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Ionizing radiations increase the activity of the cell surface glycohydrolases and the plasma membrane ceramide content

Massimo Aureli · Rosaria Bassi · Alessandro Prinetti · Elena Chiricozzi · Brigida Pappalardi · Vanna Chigorno · Nadia Di Muzio · Nicoletta Loberto · Sandro Sonnino

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Abstract We detected significant levels of β-glucosidase, β-galactosidase, sialidase Neu3 and sphingomyelinase activities associated with the plasma membrane of fibroblasts from normal and Niemann-Pick subjects and of cells from breast, ovary, colon and neuroblastoma tumors in culture. All of the cells subjected to ionizing radiations showed an increase of the activity of plasma membrane β-glucosidase, β-galactosidase and sialidase Neu3, in addition of the well known increase of activity of plasma membrane sphingomyelinase, under similar conditions. Human breast cancer cell line T47D was studied in detail. In these cells the increase of activity of β-glucosidase and β-galactosidase was parallel to the increase of irradiation dose up to 60 Gy and continued with time, at least up to 72 h from irradiation. β-glucosidase increased up to 17 times and β-galactosidase up to 40 times with respect to control. Sialidase Neu3 and sphingomyelinase increased about 2 times at a dose of 20 Gy but no further significant differences were observed with increase of radiation dose and time. After irradiation, we observed a reduction of cell proliferation, an increase of apoptotic cell death and an increase of plasma membrane ceramide up to 3 times, with respect to control cells. Tritiated GM3 ganglioside has been administered to T47D cells under conditions that prevented the lysosomal catabolism. GM3 became component of the plasma membranes and was transformed into LacCer, GlcCer and ceramide. The quantity of ceramide produced in irradiated cells was about two times that of control cells.

Keywords Ceramide · Glycosidases · Plasma membrane · Apoptosis · Radiations · Radiotherapy

M. Aureli · R. Bassi · A. Prinetti · E. Chiricozzi · V. Chigorno · N. Loberto · S. Sonnino
Department of Medical Chemistry, Biochemistry and Biotechnology, University of Milan,
Milan, Italy

B. Pappalardi · N. Di Muzio
Department of Radiotherapy, Hospital San Raffaele,
Milan, Italy

S. Sonnino (✉)
Dipartimento di Chimica, Biochimica e Biotecnologie per la Medicina, Università degli Studi di Milano,
Via Fratelli Cervi 93,
20090 Segrate, Italy
e-mail: sandro.sonnino@unimi.it

Introduction

Cancer is usually characterized by aberrant glycan expression ("aberrant glycosylation") at the cell surface [1–3]. This event contributes to the deep alterations in the interactions between tumor cells and the extracellular environment, which underlie many of the features of the tumor phenotype, including self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion from programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Aberrant glycosylation in cancer is usually regarded as the consequence of alterations in the metabolic machinery of glycoconjugates [4, 5]. The metabolic pathway of glycoconjugates is very complex and the final glycosphingolipid and glycoprotein plasma membrane (PM) composition is the result of several processes comprising biosynthesis, catabolism and intracellular trafficking [6]. In addition to these events, changes of the glycoconjugate oligosaccharide structures, in particular changes of glycosphingolipids, can be obtained at the cell surface by the activities of PM-associated glycosidases [7, 8]. Moreover changes of expression/activity of these enzymes seem to have specific roles in signaling processes [9, 10] involved in the control of cell

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proliferation and survival. In the last years many studies confirmed that several glycosidases, like sialidase Neu3, β-hexosaminidase, β-galactosidase and β-glucosidase, working on the oligosaccharide chain of glycosphingolipids, and in some cases on the oligosaccharides linked to proteins, are associated with the external leaflet of the plasma membrane, where they display both *cis* and *trans* activity, being capable to act as well on surface components of the neighboring cells [11–13]. In particular, a specific role for sialidase Neu3 has been proposed in intestinal and prostatic tumor cells resistant to therapy. These cells express higher quantity of Neu3 with respect to those belonging to the tissue around the tumor. Neu3 hydrolyzes the PM ganglioside GM3, maintaining high PM lactosylceramide level. Lactosylceramide has been shown to act as antiapoptotic compound and this is believed to be a system devised by the tumor to escape apoptosis and to proliferate [14, 15].

Radiotherapy represents the therapeutic strategy for the treatment of an increasing number of tumors. Thanks to the application of conformal/intensity-modulated techniques in combination with precise image-guided localization of the target, radiotherapy dose escalation has been shown to improve disease control [16, 17]. Therefore, it should be possible to use a larger dose per fraction without increasing the risk of subsequent serious injury to the normal tissue [18, 19]. Irradiation of cancer cells leads to cell death by different mechanisms such as direct DNA and mitochondrial damage, superoxide production and ceramide-induced apoptosis [20]. Sphingomyelin is one of the main sphingolipid components of plasma membranes, and the production of pro-apoptotic ceramide by sphingomyelin hydrolysis due to the activation of sphingomyelinase has been reported to participate to the tumor reduction upon treatment with ionizing radiations [21]. The ceramide produced by sphingomyelin hydrolysis forms large ceramide-enriched membrane platforms, instrumental for the operation and modulation of cell signaling processes. Several sphingomyelinases belonging to different subcellular fractions have been described [22, 23], but a specific role of PM sphingomyelinase has been reported in the above described apoptotic process [24].

So far, an effect of ionizing radiations on the PM activities of glycosidases has never been reported, however some data in the literature suggested that these enzymes could contribute to the formation of pro-apoptotic ceramide under some circumstances. It has been demonstrated that in human fibroblasts the over-expression of Neu3 causes the onset of apoptosis due to an increase in the PM ceramide production by a parallel increase of expression/activity of β-galactosidase and β-glucosidase as cellular response [10, 11]. Activation of glycosidases by ionizing radiations could change the ratio between the apoptotic ceramide and the antiapoptotic/proliferative lactosylceramide. This could explain why some tumors are more responsive to irradiation than others.

In this manuscript we present the first detailed information on the activation of several cell surface glycosidases in normal and pathological cells subjected to irradiation, an event accompanied by the production of plasma membrane ceramide and the onset of apoptotic cell death.

Materials and methods

Commercial chemicals

Commercial chemicals were of the highest purity available, common solvents were distilled before use and water was doubly distilled in a glass apparatus. Trypsin, KCl, Trypan blue, reagents for cell culture were from Sigma Chemical Co. (St Louis, MO, USA). MEM, DMEM and RPMI-1640 as well as fetal calf serum (FCS) were purchased from EuroClone (Leeds, UK); AM-calcein and DMEM-F12 were from Invitrogen (Carlsbad, CA, USA). 6-hexadecanoylamino-4-methylumbelliferone (H-MUB) 4-methylumbelliferone (MUB), 4-methylumbelliferyl-β-D-galactopyranoside (MUB-Gal) and 4-methylumbelliferyl-β-D-glucopyranoside (MUB-Glc) were from Glycosynth (Warrington, UK).

