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Gangliosides protect human melanoma cells from ionizing radiation-induced clonogenic cell death*

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With an experimental model of spontaneous lung metastases of melanoma developed in this laboratory, a range of sublines (variants and clones) with different metastatic potential and ganglioside expression was established from a single human melanoma cell line M4Be. Using an *in vitro* clonogenic assay and provided that cells were cultured for no more than five passages, variations in cellular radioresistance of M4Be and seven sublines derived from M4Be were detected. This study shows a positive correlation between the cell intrinsic radioresistance of M4Be and its seven sublines and their total ganglioside content. More precisely, the proportion of radioresistant cells in M4Be and the seven sublines correlated with the number of cells determined by flow cytometry that were positively labelled with a monoclonal antibody directed to GD3 disialoganglioside. Blocking the cellular biosynthesis of gangliosides with the inhibitor Fumonisin B1 or cleaving with *Vibrio cholerae* neuraminidase the cell surface ganglioside-bound sialic acid in a radioresistant poorly metastatic subline increased its radiosensitivity *in vitro*. In contrast, enrichment of a radiosensitive metastatic subline with exogenous bovine brain GM1 increased its radioresistance *in vitro*. These results suggest that, in the radiation dose range important for radioprotection (0–1 Gy), membrane gangliosides radioprotect human melanoma cells *in vitro*.

Keywords: human melanoma clones, spontaneous metastatic potential *in vivo*, radiosensitivity *in vitro*, gangliosides, sialic acid

Introduction

Human tumours, even small ones, are heterogeneous with regard to biological properties *in vitro* and *in vivo*. For example, large variations in intrinsic radiosensitivity [1–6] and metastatic potential [7, 8] exist among sublines derived from a single parental tumour cell line. These two independent biological variables, that are thought to represent a major obstacle to radiotherapy, have never been correlated (see review in [9]). However, using an experimental model of spontaneous lung metastases of human melanoma in immunosuppressed newborn rats, we have recently found that the higher the metastatic potential *in vivo* of seven clones and variants derived from a single human melanoma cell line (M4Be), the higher their intrinsic cellular radiosensitivity, estimated using a clonogenic assay *in vitro* [10]. Ramakrishnan *et al.* have showed that plasma membranes are sensitive targets in cell death induced by ionizing radiation [11] and other studies have observed that surface glycolipids may be involved in radiosensitivity [12, 13]. These observations prompted us to characterize clones and variants derived from M4Be by their ganglioside expression. The increase in their metastatic potential was found to be associated with a loss of the major melanoma disialoganglioside GD3 together with an increase in lactosylceramide (CDH) [14]. We show here a significant relationship between the intrinsic radioresistance of M4Be and its seven sublines and the ganglioside expression at their cell surface. The

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effect of the modification of the ganglioside status of the melanoma cells on their intrinsic radioresistance was studied.

Materials and methods

CELLS

The selection and characterization of the seven sublines derived from the human melanoma parental cell line M4Be have already been described in detail [10]. They have all been characterized for their ability to give low, intermediate and high numbers of spontaneous metastases in the lungs of immunosuppressed newborn rats (Table 1). The lung metastatic potential was measured using a standardized protocol described previously [15]. Briefly, cells taken from the frozen stock were cultured for at least one passage and, at day 0, Wistar rats not older than 24 h were injected subcutaneously (sc) with 10^6 cells in 0.1 ml PBS in the ventral area, together with an optimal dose of anti-thymocyte serum (ATS) in the dorsum (0.05 ml). The ATS injection was repeated on days 2, 7 and 14. All sublines derived from M4Be, whatever their metastatic potential, produced sc tumours at the injection site. Animals were killed on day 21 and the metastatic potential was determined by direct counting of the pulmonary nodules. Absence of occult metastases in the lungs with no visible metastases was confirmed by histological examination. All experiments were performed according to the national regulations for animal welfare.

