Glycoconj J (2014) 31:449–459
DOI 10.1007/s10719-014-9541-y

Exploring the link between ceramide and ionizing radiation

Massimo Aureli · Valentina Murdica · Nicoletta Loberto · Maura Samarani · Alessandro Prinetti · Rosaria Bassi · Sandro Sonnino

Published online: 17 August 2014

Abstract The aim of radiotherapy is to eradicate cancer cells with ionizing radiation; tumor cell death following irradiation can be induced by several signaling pathways, most of which are triggered as a consequence of DNA damage, the primary and major relevant cell response to radiation. Several lines of evidence demonstrated that ceramide, a crucial sensor and/or effector of different signalling pathways promoting cell cycle arrest, death and differentiation, is directly involved in the molecular mechanisms underlying cellular response to irradiation. Most of the studies strongly support a direct relationship between ceramide accumulation and radiation-induced cell death, mainly apoptosis; for this reason, defining the contribution of the multiple metabolic pathways leading to ceramide formation and the causes of its dysregulated metabolism represent the main goal in order to elucidate the ceramide-mediated signaling in radiotherapy. In this review, we summarize the current knowledge concerning the different routes leading to ceramide accumulation in radiation-induced cell response with particular regard to the role of the enzymes involved in both ceramide neogenesis and catabolism. Emphasis is placed on sphingolipid breakdown as mechanism of ceramide generation activated following cell irradiation; the functional relevance of this pathway, and the role of glycosphingolipid glycohydrolases as direct targets of ionizing radiation are also discussed. These new findings add a further attractive point of investigation to better define the complex interplay between sphingolipid metabolism and radiation therapy.

M. Aureli · V. Murdica · N. Loberto · M. Samarani · A. Prinetti · R. Bassi · S. Sonnino (✉)
Department of Medical Biotechnology and Translational Medicine, University of Milano, Via Fratelli Cervi 93, 20090 Segrate, Italy
e-mail: sandro.sonnino@unimi.it

Keywords Glycosphingolipids · Apoptosis · Glycohydrolases · Sphingomyelin · Sphingomyelinase · Ceramide

Introduction

In radiation therapy, high energy liberation of electrons is used to activate different cell death pathways in malignant tumor cells. The classical theory on the cellular effects of ionizing radiation (IR) identifies DNA as the primary target for initiating death pathways [1]. DNA double-strand breaks (DSBs) are lethal lesions, and unrepaired or misrepaired breaks lead to mitosis-associated (also termed reproductive or postmitotic) cell death or to p53-mediated apoptosis. Interestingly, the widely described p53-indipendent cell death pathway is mainly regulated by the sphingolipid metabolism through the production of apoptotic ceramide (Cer) [2, 3]. Cer is a key molecule in several signaling pathways and different molecules, such as kinase suppressor of RAS (KSR; identical to ceramide-activated protein kinase) [4], ceramide-activated protein phosphatase (CAPP) [5], protein kinase C [6], c-RAF-1 [7], the small G proteins RAS and RAC [8], phospholipase A2 and cathepsin D [9], are direct targets of Cer. The mechanisms of the interaction between ceramide and these targets are not completely known. In some cases a direct interaction has been demonstrated, as for cathepsin D [10], thus promoting its catalytic cleavage in the active form [11]. Similarly, Cer has also been shown to direct bind the C1B domain of PKCd, for which it exhibits a higher affinity than the phorbol ester TPA. As it has been demonstrated by Kashiwagi in COS-7 and CHO-K1 cells, the interaction between ceramide and C1B domain causes the translocation of the latter to the intracellular membrane compartments [6]. The C1B domain is also present in other ceramide responsive factors, such as KSR and c-RAF-1; however, for these

molecules, it is still unknown if the presence of the C1B domain causes the targeting of these proteins to the sites of ceramide generation, such as plasma membrane rafts. In addition, different studies revealed several signaling proteins, which are indirect effectors of Cer. For example, Cer indirectly activates the Src-like tyrosine kinase p56lck that blocks the n-type Kþ channel (n-Kþ, Kv1.3) [12], the calcium release activated calcium channel (CRAC) [13] in lymphocytes or the c-Jun N-terminal kinases (JNKs), crucial regulators of apoptosis in a variety of cell types [14]. Moreover, Cer induces cell death via BAD activation involving RAS, KSR, c-RAF-1, and MEK-1 pathway [15] and causing Akt inactivation. Since Akt activity maintains BAD in the inactive form, Akt inhibition rescues the proapoptotic function of BAD.