6-hexadecanoylamino-4-methylumbelliferyl-phosphorylcholine (H-MUB-PC) was from Moscerdam Substrates (Oegstgeest, Netherland). Hoechst 33258 (2′-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5′-bi-1H-benzimidazole trihydrochloride trihydrate) was from Thermo Scientific (Rockford, IL, USA). High-performance silica gel thin-layer plates (HPTLC Kieselgel 60) were from Merck GmbH (Milan, Italy).

The preparation of isotopically labeled [3-^{3}H(sphingosine)]GM3 (specific radioactivity, 2.3 Ci/mmole) and of [1-^{3}H]sphingosine (specific radioactivity, 2.2 Ci/mmole) has been described in detail [25].

Human normal and pathological fibroblasts were prepared as described [26]. Pathological cells were prepared from a 7 year old Niemann-Pick type A patient (NPD). These cells had an acidic sphingomyelinase activity on the artificial substrate H-MUB-PC lower than 9 % of the activity present in normal human fibroblasts and a six fold increase of sphingomyelin content [27]. Tumor cell line T47D from breast tumor, HT-29 and Caco-2 from colon carcinoma, SHSY-5Y from neuroblastoma tumor; A2780, A2780/HPR and A2780/Cis from ovary tumor [28–30] were available in the laboratory.

Cell cultures

Human fibroblasts and Caco-2 were cultured and propagated in MEM; SHSY-5Y and HT-29 in DMEM; A2780 and T47D in RPMI-1640; A2780/HPR and A2780/Cis in RPMI-1640 in presence of 5 μM HPR (*N*-(4-hydroxyphenyl)

retinamide) and 1 μM cisplatin, respectively [31, 32]. All of the cultures medium were supplemented with 10 % FCS (heat-inactivated for all cell lines except fibroblasts), 1 % glutamine, 1 % penicillin/streptomycin. The cells were cultivated as monolayer in a humidified atmosphere at 37°C and 5 % CO_2.

Irradiation of cells

The most widely used technique for mathematically modeling the effect of radiation on cancer cell survival, is linear quadratic (LQ) formalism. The core formula involved is an expression, which correlates the probability of survival, S, of a population of mammalian cells following exposure to an individual radiation dose, d, as follows:

$$S = \exp[-\alpha D - \beta D2]$$

where α and β are tumor- or normal tissue-specific parameters. Rearrangement of the equation to account for the total impact of n individual fractions of radiation exposure yields another common expression for the biologically effective dose (BED):

$$BED = nd(1 + /\alpha/\beta)$$

The units of BED are Gray (Gy), the standard unit of radiation dose. The BED is a convenient metric for comparing the relative impact of a given schedule of radiation dose on a given tumor or tissue, as long as the α/β ratio is known or closely estimated. The usual value for tumors is approximately 10 Gy, but intraoperative methods for delivering hypofractionated partial breast irradiation suggested that single doses of up to 21 Gy were tolerated for small tumors (≤2.5 cm) with excellent results regarding tumor control. In this study, preliminary experiments allowed to increase this dose without a very acute toxicity effect on the mammalian cells irradiated. We irradiated the cell lines with 6 Mev photons (direct field) with a linear accelerator. We utilized a dose escalation scheme delivering four doses of 10 Gy, 20 Gy, 40 Gy and 60 Gy, with enzymatic activity evaluated after every fraction (from 10 min to 96 h after irradiation). The same enzymatic analysis was performed simultaneously on non-irradiated cells.

Determination of cell viability and apoptosis

At the end of the enzyme assays and at different times after irradiation, cell viability was assessed by calcein staining and by Trypan blue exclusion [33], whereas cell apoptosis was determined by Hoechst 33258 fluorescent staining. For the calcein staining, cells were washed with PBS, the plate was then put on ice and 200 μl of a calcein-AM solution (6.25 μg/ml in PBS) were added to each well. Cells were incubated for 15 min at 37°C, 5 % CO_2. Calcein-AM was then removed and 100 μl of PBS and 50 μl of 1 % Triton X-100 were added to each well. Plate was stirred at RT for 15 min and then the fluorescence was detected by a microplate reader (Victor, Perkin-Elmer).

The number of living and dead cells has been determined by counting cells after Trypan blue staining, as previously described [34].

For the detections of apoptosis, control and irradiated cells were stained with a PBS solution containing 10 μg/ml of Hoechst 33258 at room temperature for 10 min in the dark and then cells were washed three times with PBS and analyzed by fluorescence microscope. Due to the chemical properties of the Hoechst 33258 in the conditions mentioned above, only the apoptotic cells show the nuclear staining [35]. This was confirmed by the evaluation of the changes of nuclear chromatin morphology. A minimum of 700 cells were scored for the incidence of chromatin apoptotic changes (condensation, compaction along the periphery of the nucleus and segmentation) as previously described [21].

Plasma Membrane (PM) associated hydrolase assays

We recently described a simple method [33], that allows to measure the activity of several glycohydrolases associated with the plasma membranes of intact living cells. This method is based on the observation that the fluorogenic substrates commonly used for the *in vitro* assay of glycohydrolase activity (*i.e.*, MUB-Glc, MUB-Gal) are not taken up by living cells [36]. In fact, under the appropriate experimental conditions, we did not observe any fluorescence associated with the cells. Moreover, the artificial substrates were not subjected to spontaneous, non-enzymatic hydrolysis nor hydrolyzed in observable entity by secreted enzymes. Thus, their hydrolysis under these experimental conditions is due exclusively to the plasma membrane associated enzymatic activities [8, 33].

PM associated β-galactosidase (β-Gal-ase), β-glucosidase (β-Glc-ase) and sphingomyelinase (SM-ase) activities were determined in control or irradiated living cells plated in 96-well microplate at the density of $33X10^3$ cells/cm^2 by a high throughput assay (HTA). The β-Gal-ase and β-Glc-ase activities were assayed using the artificial substrates MUB-Gal and MUB-Glc solubilized in DMEM-F12 without phenol red at pH 6 at the final concentrations of 250 μM and 6 mM respectively. The SM-ase activity was assayed using the artificial substrate H-MUB-PC solubilized in the same DMEM-F12 reported for the other enzymes at the final concentration of 100 μM. At different times (from 30 min to 2 h) aliquots of the medium were analyzed by fluorimetry in a microplate reader (MUB: λ_{ex}: 355 nm /λ_{em}: 460 nm ; H-MUB: λ_{ex}:405 nm /λ_{em}:460 nm) after adding 15 volumes of 0.25 M glycine (containing 0.3 % Triton X-

100 for SM-ase assay), pH 10.7. MUB and H-MUB were used to establish the calibration curves in order to quantify the substrates hydrolysis.