EXPRESSION OF THE CELL SURFACE GANGLIOSIDES GD3 AND GM3

Cultures obtained from the M4Be melanoma cell line and its seven sublines were detached with 0.02% EDTA and the expression of gangliosides in 10^6 cells was detected by immunofluorescence using murine monoclonal antibodies (45 min of incubation) and a mouse antibody coupled to fluoroscein (30 min of incubation). Antibody 4F6 (IgG3) was produced after immunization of Balb/C mice with GD3 purified from human melanoma tumours and fusion with SP2 mouse myeloma. As shown by ELISA [16] and TLC-immunostaining [17], the 4F6 antibody reacts with GD3, *O*-acetylGD3 and GT1a, but not with GM3, GD2 and GT3. Antibody GMR6 (IgM) directed to GM3 ganglioside was kindly provided by Dr Tadashi Tai (Tokyo Metropolitan Institute of Medical Science). A flow cytometer FACScan was used to compare the number of fluorescent cells detected in PBS with fluoroscein and no antibody (control) with the number of fluorescent cells detected in presence of the antibody and fluoroscein. Two thousand cells were analysed.

Table 1. Spontaneous lung metastatic potential in immunosuppressed new-born rats, plating efficiencies *in vitro* and radiation cell survival curve parameters of the human melanoma cell line M4Be and seven sublines derived from M4Be. Data were obtained after no more than five passages in culture after cell defrost.

Cells	*Metastatic potential*[a]	*PE*[b] *(± SD) (%)*	$\overline{D}_T$ (Gy)[c]	λ[d] *(95% CI) (%)*	*1-*λ[e] *(%)*
M4Be (parental line)	Low	44 (± 17)	3.17	110 (99–122)	0
Clone 1	Low	42 (± 13)	3.2	109 (88–134)	0
Clone 2	Medium	54 (± 10)	2.63	94 (85–104)	6
Clone 3	Medium	46 (± 4)	2.59	90 (84–96)	10
Subvariant 1−	Medium	40 (± 8)	3.34	96 (90–101)	4
Variant 1	High	33 (± 8)	2.37	82 (73–93)	18
Subvariant 1+	High	34 (± 5)	1.96	67 (61–73)	33
Clone 4	High	25 (± 13)	1.44	60 (47–74)	40

[a]Data from [10].
[b]Plating efficiency *in vitro*.
[c]Mean inactivation dose of the whole population. Data from [10].
[d]Proportion of radioresistant cells and estimated 95% confidence intervals.
[e]Proportion of radiosensitive cells.

GANGLIOSIDE EXTRACTION AND THIN-LAYER CHROMATOGRAPHY (TLC)

Gangliosides were extracted as previously described [18] and separated by HPTLC on aluminium-backed silica gel 60 HPTLC plates using chloroform:methanol:0.2% aqueous $CaCl_2$ (55:45:10) as solvent. Gangliosides, visualized by spraying with orcinol-sulfuric acid and heating at 160 °C, were identified as previously described [14]. Their relative amount was estimated by scanning densitometry at 480 nm on a Chromatoscan CS-930 (Shimadzu, Tokyo). The ganglioside sialic acid content was determined by the periodate-resorcinol method [19]

IRRADIATION AND CELL RADIOSENSITIVITY MEASUREMENT

The method has been published previously [10]. Briefly, radiosensitivity experiments were performed using an *in vitro* colony assay with cells obtained from the frozen stock; cells were not cultured for more than 3 months after defrost, i.e. no more than 15 passages. Cells were cultured as monolayers in plastic flasks (Corning) and maintained at 37 °C in an atmosphere of 5% CO_2 in air. Cells were grown in McCoy 5A medium (GIBCO) supplemented with 10% (v/v) decomplemented foetal calf serum (GIBCO), 1% (v/v) antibiotics (penicillin, streptomycin), 1% (v/v) Hepes 1 M and 1% (v/v) glutamine. Cultures were routinely checked and found free of mycoplasma. In the growth phase, the doubling time of the clones and variants was about 24 h without any apparent relationship with the metastatic ability [20]. Clones 1 and 4, variants 1 and subvariants 1– and 1+ were irradiated with γ rays from a ^{60}Co source that provided a mean dose rate of 58 ± 1 cGy per min to the culture flasks. Clones 2 and 3 as well as the M4Be cell line were irradiated with high energy X-rays produced from a 5 MV linear accelerator that provided a mean dose rate of 4 Gy per min to the culture flasks. Experiments were carried out with early confluent cultures, i.e. at a time where 80% of the cells are blocked in the G1 phase. Cells were detached with 0.05% trypsin, 0.02% EDTA in PBS and single cell suspensions were seeded on 25 cm^2 plastic flasks (Corning) at cell numbers appropriate for colony formation. Two flasks were used for each radiation dose and the number of radiation doses was eleven – varying from 5 cGy to 7 Gy. Twenty-four hours after plating, cultures were irradiated after electronic equilibrium and at room temperature under aerobic condition. At this time, the cell multiplicity (number of cells per potential colony-forming unit) was one. After 2 weeks of incubation without changing the medium in an atmosphere of 5% CO_2 in air, colonies were rinsed with PBS, fixed and stained with a mixture of alcohol 20%:crystal violet (1:1 by vol). Colonies containing more than 50 cells were scored and cell survival curves were established. The surviving fraction S (D) after a radiation dose D was calculated as the plating efficiency (PE) of the irradiated cells over that of the unirradiated cells; PE of the unirradiated cells, which was calculated as the number of colonies counted over the number of cells inoculated at day 0, varied from 25% (clone 4) to 54% (clone 2) (Table 1).

