irradiation induced a larger increase of AVO in HLA-G1 expressing cells in comparison with HLA-G1 negative cells in both M8 and K562 cells (Fig. 2). This result could postulate the induction of autophagy as one of the mechanisms responsible of the differential radiosensitivity between cells that express or not the HLA-G1 molecule.

We then analyzed the percentage of apoptotic cells to determine if the differences on survival after irradiation were also due to an enhanced cell death by apoptosis in HLA-G1 expressing cell lines. Two different types of radiation induced apoptosis have been described [23]: some cell types undergo apoptosis quickly after irradiation (2–6 h) before the first post-irradiation mitosis, whereas for other cell systems apoptosis occurs as a consequence of reproductive cell death. In this latter case, apoptotic cells appear at longer times after irradiation (24–96 h). In our experimental model we could observe the second type of cell death: apoptosis at latter times after irradiation (24 and 48 h; Fig. 3A and B). We could not find any differences in the percentage of apoptotic cells between M8-HLA-G1 positive and negative cells (Fig. 3A), whereas with K562 cells we obtained the opposite result: after 24 and 48 h of irradiation, the level of apoptosis in K562-HLA-G1 cells was significantly higher than the obtained in K562-pRc/RSV cells (Fig. 3B).

Another effect of ionizing radiation is the alteration of cell cycle progression, causing arrest in G1, S or G2/M phases. These cell cycle checkpoints provide an opportunity for cells to repair DNA damage before entering the mitotic phase [24]. The G1 arrest is absent in many cell lines, whereas the G2 arrest is seen in virtually all eukaryotic cells, after high or low doses of radiation, and is followed by apoptotic cell death [24]. We analyzed the cell cycle of both cell lines, under control and irradiated conditions. For M8 cells, we could observe a significant arrest in the G2/M phase after 24 h of irradiation with 5 Gy, with a higher percentage of arrest in M8-HLA-G1 cells with respect to M8-pcDNA cells (Table 2). For the K562 erythroleukemia cell line, G2/M arrest occurred but without statistically significant differences between K562-pRc/RSV and K562-HLA-G1 cells (Table 2).

It is well known that tumors from the same histological group and stage of development are extremely heterogeneous in their sensitivity to radiotherapy [25]. There are many factors that could affect the sensitivity of tumoral cells to ionizing radiations, so is not surprising that two different cell lines with different histological origin (M8 and K562 cell lines) do not share the same mechanism to regulate radiosensitivity, as we could observe in the present work.

Previous data from our laboratory have determined that ionizing radiation causes the cleavage of surface HLA-G1 in FON melanoma cells, releasing the soluble form of this molecule (sHLA-G1) to the culture medium [8]. We tested if the same phenomenon was occurring in our experimental system, and we found that gamma irradiation also caused the decrease of surface HLA-G1 in M8 and K562 HLA-G1 expressing cells (Fig. 4A), with the concomitant increase in sHLA-G1 levels in the culture medium (Fig. 4B). It has been shown that sHLA-G cause inhibition of cell proliferation in UT7/EPO and HEL erythroleukemia cells [26] and in Vγ9Vδ2 T cells [27]. In addition, *in vitro* studies indicate that sHLA-G1 induce apoptosis in activated endothelial cells [28] and in activated CD8+ cells [29]. With this background, we wondered whether sHLA-G1 could be involved in the inhibition of the growth rate in irradiated HLA-G1 positive cells by an autocrine signaling process. In order to test this hypothesis, we studied the effect of the incubation of N-IR HLA-G1 expressing cells with the culture medium of N-IR or IR HLA-G1 positive cells. We could not find any significant differences in the final cell number in any cell line, indicating that sHLAG-1 was not responsible for the reduction in the reproductive capacity of irradiated HLA-G1 positive cells. Shedding of HLA-G from the cellular surface of tumoral cells is a common mechanism of tumor dissemination due to its immunosuppressive action by binding to inhibitory receptors present on immune cells [30,31] {Carosella, 2003 #1}. Three HLA-G-recognizing immunoglobulin-like receptors have been identified, including ILT-2, ILT-4 and KIR2DL4 [32–34]. These receptors are differentially expressed by B and T lymphocytes (ILT-2), decidual and peripheral NK cells (KIR2DL4 and ILT-2) and by monocytes/macrophages/dendritic cells (ILT-2 and ILT-4) [31,35].

In conclusion, the results reported in the present study reveal the involvement of HLA-G1 in modulating the radiosensitivity of M8 and K562 cells. This modulation was independent of the dose of radiation and of the cellular origin, although was mediated by different mechanisms for each cell line.

Although further investigations are needed to elucidate why HLA-G1 exerts this regulation on radiation sensitivity, our data could be an important contribution for the development of new therapeutic approaches in anti-tumoral treatments.

Acknowledgments

We want to thank to Dr. Pawlak of the National Atomic Energy Commission for her collaboration in γ-irradiation of the cells, and Dr. LeMaoult by the critical reading of the work. This work was supported by the Commissariat à ÍEnergie Atomique, the Nuclear Regulatory Authority, and the MINCyT-ECOS (Argentine-France) Scientific-Technological Cooperation Program (A07SO2).

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