Control experiments were performed also in irradiated cells in order to exclude any activity released from lysosomes and/or from other intracellular sites. To this purpose, each enzymatic assay was performed on aliquots of medium previously maintained in contact with cells for different times. Moreover, to exclude any intracellular enzymatic degradation of the fluorigenic substrates, the absence of fluorescence associated with the cells was evaluated at the end of each enzymatic assay by fluorescent microscopy and by fluorimetric analysis of the lysed cells in 0.25 M glycine (pH 10.7) [11, 33].

Activity of plasma membrane sialidase Neu3 was determined on the total homogenate using 6 μM [3-^{3}H(*sphingosine*)]GM3 as a substrate in the presence of detergent Triton X-100 [10]. This experimental condition strongly reduced the activity of the other sialidases [37]. The enzymatic reaction was stopped by adding chloroform/ methanol (2:1 by vol) and the reaction mixture was analyzed by HPTLC using the solvent system chloroform/methanol/0.2 % aqueous $CaCl_2$, 55:45:10 by vol. After chromatography, the separated radioactive lipids were detected and quantified by radioactivity imaging performed with a Beta-Imager 2000 instrument (Biospace, Paris, France), and the radioactivity associated with individual lipids was determined with the specific β-Vision software provided by Biospace.

Cell treatments with tritiated lipids

Treatment of cell cultures with [1-^{3}H]sphingosine. [1-^{3}H] sphingosine was administered to 20, 40 and 60 Gy irradiated and control T47D cells in order to metabolically label all sphingolipids as previously described [38]. [1-^{3}H]sphingosine dissolved in methanol was transferred into a sterile glass tube, and dried under a nitrogen stream; the residue was then solubilized in an appropriate volume of pre-warmed (37°C) cell medium to obtain the desired final concentration (3×10^{-9} M). After 2 h incubation (pulse), the medium was removed, cells were washed and incubated up to 72 h (chase) with cell medium not containing radioactive precursor. After chase the cells were collected and radioactive lipids were analyzed as described below.

Treatment of cell cultures with [3-^{3}H(sphingosine)]GM3. Isotopically labeled [3-^{3}H(*sphingosine*)]GM3 was administered to both T47D control and irradiated cells (40 Gy dose) after 24, 48 and 72 h from irradiation. [3-^{3}H(*sphingosine*)] GM3 dissolved in propan-1-ol/water, 7:3 (v/v), was transferred into a sterile tube and dried under a nitrogen stream. The residue was solubilised in the cell culture medium without serum at the final concentration of 4.5×10^{-6} M. In order to follow both the lysosomal and PM metabolism of the gangliosides a part of the cells were pre-incubated with 50 μM chloroquine for 30 min in complete cell culture medium in agreement with previously reported protocol [10]. After removal of the medium and rapid washing of cells, 1 ml of the medium containing the radioactive lipid were added to each dish and the cells were incubated at 37° C in the presence or not of 50 μM chloroquine for 4 h. At the end of incubation, cells were washed four times with complete cell culture medium and then collected with PBS.

Radioactive lipid analyses

Cells were lyophilized and subjected to lipid extraction and sphingolipid analysis. Total lipids from lyophilised cells were extracted with chloroform/methanol/water 20:10:1 by vol. followed by chloroform/methanol 2:1 by vol. The total lipid

Table 1 Specific cell surface enzymes activity in non irradiated tumor cell lines. Each value is the mean of three independent experiments performed in five-fold replicate ± SD. β-Glucosidase, β-galactosidase and sphingomyelinase activities were determined in living cells on artificial substrates. Sialidase Neu3 activity was determined in the cell homogenate on ganglioside GM3. Sphingomyelinase activity was determined at pH 6 and is associated to both neutral and acidic activities. The activity of sphingomyelinase in Niemann-Pick type A (NPA) fibroblasts was too low to be statistically analysed

Cells	β-glucosidase pmoles/10^6 cells/hour	β-galactosidase pmoles/10^6 cells/hour	Neu3 pmoles/mg cell protein/hour	sphingomyelinase pmoles/10^6 cells/hour
Normal fibroblasts	8,598±342	776±87	289±9	1,100±200
NPA fibroblasts	7,645±678	634±35	198±21	/
T47D	731±120	447±53	57±4	423±51
HT-29	424±60	1,027±181	74±10	115±37
Caco-2	1,621±206	3,133±405	3,203±324	690±79
SHSY-5Y	8,090±327	6,090±567	96±12	334±15
A2780	462±90	240±50	123±16	294±21
A2780/HPR	327±35	86±12	65±11	170±14
A2780/cis	127±16	68±9	190±5	115±30

extracts were subjected to a two-phase partitioning by adding 20 % water to the lipid extract; the total lipid extract, the aqueous and organic phases were analysed by HPTLC (1000 dpm/lane). [^{3}H]sphingolipids of total extracts and organic phases were separated using the solvent system chloroform-methanol–water 110:40:6 by vol, and those of aqueous phases with chloroform-methanol-0.2 % aqueous $CaCl_2$, 50:42:11 by vol. For the specific determination of the ceramide content the radioactive lipids of the organic phases were separated using the solvent system hexane-chloroform-acetone-acetic acid 20:70:20:2 by vol. [^{3}H]sphingolipids were identified by referring to the position of standards in the