STATISTICAL ANALYSIS OF RADIATION CELL SURVIVAL CURVES

Data obtained with cells cultured for no more than five passages after defrost were adequately fitted with the linear-quadratic model if an extra parameter (λ) was introduced to account for a zero dose extrapolation that was often clearly lower than one. The equation of the model was:

$$S\ (D) = \lambda \exp -(\alpha_R D + \beta_R D^2)$$

where S is the surviving fraction, D is the radiation dose, λ is the proportion of radioresistant cells and α_R, β_R are parameters of the radioresistant cell survival curve. The hypothesis underlying this version of the linear-quadratic model is as follows: two populations of cells with different radiosensitivity coexist in the culture. Although the fraction of radiosensitive cells ($1-\lambda$) could be estimated from the shape of the radiation cell survival curve, its intrinsic radiosensitivity is too high to be properly evaluated by the colony assay. The mean inactivation dose, a parameter equal to the area under the survival curve plotted in linear coordinates, is used as an index of radiosensitivity. It is calculated by the numerical integration of S (D) [21].

CHEMICALS

Fumonisin B1 (obtained from Sigma)

The poorly metastatic and radioresistant cell line M4Be of which the number of cells positively labelled with the monoclonal antibody directed to GD3 is on average 40% was treated with 10 μM Fumonisin B1 for 6 days. After treatment, cells were detached, seeded for colony formation, exposed again to 10 μM Fumonisin B1 and irradiated 24 h later in the presence of the mycotoxin, which was left in the medium for the 15 days colony formation period. Fumonisin B1 alone produced a non-significant reduction of plating efficiency from 37% ± 8% (control) to 28% ± 6%.

Vibrio cholerae neuraminidase (obtained from Sigma)

Poorly metastatic and radioresistant cells obtained from an early confluent clone 1 culture were detached and seeded for colony formation. Twenty-two hours later, *Vibrio cholerae* neuraminidase was added to the culture medium over 2 h at a concentration of 0.05 U per ml of medium and at 37 °C. The ganglioside-bound sialic acid was assayed before and after treatment by the periodate-

resorcinol method [19]. Cells were irradiated in the presence of the enzyme which was not removed during the 15 days colony formation period. Neuraminidase alone produced a non-significant reduction in PE from 45% ± 9% (control) to 35% ± 6%.

Bovine brain monosialoganglioside GM1

The highly metastatic and radiosensitive cells obtained from clone 4 were incubated in a culture medium containing 4 μM GM1 for 3 days. After treatment, cells were detached, seeded for colony formation, exposed again to 4 μM GM1 and irradiated 24 h later in the presence of the ganglioside, which was left in the medium for the 15 days colony formation period. In our experimental conditions, GM1 alone produced a non-significant reduction in PE from 23% ± 9% (control) to 14% ± 12%. Neither the cell cycle distribution nor the cell doubling time *in vitro* was changed by GM1.

Results and discussion

Ionizing radiation cell survival curves vary from one subline to another

The survivial data obtained with sublines derived from the human melanoma cell line M4Be have been published previously [10]. Examination of the parameters of the eight survival curves shows that clones and variants derived from the M4Be parental cell line differ in radiosensitivity (Table 1). Clone 1, subvariant 1– and the M4Be cell line are the most radioresistant cells, clone 4 and subvariant 1+ are the most radiosensitive cells while clones 2 and 3 and variant 1 showed an intermediate response to radiation. This result is comparable to previous observations showing clonal variation in radiosensitivity inside tumour cell lines [1–6]. Of interest is that the radiation cell survival curves obtained from M4Be and the seven sublines derived from M4Be differ essentially in the proportion of radioresistant cells determined from the shape of the survival curves (Table 1).