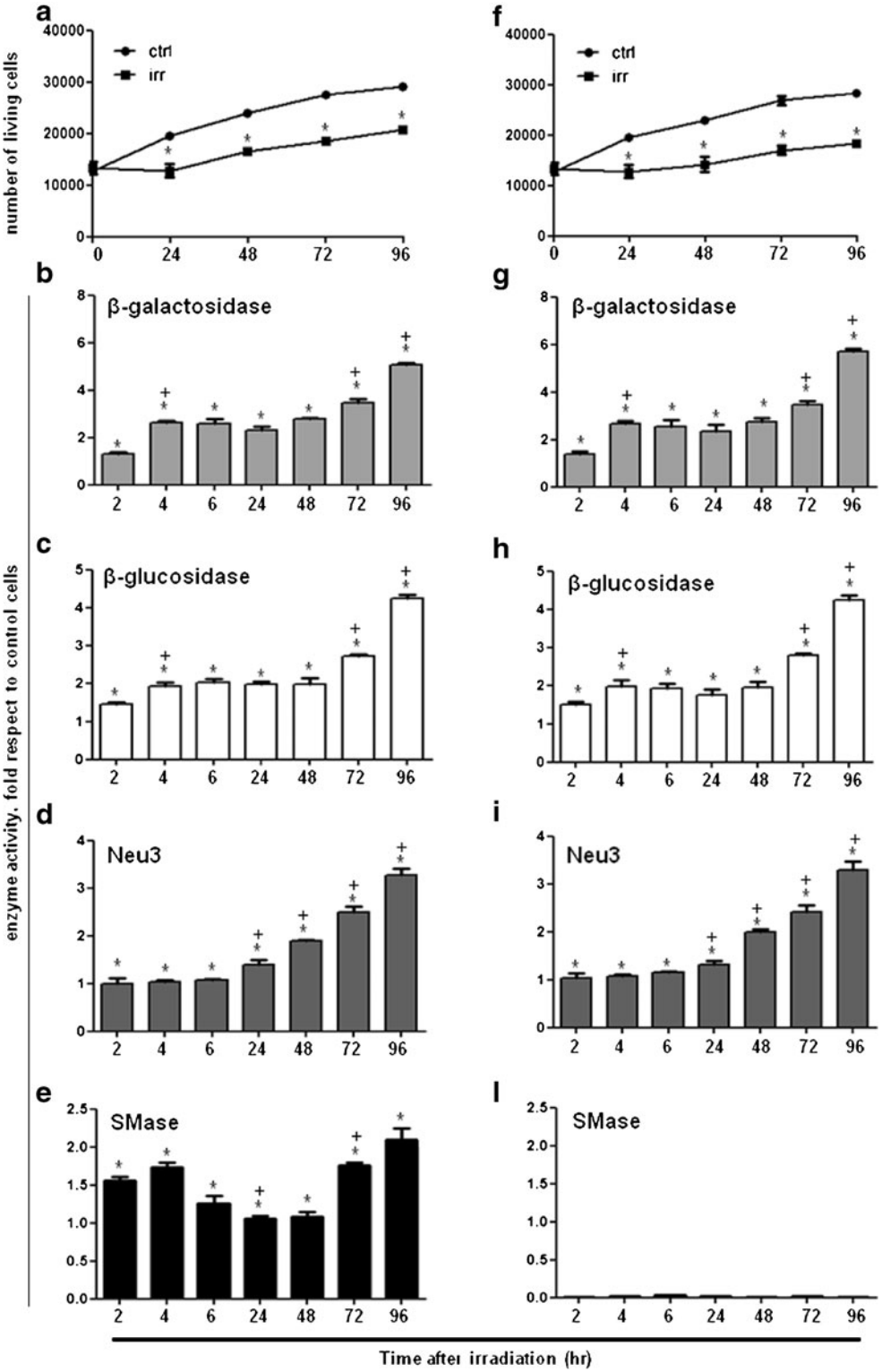

Fig. 1 Effect of ionizing radiations on human fibroblasts derived from a normal subject and from a patient affected by Niemann-Pick type A disease. Effect of a dose of 10 Gy on the growth of fibroblasts in culture and on their cell surface associated hydrolase activities. Panels a, b, c, d and e: normal human skin fibroblasts prepared from a young subject (features of this cell line have been reported in several previous papers [11, 27, 54–56]; f, g, h, i and l, fibroblasts from a Niemann-Pick type A subject with total residual sphingomelinase activity of 9 % (features of this cell line have been reported previously [27]). The number of living cells was determined by both trypan-blue exclusion assay and Calcein-AM assay reported data are those obtained with Calcein-AM that are confirmed by trypan-blue exclusion. Plasma membrane β-galactosidase, β-glucosidase, and sphingomyelinase activities were determined on living cells using artificial substrates. Sialidase Neu3 was determined on the cell homogenate using GM3 ganglioside as substrate. Cell growth and enzyme activity were determined up to 96 h from cells irradiation. Enzyme activity of cell subjected to irradiation is expressed as increase of activity with respect of non irradiated cells. Basal activities of plasma membranes enzymes are reported in Table 1. Data represent the mean ± SD, n=5 independent experiments, 2–3 replicates for each experiment. Two Way ANOVA followed by Bonferroni post-tests *p<0.05 versus control cells, + p<0.01 vs precedent time

chromatogram and quantified by radioimaging after 48–96 h of acquisition (β-Imager 2000, Biospace, Paris, France).

Other analytical methods

The radioactivity associated with lipid extracts was determined by liquid scintillation counting.

The protein assays were carried out using the DC Protein Assay kit (Biorad).

Statistics

All the experiments have been performed in fivefold and repeated three times. Data are presented as the mean values ± standard deviations and were tested for significance employing ONE or Two way ANOVA with Bonferroni post-test analysis, as specified in figure legend. The level of significance was set at $p<0.05$.

Results

Nine cell lines (human skin fibroblasts from normal subject, fibroblasts from a Niemann-Pick type A subject, T47D cells from breast cancer, HT29 and CaCO2 from colon carcinoma, SHSY-5Y from neuroblastoma tumor; A2780, A2780/HPR and A2780/Cis from ovary tumor) were analyzed for the presence of β-glucosidase, β-galactosidase, sialidase Neu3, and sphingomyelinase activities associated with the PM. The plasma membrane enzyme activities were determined in living cells under conditions that excluded contamination from intracellular activities. All of the tested cells showed a characteristic PM activity pattern as reported in Table 1. This confirms our previous data on the association of glycohydrolases activity to the plasma membrane [7, 11, 33], increases the number of cell lines presenting these activities and suggests that probably all of the mammalian cells display these activities at the plasma membrane level.

Fibroblasts derived from normal subjects and from a patient affected by a Niemann-Pick type A disease were exposed to a radiation dose of 10 Gy. As shown in Fig. 1, the normal fibroblasts submitted to ionizing irradiation underwent a growth arrest at the first day after irradiation and were characterized by a progressively lower growth rate with respect to control cells. After 4 days in culture, the number of living cells was reduced by about 30 %, in comparison with control cells. The Hoechst staining assay showed DNA condensation and fragmentation, suggesting that cells entered into apoptosis. We determined that the apoptotic onset was 20-25 % in treated cells *vs*. 5-7 % in control cells. Similar results were obtained on fibroblasts prepared from a Niemann-Pick type A patient that displayed very low residual activity of sphingomyelinase (9 % of residual activity). Also in these cells we observed a reduction of living cells and an increase in apoptosis, from 7 % to 25 % with respect to non-irradiated NPD fibroblasts.

Treated cells, both normal and NPD fibroblasts, were analyzed for the PM activity of the three glycohydrolases β-Glc-ase, β-Gal-ase and sialidase Neu3. The enzyme activities increased upon cell irradiation. The activity of glycosidases doubled rapidly after irradiation and then progressively increased up to 96 h. As internal control, we analyzed the activity of PM SM-ase, that it is known to be activated by ionizing radiations. In normal fibroblasts PM SM-ase activity rapidly increased following irradiation but then remained almost constant, while we could not verify any activity change in Niemann-Pick cells.

Afterwards we analyzed several tumor cell lines to assess the effect of irradiation on the PM activities of the four above mentioned enzymes. As reported in Table 1, all of the analyzed cell lines showed a basal activity of the four PM associated hydrolases, and we observed a general increase (even if at different level for each cell line tested) at 24 h after 20 Gy dose irradiation, with a significant difference for each cell line considered (Fig. 2) with respect to non-irradiated cells. Afterwards we concentrated our study on the T47D cell line derived from human ductal breast cancer, a tumor that is often treated with radiotherapy.

Cells were irradiated up to 60 Gy and microscopically analyzed by Trypan-blue exclusion assay or by calcein

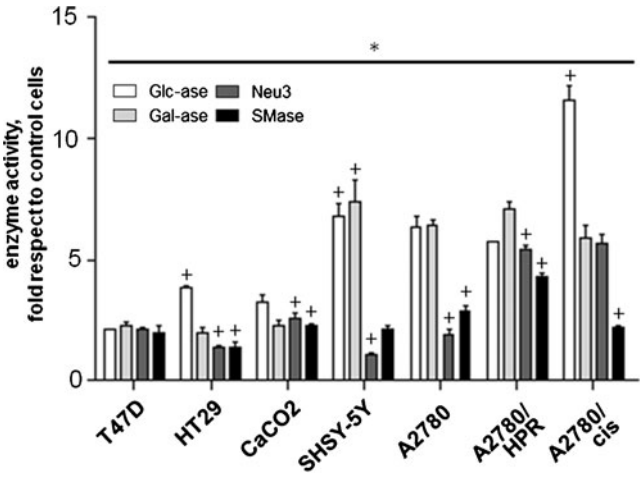

Fig. 2 Effect of ionizing radiations on cell PM associated activity in tumor cells. Effect of a dose of 20 Gy on the enzymes β-glucosidase, β-galactosidase, sialidase Neu3 and sphingomyelinase associated to the cell surface of breast tumor line T47D, colon carcinoma cell lines HT-29 and Caco-2, neuroblastoma cell line SHSY-5Y and ovary tumor cell lines A2780, A2780/HPR and A2780/cis. Enzyme activity associated to the plasma membranes was determined 24 h from cell irradiation and is expressed as increase of activity with respect to non irradiated cells (control cells). Basal enzyme activities are reported in Table 1. Data represent the mean ± SD, $n=3$ independent experiments, 3 replicates for each experiment. Two Way ANOVA followed by Bonferroni post-tests *$p<0.0001$ versus control cells, + $p<0.001$ vs the same enzyme in the previous cell line

staining. Figure 3 shows that the number of living cells progressively decreased along 96 h in culture after receiving 20, 40 and 60 Gy, respectively. Correspondently, the percent of apoptosis moved from 5 % in control cells to 20-25 % after 24 h and 30-35 % after 48/72 h after irradiation. The results on cell growth and apoptosis suggest that the maximum effect due to irradiation is already obtained at the dose of 20 Gy.

The PM activity of β-galactosidase and β-glucosidase increased progressively with the dose and in less extent with time (the increased activity was detectable from 2 h post irradiation) after the cell irradiation. The two enzymes reached an activity that was 40 and 15 times higher with respect to that of control cells. Instead, the behavior of Neu3 and sphingomyelinase was somehow different. In irradiated cells, both enzymes showed activity about two times higher than in control cells. Nevertheless, the change of activity could not be modified by increasing dose or time in culture (Fig. 3).

Cell sphingolipids were metabolically labeled with [1-^{3}H] sphingosine, submitted to irradiation, and the radioactive lipids analyzed for changes in their content with respect to the control. Specific analytical procedures were applied to assess the ceramide levels. Figure 4 shows the tritium-imaging of TLC separation of lipids extracted from irradiated and control T47D cells after metabolic labeling with [1-^{3}H]sphingosine. The double band corresponding to ceramide, (due to heterogeneity in acyl chains) displays a higher intensity in irradiated cells with respect to control cells. Quantification of the spots showed that ceramide significantly increased. We calculated that ceramide increased 3 times with respect to control, independently from the radiation dose. No significant differences were detected for the other sphingolipids between irradiated