Ganglioside content and radioresistance are correlated in human melanoma cells

The number of cells positively labelled with the monoclonal antibodies directed to surface disialoganglioside GD3 and monosialoganglioside GM3 in the M4Be melanoma cell line and seven clones and variants derived from M4Be are shown in Table 2 along with the total ganglioside contents. The higher the ganglioside content, the higher the intrinsic cellular radioresistance of M4Be and its seven sublines ($R = 0.78$, $p < 0.05$) (Fig. 1a). More precisely, it was found that the higher the number of cells expressing GD3 at their surface, the higher the proportion of radioresistant cells ($R = 0.97$, $p < 0.001$) (Fig. 1b). The number of cells expressing GM3 at their surface also increases with the proportion of radioresistant cells, although the correlation was not significant in this case ($R = 0.65$, $p > 0.05$). A previous report from Kono *et al.* [12] has shown, using a panel of human melanoma cell lines, that the higher the GM3, the higher the cellular radioresistance *in vitro* – but GD3 was not found to be correlated to radioresistance. This apparent discordance with our results may be due to increased radioresistance

Table 2. Gangliosides expression in the M4Be melanoma cell line and seven sublines derived from M4Be.

Cells	*Total gangliosides*[a]	*GD3 fraction (%)*	*GM3 fraction (%)*	*Number of cells GM3+*[b] (± SEM) *(%)*	*Number of cells GD3+*[b] (± SEM) *(%)*
M4Be (parental)	0.943	38	35	27 (± 10)	40 (± 8)
Clone 1	1.511	35	35	57 (± 20)	41 (± 9)
Clone 2	1.148	33	39	57 (± 13)	28 (± 6)
Clone 3	0.924	32	27	47 (± 16)	20 (± 6)
Subvariant 1–	0.883	34	38	44	28 (± 7)
Variant 1	ND[c]	ND[c]	ND[c]	40 (± 19)	17 (± 4)
Subvariant 1+	0.607	34	40	28 (± 10)	15 (± 1)
Clone 4	0.322	16	70	7 (± 1.5)	2 (± 0.3)

[a]Expressed in μg neuraminic acid per mg protein.
[b]Determined by flow cytometer with monoclonal antibody.
[c]ND, not determined.

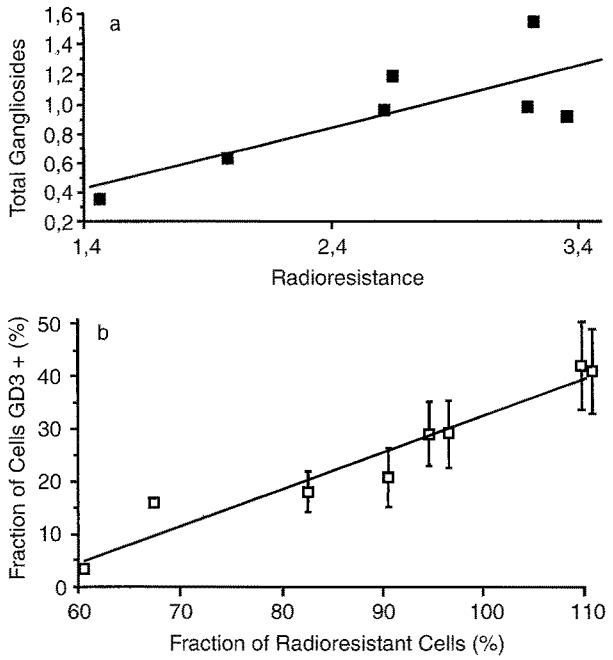

Figure 1. (a) Total gangliosides content (expressed in μg neuraminic acid per mg protein) in the human melanoma cell line M4Be and seven sublines derived from M4Be is correlated to radioresistance estimated by the mean inactivation dose ($R = 0.78$, $p < 0.05$). (b) The proportion of cells positively labelled with the monoclonal antibody directed to the disialoganglioside GD3 is correlated to the proportion of radioresistant cells in the human melanoma cell line M4Be and seven sublines derived from M4Be ($R = 0.97$, $p < 0.001$). Mean and SEM of data from four experiments are represented.

along with culture passages *in vitro* [10]. Indeed, our highly metastatic human melanoma cells increase their radioresistance after less than 10 passages *in vitro* (manuscript submitted), so that at this time radioresistance was not correlated to GD3 content in the sublines derived from the M4Be cell line.