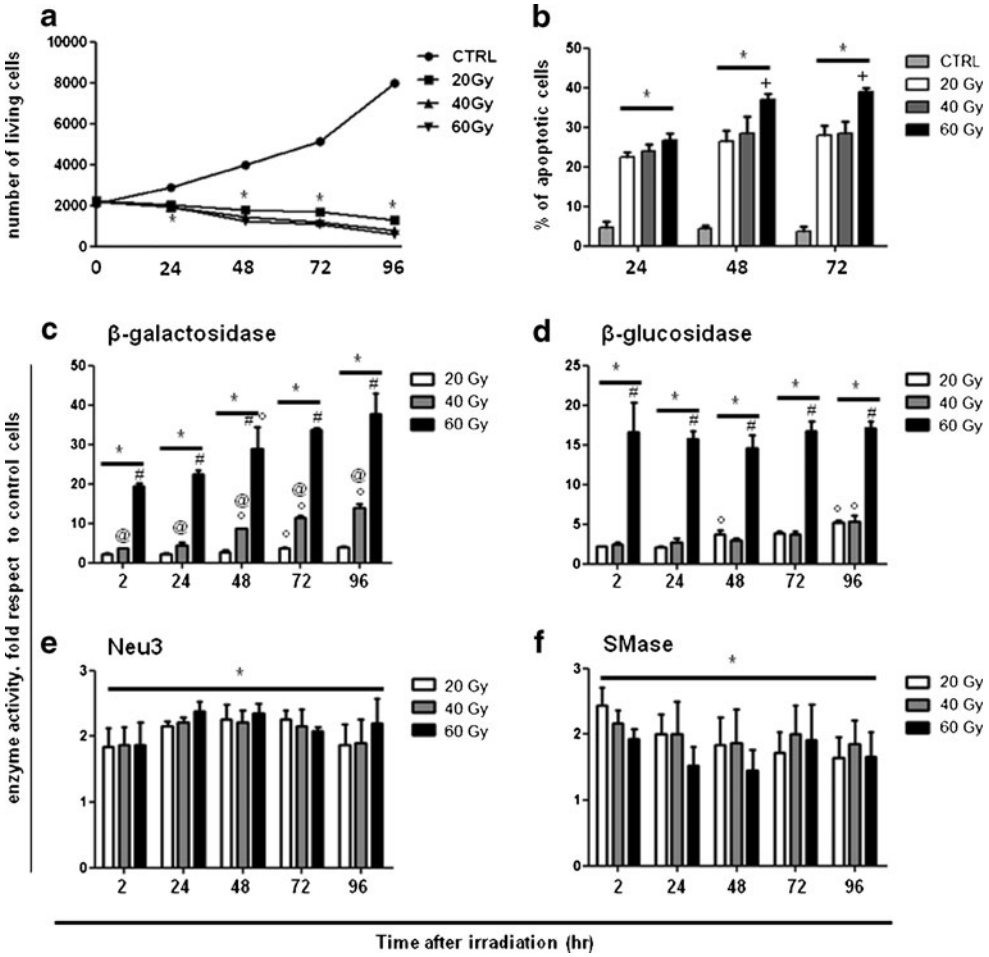

Fig. 3 Effect of ionizing radiations on cell death and PM activity in breast tumor cell line T47D. Cells were irradiated with doses of 20, 40 and 60 Gy and assayed for cell growth, apoptosis and plasma membrane sphingolipid hydrolases. Panel a, number of living cells determined by trypan-blue exclusion assay up to 96 h from cell irradiation. Panel b, percent of apoptosis determined at 24, 48 and 72 h from cell irradiation. Panels c, d, e and f, activity of β-galactosidase, β-glucosidase, sialidase Neu3 and sphingomyelinase determined up to 96 h from cell irradiation and expressed as increase of activity with respect to non irradiated cells. Basal activities are reported in Table 1. Data represent the mean ± SD, n=2 independent experiments, 3 replicates for each experiment. Two Way and one Way (in separated graph not shown) ANOVA followed by Bonferroni post-tests *p<0.0001 versus control cells, + p<0.003 vs the previous dose, ° p<0.01 vs the same dose in the previous time, @ p<0.002 vs 20 Gy, # p<0.0001 vs 40 Gy

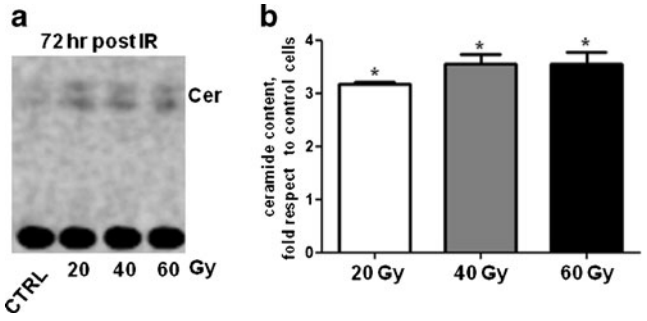

Fig. 4 Metabolic tritium labeling of breast tumor cells T47D sphingolipids. Cells were metabolically labeled with [1-^{3}H]sphingosine and submitted to irradiation up to 60 Gy. Panel A, TLC chromatographic separation of the total radioactive lipids extracted from cells tritium labeled and subjected to irradiation. Extraction was performed 72 h from irradiation. Total extract was separated on HPTLC silica gel 100 plates using the solvent system hexane-chloroform-acetone-acetic acid 20:70:20:2 by volume optimal for the ceramide separation. 1000 dpm were applied in each lane and after separation were submitted to 3 day counting for digital radio-imaging. Panel B, radioactivity quantification of ceramide spots reported in panel A; data are expressed as increase of radioactivity with respect to non irradiated cells. Data represent the mean ± SD, n=2 independent experiments, 3 replicates for each experiment. Two Way ANOVA followed by Bonferroni post-tests *p<0.004 versus control cells

and control cells (data not shown), suggesting that the majority of this ceramide is likely produced in lysosomes. To obtain information on the ceramide production at the plasma membrane level we loaded cells with isotopically tritium labeled ganglioside GM3 in the presence of chloroquine (Fig. 5), a well known inhibitor of lysosomal activity. After a pulse with radioactive GM3, total lipids were extracted and analyzed for radioactivity content after TLC separation. The administered GM3 is taken up by the cells becoming component of the plasma membrane. In the absence of chloroquine, GM3 enters the cell metabolic pathway of sphingolipids and reaches lysosomes by membrane endocytosis. Under these conditions we found, in addition to a relatively large amount of unmetabolized GM3, LacCer, GlcCer, and Cer as catabolic products. We found also radioactive sphingomyelin, due to the recycling of sphingosine. This latter result is the proof that GM3 reached the lysosomes. Cells subjected to 40 Gy irradiation showed a similar catabolic pattern and a quantity of ceramide 10–35 % higher than control cells.

In the presence of chloroquine, lysosomes are not active and GM3 cannot be intracellularly catabolysed. Under these conditions, GM3 was again transformed into LacCer, GlcCer and Cer, but no radioactive sphingomyelin could be observed in the radioactive lipid mixture. This result confirms that any change of the GM3 structures occurs at the cell surface, likely by sequential activity of sialidase Neu3, β-Gal-ase and β-Glc-ase. These cells showed a 150 % increase of ceramide after 40 Gy irradiation, reaching 200 % after 72 h irradiation. The increase of the cell ceramide content in irradiated cells was followed by a significant reduction of glucosylceramide. This confirms our previous results obtained in fibroblasts [10] indicating that ceramide is produced also at the PM level and that its quantity depends on the activity of PM hydrolases.

Discussion

Sphingolipid hydrolases are known since a long time as lysosomal catabolic enzymes necessary to remove the hydrophilic head from sphingolipids yielding ceramide. Ceramide is then hydrolyzed by ceramidase to sphingosine that can be recycled or completely catabolysed, outside of lysosomes, to water and carbon dioxide [39]. Hydrolases of complex sphingolipids encompass glycosidases, specifically recognizing the different oligosaccharide glycosidic linkages, and sphingomyelinase working on sphingomyelin.

In recent years a more detailed information became available on the association of some sphingolipid hydrolases to the plasma membranes [12]. The availability of a simple methodology capable to measure the enzyme activities associated with plasma membrane in living cells, with no interference of the lysosomal activities, allowed to better understand the behavior of these enzymes [33]. Some of these enzymes become plasma membrane components as a consequence of fusion processes between lysosomes and PM, but some have a structure different from that of the lysosomal enzymes as described for glucocerebrosidase GBA2, sialidase Neu3 and probably the neutral sphingomyelinase [12]. The action of these enzymes to modify the sphingolipid structure has been considered a system to modify the plasma membrane organization and properties, mainly related to the cell signaling processes [6]. An increase of activity of PM sphingomyelinase is claimed to be responsible for the formation of large ceramide-rich platforms instrumental to the cell signaling [40]. Activation of sphingomyelinase by TNF-α leads to the ceramide dependent phosphorylation cascade ending with cell apoptosis [41]. Activation of the PM sphingomyelinase by the ionizing radiations used for radiotherapy of tumors, is well described to participate to the cell death and tumor reduction, through cell apoptosis caused by an increase of PM ceramide [21, 24]. Previously we showed that in human fibroblasts an increased expression of sialidase Neu3 is associated with an increase of activity of β-glucosidase and β-galactosidase, resulting in an increase of PM ceramide and cell death by apoptosis [10].

In this manuscript we present data on the behavior of PM glycosidases in cells treated with ionizing radiations, and present the first information on their possible involvement in ceramide production and cell death after radiotherapy treatments.