Influence of modifications of the ganglioside content of human melanoma cells on their radioresistance

These results suggest that gangliosides contribute to the radioresistance of human melanoma cells *in vitro*. This hypothesis was tested by blocking, at the time of irradiation, the biosynthesis of gangliosides with the inhibitor of ceramide biosynthesis Fumonisin B1, a mycotoxin produced from *Fusarium moniliforme* [22]. After treatment with 10 μM Fumonisin B1 for 6 days, human melanoma cells M4Be do not express GD3 at their surface (Fig. 2). This effect is transient since, in pilot experiments, it was found that 24 h after removal of Fumonisin B1 from the medium, cells begin to synthesize GD3 again (data not shown). The influence of Fumonisin B1 on the intrinsic radioresistance of the M4Be cell line is shown in Fig. 3. The radiation cell survival curves differ essentially by the radioresistant cell fraction (λ) which is

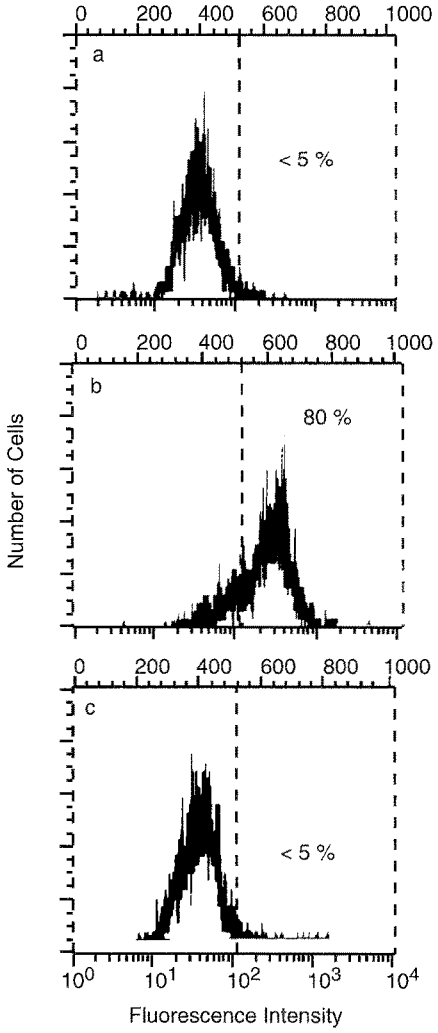

Figure 2. Number of cells positively labelled with the monoclonal antibody against the disialoganglioside GD3 in the M4Be cell line (b) and in M4Be cells treated with Fumonisin B1 at the time of irradiation (c). The number of cells positive was determined from the fluorescence intensity detected at the right of the threshold that was fixed on the X axis (dashed line). The background level of fluorescence is given in panel (a).

102% (range 91–114%) for control cells and 84% (range 80–88%) [95% confidence interval (CI)] for cells treated with Fumonisin B1. The mean inactivation dose is 2.75 Gy for control cells and 2.04 Gy for treated cells, indicating that Fumonisin B1 reduces the intrinsic radioresistance of the M4Be cell line. Therefore, since Fumonisin B1 prevents gangliosides biosynthesis, data from Figs 1–3 suggest that gangliosides (particularly GD3) are involved in the intrinsic radioresistance of melanoma cells.

This possibility was further investigated by treating clone 1 in culture with neuraminidase from *Vibrio cholerae* 2 h before irradiation. The treatment released more than 50% of ganglioside-bound sialic acid from these cells. The influence of neuraminidase on the

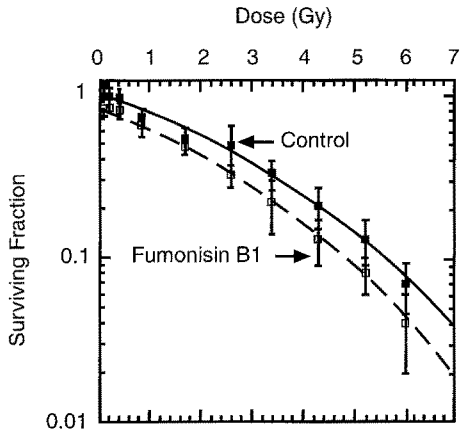

Figure 3. Effect of Fumonisin B1 on the intrinsic cellular radioresistance of the human melanoma cell line M4Be. Mean and standard deviation from three experiments are represented for control cells (■) and treated cells (□). Data were adequately fitted with the linear-quadratic model if an extra parameter (λ) is introduced to account for a zero dose extrapolation which was significantly lower than 100% after treatment (see Materials and methods).