Our first experiments were carried out on human normal fibroblasts. They were used to establish the experimental

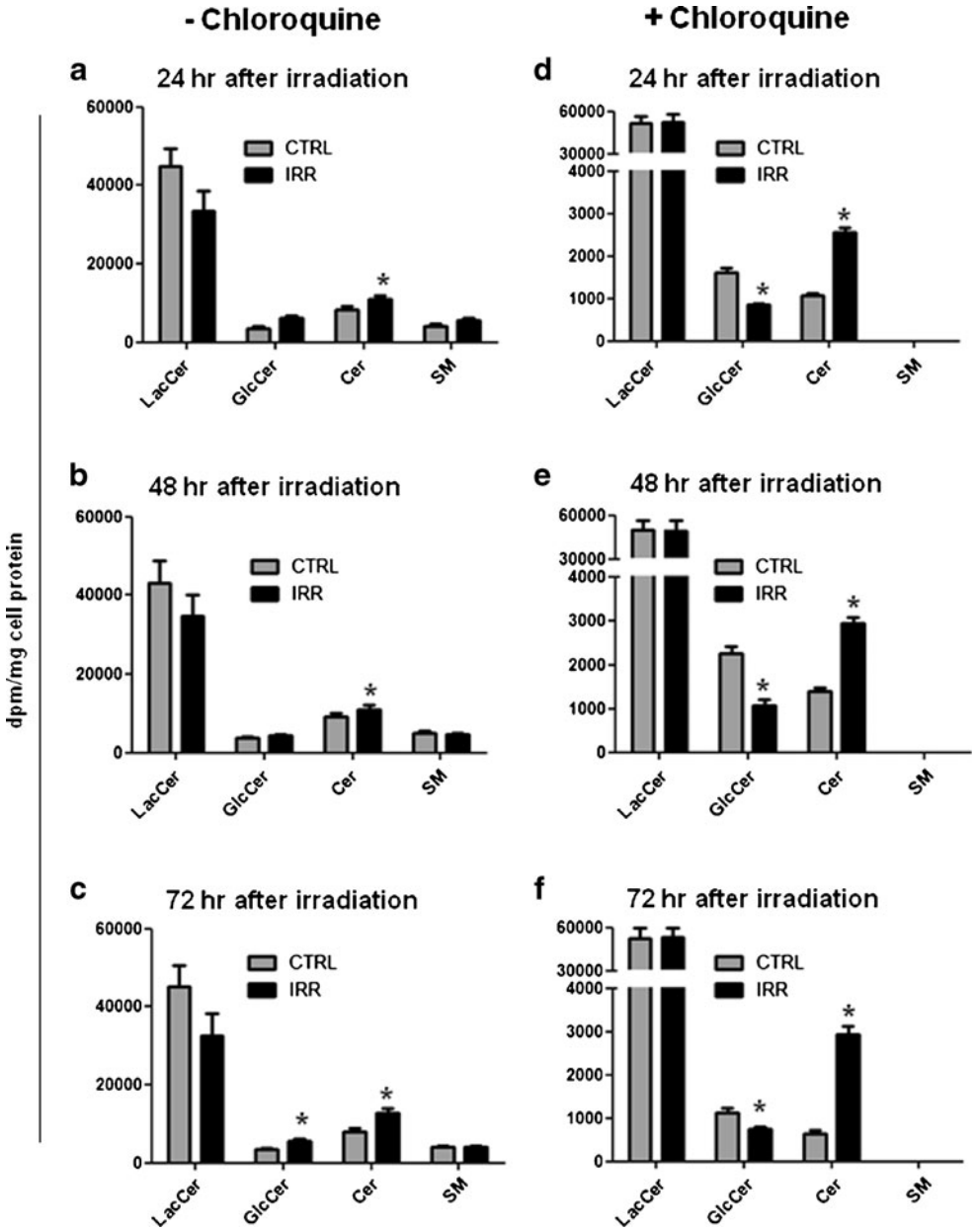

Fig. 5 Administration of isotopically tritium labeled GM3 to breast tumor cells T47D. Cells were maintained in culture in the presence of chloroquine to prevent lysosomal activity, and incubated with [3-^{3}H (*sphingosine*)]GM3 in the absence of serum. After a short pulse and irradiation with a dose of 40 Gy, the radioactive lipids were extracted and separated by TLC. Separation was carried out using the solvent system chloroform: methanol:water, 110:40:6 by vol. After TLC separation and radio-imaging, lipids were quantified for radioactivity content. Panel a, b, and c, radioactivity distribution between lipids extracted from cells cultured in the absence of chloroquine, control cells, 24, 48 and 72 h from irradiation. Panel d, e and f, radioactivity distribution between lipids extracted from cells cultured in the presence of chloroquine, 24, 48 and 72 h from irradiation. Radioactivity distribution is in comparison with non irradiated cells. Data represent the mean ± SD, $n=2$ independent experiments, 3 replicates for each experiment. Two Way ANOVA followed by Bonferroni post-tests *$p<$ 0.0003 versus control cells

conditions necessary to verify the possible changes of enzyme activities after cell irradiation.

As expected, fibroblasts submitted to ionizing radiations, as expected, progressively died. The four enzymes sialidase Neu3, β-glucosidase, β-galactosidase and sphingomyelinase displayed increased activity after treatment. The activity of glycosidases progressively increased and 96 h after treatments they were 3–4 times higher than control cells. On the contary, the activity of sphingomyelinase remained constant during the four days. Very similar results were obtained in fibroblasts prepared from a Niemann-Pick patient, having a very low residual sphingomyelinase activity. The PM activity of sphingomyelinase in these cells was too low to detect any difference after treatment, but no statistical different mortality between normal and pathological cells was observed. It has been reported that the ceramide-induced apoptosis consequent to the activation of the acid sphingomyelinase is strictly dependent on the tissue considered. Our results suggest that in NPA fibroblasts, the ceramide production by increased glycosidase activities is sufficient to compensate the lack of sphingomyelinase activity.

Following the results obtained in fibroblasts, we treated several cell lines from breast, ovary, colon and neuroblastoma tumors. All of these cells showed basal plasma membrane activities of sialidase Neu3, β-glucosidase, β-galactosidase and sphingomyelinase (Table 1) that increased after cell irradiation. Nevertheless, the quantitative changes of these activities were different in the cell lines as reported in Fig. 2.

The reasons leading to the increase in the PM associated glycohydrolase activities are not yet well clarified being

attributable to an increased expression, an augmented PM translocation or conformational changes of the enzyme structures. This latter hypothesis has been already postulated for the sphingomyelinase by Haimovitz-Friedman *et al*. [24] and, considering the irradiation energy it is not surprising that it could modify the enzyme conformation and activity.

The determination of possible changes of the expression levels of the glycohydrolases could be not completely informative since also the glycohydrolases typically associated to the plasma membrane, the sialidase Neu3 and the β-glucosidase GBA2, have been demonstrated to be not exclusively associated to the PM being in part intracellular components.