intrinsic radioresistance of clone 1 is shown in Fig. 4. Clone 1 is a subline derived from the human melanoma cell line M4Be that has a biological behaviour similar to M4Be with regards to radioresistance, metastatic potential and gangliosides expression [10, 14]. The survival curves differ only by the radioresistant cell fraction (λ) which is 107% (96–119%) for control cells and 80% (64–101%) (95% CI) for cells treated with neuraminidase. The mean inactivation dose is 2.87 Gy for control cells and 2 Gy for neuraminidase-treated cells. This result which is almost

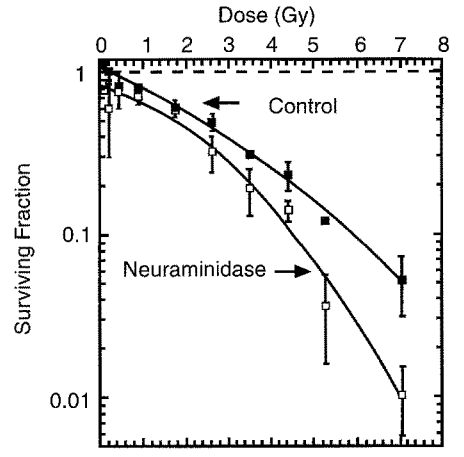

Figure 4. Effect of neuraminidase from *Vibrio cholerae* on the intrinsic cellular radioresistance of clone 1. Mean and standard deviation from two experiments are represented for control cells (■) and treated cells (□). Data were adequately fitted with the linear-quadratic model if an extra parameter (λ) is introduced to account for a zero dose extrapolation which was significantly lower than 100% after treatment (see Materials and methods).

the same as that observed after Fumonisin B1 treatment suggests that sialic acids from gangliosides are involved in the radioresistance of human melanoma cells *in vitro*.

The hypothesis that gangliosides can protect from ionizing radiation-induced clonogenic cell death in melanoma cells was further tested by adding exogenous gangliosides to the medium prior to and during irradiation. Pilot experiments have shown that 4 μM of bovine brain monosialogangliosides GM1 added to the culture medium 4 days prior to and during irradiation produced optimal radioprotection *in vitro* of cells obtained from clone 4 (Fig. 5a). The influence of the time of incubation of GM1 (4 μM) prior to irradiation on the radioresistance of clone 4 is shown in Fig. 5b. GM1 was added to the culture medium for 5, 24, 48, 72 or 96 h; after GM1 treatment, cells were detached, seeded in the medium without GM1 and irradiated 24 h later. No radioprotection occurred at 5 and 24 h. The maximal radioprotective effect occurred when GM1 was present in the culture medium for 96 h but a significant increase in radioresistance was also observed at 48 and 72 h. Similar results were obtained when GM1 was added every 3 days during the 15 days colony formation period but when GM1 was given immediately after irradiation, no radioprotection *in vitro* occurred (results not shown). The influence of GM1 on the intrinsic cellular radiosensitivity of clone 4 in the dose range 5 cGy–7 Gy is shown in Fig. 5c. Clone 4 is characterized by high metastatic potential and high intrinsic radiosensitivity [10] and a deficiency in surface gangliosides [14]. The radiation cell survival curves differ only by the radioresistant cell fraction (λ) which is 70% (65–76%) for control cells and 115% (107–124%) (95% CI) for cells treated with GM1. The values above 100% for the proportion of radioresistant cells may be due to an increase in cloning efficiency after GM1 treatment and irradiation. The mean inactivation dose is 2.05 Gy for control cells and 3.03 Gy for GM1 treated cells. In contrast, the intrinsic cellular radioresistance of clone 1 remained unchanged after similar GM1 pretreatment (results not shown). Thus, exogenous GM1 given at low concentration *in vitro* (4 μM) protected only radiosensitive melanoma cells against cell death induced by ionizing radiation. Comparable radioprotection occurred with exogenous bovine brain disialoganglioside GD1b (1.5 μM), trisialoganglioside GT1b (0.7 μM) added to the medium 4 days before irradiation of radiosensitive clone 4, but no protective effect was seen using asialoGM1 at 4 μM. When human melanoma GD3 disialoganglioside was incubated with the cells, the radioprotective effect seen with a high concentration of GD3 (up to 17 μM) was lower than that obtained with 4 μM GM1, although more than 90% of cells became GD3-positive as monitored by flow cytometry (results not shown). Thus the radioprotective effect of gangliosides is dose-dependent.

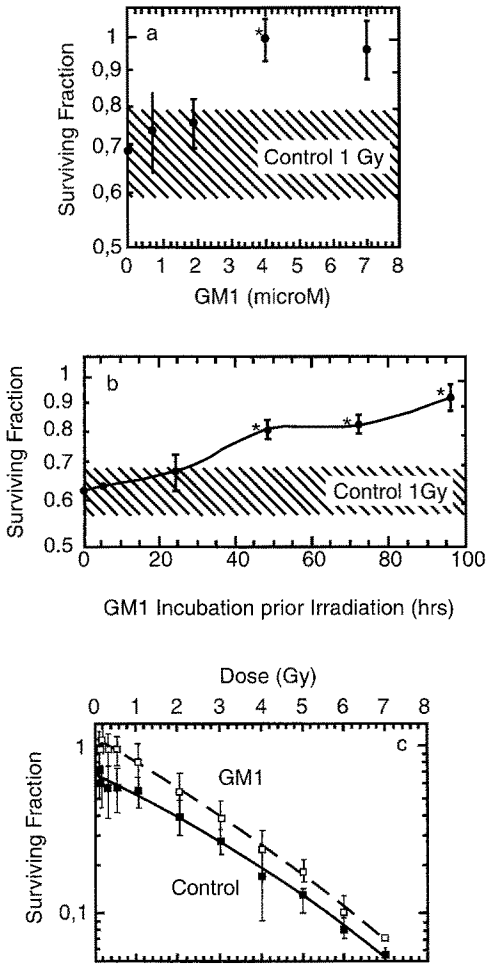

Figure 5. (a) Effect of the concentration of ganglioside GM1 on the radiosensitivity *in vitro* of cells obtained from clone 4. GM1 was added to medium 4 days prior to and during irradiation. (b) Effect of the time of incubation of 4 μM GM1 prior irradiation on the radiosensitivity *in vitro* of cells from clone 4. (c) Radiation cell survival curves of clone 4 treated with 4 μM GM1 4 days prior to and during irradiation. Mean and standard deviation from two experiments are represented for control cells (■) and treated cells (□). Data were adequately fitted with the linear-quadratic model if an extra parameter (λ) is introduced to account for a zero dose extrapolation which was significantly lower than 100% in control cells (see Materials and methods). (*) $p < 0.05$ compared to control (*t*-test).

In summary, our data show evidence that gangliosides protect from ionizing radiation-induced clonogenic death of human melanoma cells *in vitro* and that sialic acid residues of cell surface gangliosides are involved. In addition, gangliosides prevent cell death induced by glutamate [23], TNFα [24] and serum deprivation [25]. How gangliosides prevent cell death remains unknown but their bifunctional role as regulators of cell proliferation and receptors for cellular interactions may be involved [26]. One of the first events in the protection mechanism may involve an increase in cell attachment, possibly mediated by the electrostatic environment due to the negatively charged sialic acid residues of cell surface gangliosides [27]. The fact that plating efficiency *in vitro* is correlated to GD3 content in our melanoma cells ($R = 0.73$. $p < 0.05$) but not to GM3 content ($R = 0.58$, $p > 0.05$) and their radioresistance was measured with a colony assay would support this hypothesis.

Gangliosides are shed from tumour cells in the serum [28]. Radiosensitive highly metastatic cells escaping from primary tumours could become radioresistant again once they incorporate at their surface gangliosides shed from tumours cells. Radiosensitivity of metastatic cells *in vivo* may be a dynamic process that depends on the presence of tumour gangliosides in the serum. This may be true for metastatic potential as we show that highly metastatic radiosensitive cells with low GD3 treated with exogenous GM1 (4 μM for 4 days) are radioresistant (this study) and non-metastatic compared with control cells (manuscript submitted). On the other hand, modifying permanently (and not transiently as in this study) the GD3 content of a radioresistant poorly metastatic cell line to increase its metastatic potential would strengthen our hypothesis.

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