Moreover, we found that changes of plasma membrane enzyme activities occur after only 40 min from cell irradiation (data not shown), a time for which it is difficult to hypothesize that the increase of activity is due to the induction of mRNA synthesis, followed by the protein translation and its transfer to the plasma membrane.

We analyzed in deeper detail the effects of radiations on the T47D cell line derived from breast cancer, with particular interest for the behavior of the above enzymes and production of ceramide at the plasma membranes. Patients with breast tumor are often subjected to radiotherapy [42, 43].

A marked increase of activity of β-glucosidase and β-galactosidase was observed after treatment. The activity increased with the radiation dose and with time after the treatment. Neu3 and sphingomyelinase reached the maximum value already at 20 Gy remaining then constant up to 60 Gy. At different dose or time after the treatment cells, showed a similar mortality and similar percentage of apoptotic cells. This suggest that over a certain increase of dose and time after the treatment, the role of PM hydrolase activities, become less relevant. As described for the sphingomyelin pathway, these signaling mechanisms may be distinct from the p53-dependent apoptosis. Remarkably, the T47D cell line is characterized by high expression levels of mutant p53 [44].

The prevailing paradigm of the lethal effects of the radiation identifies double stranded DNA breaks, directly produced by radiations or by radiation-induced free radicals, as the critical lesions that lead to cell death [45, 46]. Whereas the great majority of the DNA breaks are rapidly repaired by constitutively expressed enzymatic mechanisms [47, 48], residual unrepaired or misrepaired breaks lead to genetic instability, increased frequency of mutation, and chromosomal aberration [49]. Lethal mutations or dysfunctional chromosomal aberration eventually lead to progeny cell death, usually after several mitotic cycles [50]. The cell lines considered in the present work present a cell duplication time around the 18–24 h and all the experiment presented are performed maximum at 96 h post irradiation. These conditions could avoid the onset of these processes.

To proof that, in parallel with an increase of the glycohydrolase activities, more ceramide is actually produced in the plasma membrane, we administered isotopically tritiated lipids to the human T47D breast carcinoma cells and we followed the fate of radioactivity under different experimental conditions. We first administered tritiated sphingosine in order to metabolically label all sphingolipids, so that we could follow changes of the radioactive sphingolipid pattern after cell irradiation. These changes were originated by changes of all the cell sphingolipid metabolic enzymes, including lysosomal hydrolases whose activities are much higher of those associated with the PM. The radioactive pattern displayed changes mainly related to the content of neutral glycolipids, with the exception of ceramide that triplicated in quantity. This suggests that sphingolipid hydrolases are probably activated by the ionizing radiations, but does not exclude an up-regulation of the ceramide synthase, as previously described [51, 52]. To better understand the site and the mechanism of ceramide production in irradiated cells, tritiated GM3 was administered to cells. Our idea was to follow the production of PM ceramide, due to the sequential activities of Neu3, β-galactosidase and β-glucosidase, under conditions that block lysosomal activity. To avoid lysosomal catabolism, we administered GM3 in the presence of chloroquine, a well known inhibitor of lysosomal activity, and followed its metabolic fate. In the absence of chloroquine, we observed a minor but statistically significant increase of ceramide. The experimental conditions used, that privileged the permanence of administered ganglioside at the PM with respect to the intracellular localization and the high dilution of radioactive ceramide

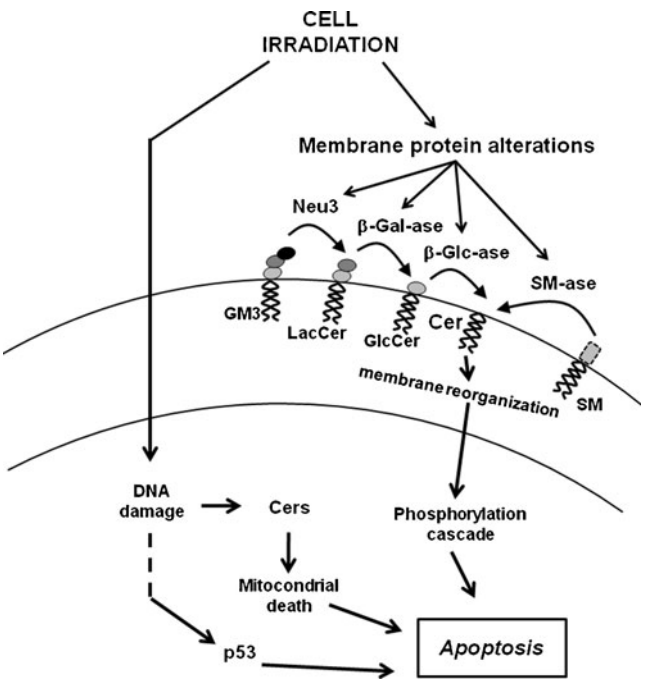

Fig. 6 Simplified scheme of the effects of ionizing radiations on cells in culture

deriving from the catabolism of exogenously administered ganglioside into non radioactive endogenous ceramide, does not allow to overlap the result obtained by steady-state metabolic labeling of all sphingolipids with tritiated sphingosine. In the presence of chloroquine, we had an high increase of ceramide. We calculated that the absolute quantitative increase of ceramide determined in the presence of chloroquine corresponds to the minor increase of total cell ceramide in the absence of chloroquine. This suggests that under our experimental conditions, the ceramide formed in lysosomes is rapidly catabolysed to sphingosine and that the observed increase of ceramide is largely due to the production of a more time existing PM ceramide.

The increase of PM ceramide in cells subjected to ionizing radiations was shown to participate to the onset of cell death [53]. The activation of sphingomyelinase has been regarded as the main mechanism for the production of pro-apoptotic ceramide. The information presented in this paper that ionizing radiations also activate PM glycohydrolases suggests that PM ceramide can derive from all PM sphingolipids. This requires additional care in the development of radiotherapy protocols. As reported in Fig. 2, tumor cells show different patterns of enzyme activities. Thus, it would be necessary to know the ratio between lactosylceramide and ceramide at the end of irradiation. Lactosylceramide is an antiapototic compound and it could cancel or reduce the apoptotic effect of ceramide.

The involvement of glycosidases in PM ceramide production increases several times the number of enzymes that can participate in cell apoptosis and adds a new knowledge on the apoptotic cell death induced by ionizing irradiations. We summarized this in Fig. 6.

The fact that PM glycosidases can participate in tumor cells apoptosis opens the possibility to design drugs capable to modulate their activity. This would allow to develop new chemotherapy treatments in combination with radiotherapy. Moreover the actions of the cell surface associated glycosidases are not confined to the glycosphingolipids metabolism but also to other cell surface associated glycoconjugates. Their modifications during the cell irradiation need to be further studied in the future.

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Conflict of interest The authors declare that they have no conflict of interest

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