All the above data support a role for ceramide as a classic second messenger. However, more recently, a new concept for ceramide function, based on its biophysical effects on membrane bilayers, has been proposed [16]. In fact, the increase in plasma membrane ceramide content can be triggered by several extracellular signals leading in turn to intracellular signaling pathways. For example, stimulation of CD95 or CD40 with the cognate ligand resulted in the translocation of the acid sphingomyelinase (ASMase) to the outer leaflet of the plasma membrane [17] where the enzyme releases ceramide from sphingomyelin (SM), which is mainly located in the glycosphingolipid enriched microdomains (GEMs) (also known as membrane lipid rafts). The massive catabolism of SM induces a spatial reorganization of GEMs resulting into large ceramide-enriched regions [18], in which ceramide molecules tend to spontaneously self-associate, via hydrogen bonding, to form aggregates that can also fuse together generating even larger platforms. The membrane reorganization in these structures seems to promote protein oligomerization that triggers the intracellular signaling. Indeed, these signaling platforms, in addition to facilitate transactivation of clustered receptors, may stabilize their interaction with ligands and recruit intracellular signaling molecules to the activated receptors [18]. These events have been proposed in different signaling pathways, as that involving Fas and its downstream effectors FADD/MORT-1 and caspase-8 [19], or the apoptotic signaling triggered by the recruitment, within ceramide-enriched rafts, of caveolin-1 to P13K-receptor complexes, which blocks the activity of P13K [20]. However, raft aggregation into platforms may also be involved in nonreceptor-mediated signaling. For instance, UV radiation triggers aggregation of cell surface CD95 [21], which might be sufficient to initiate signaling of CD95 at least under stress conditions. Similarly, UV and ionizing radiation can induce ceramide mediated apoptosis through raft aggregation without the involvement of CD95 [3]. These responses are abolished by raft disintegration induced by cholesterol depletion.

Most of the literature concerning the molecular mechanisms involved in the cell response to ionizing radiation, indicate that the ASMase/ceramide is the main pathway leading to tumor cell apoptosis. However, also Cer generated by glycosphingolipid hydrolysis could be the target of irradiation, as demonstrated in different cell types of both tumoral and non tumoral origin; these cells respond to radiation exposure by increasing the activity of different glycohydrolases at the cell surface with the consequent activation of ceramide production by glycosphingolipids (GSL) and the onset of apoptosis [22].

Here, we review the molecular pathways involved in the ceramide-induced apoptosis after exposure to ionizing radiations. In particular, we describe the contribution of the different metabolic routes leading to ceramide production (de novo biosynthesis or catabolism from sphingomyelin and glycosphingolipids) in radiation-induced cell response.

Involvement of ceramide metabolism in radiation-induced cell response

Several studies demonstrated the crucial role of ceramide in the mechanisms involved in radiation-induced cell death in different tumor models and targeting its metabolic pathway continues to be actively investigated to improve cancer therapies. As shown in Fig. 1, Cer biosynthesis requires the coordinated activity of the serine palmitoyl transferase and the ceramide synthases (CerS), a family of six isoenzymes showing a different acyl chain specificity [23, 24]. The serine palmitoyl transferase catalyzes the condensation of serine and palmitoyl-CoA to form 3-ketosphinganine, which is reduced to sphinganine and then acylated by CerS to yield dihydroceramide. This molecule is oxidized to ceramide by the introduction of a trans-4,5 double bond, thus converting the sphinganine moiety to sphingosine [25]. Under normal conditions, ceramide synthesis occurs in the endoplasmic reticulum and provides the basic unit for the synthesis of more complex sphingolipids [26]. However, CerS are also found in mitochondria and perinuclear membranes [26, 27], where they may be activated by stress stimuli, such as phorbol ester, daunorubicin and radiation [28]. Moreover, the high specificity and affinity of each CerS for a unique set of fatty acid chains, as well as their different distribution among organs, implicates that these enzymes are crucial regulators of specific ceramide pools determining cell-type and/or intracellular specific signaling outcomes in response to apoptotic stimuli. Studies performed by Liao et al. (1999) showed that DNA damage-induced apoptosis is mediated by CerS activation and subsequent increased ceramide neosynthesis. In particular, in bovine aortic endothelial cells, as well as in HeLa cells, ionizing radiation-induced CerS-mediated ceramide production, specifically occurs in the mitochondrial compartment, without affecting Cer metabolism in the endoplasmic reticulum [28]. Moreover, a recent paper reports that ionizing radiation induces de novo synthesis of ceramide and HeLa cells

apoptosis by the activation of CerS isoform 2, 5 and 6. Interestingly, CerS2 activation resulted in the partial protection from IR-induced apoptosis, whereas CerS5 and 6 are responsible of the production of proapoptotic ceramide [29] which, in turn, regulates the integration of BAX into the outer mitochondrial membrane, the cytochrome c release and the caspase-3 activation. All these events are blocked by suppressing Cer generation with CerS inhibitor Fumonisin B1 (FB1) before irradiation exposure. Mitochondria isolated from unirradiated cells pretreated with high doses of FB1 displayed depletion of ceramide, resisted the BAX integration and did not release cytochrome c in response to exogenous BAX, while the addition of exogenous ceramide restored the ability to respond to BAX. In the same paper, a role for the ataxia telangiectasia-mutated (ATM) gene product in the regulation of the apoptotic pathway activated by radiation-induced DNA damage is also proposed [28]. In fact, when Epstein Barr Virus (EBV)-immortalized B-cell lines, derived from several patients affected by ataxia telangiectasia (AT) carrying different mutations in the ATM gene, were subjected to irradiation, CerS activation, ceramide generation and apoptosis were observed. These data indicate that, upon irradiation exposure, both signals derived from DNA damage and ATM mutations contributed to the regulation of CerS activity and to the outcome of apoptosis [30].

However, the biosynthesis of ceramide in mitochondria is considered a late event upon the cell irradiation, since it follows the early phase of Cer accumulation via degradation of the sphingomyelin at the cell plasma membrane (see below).

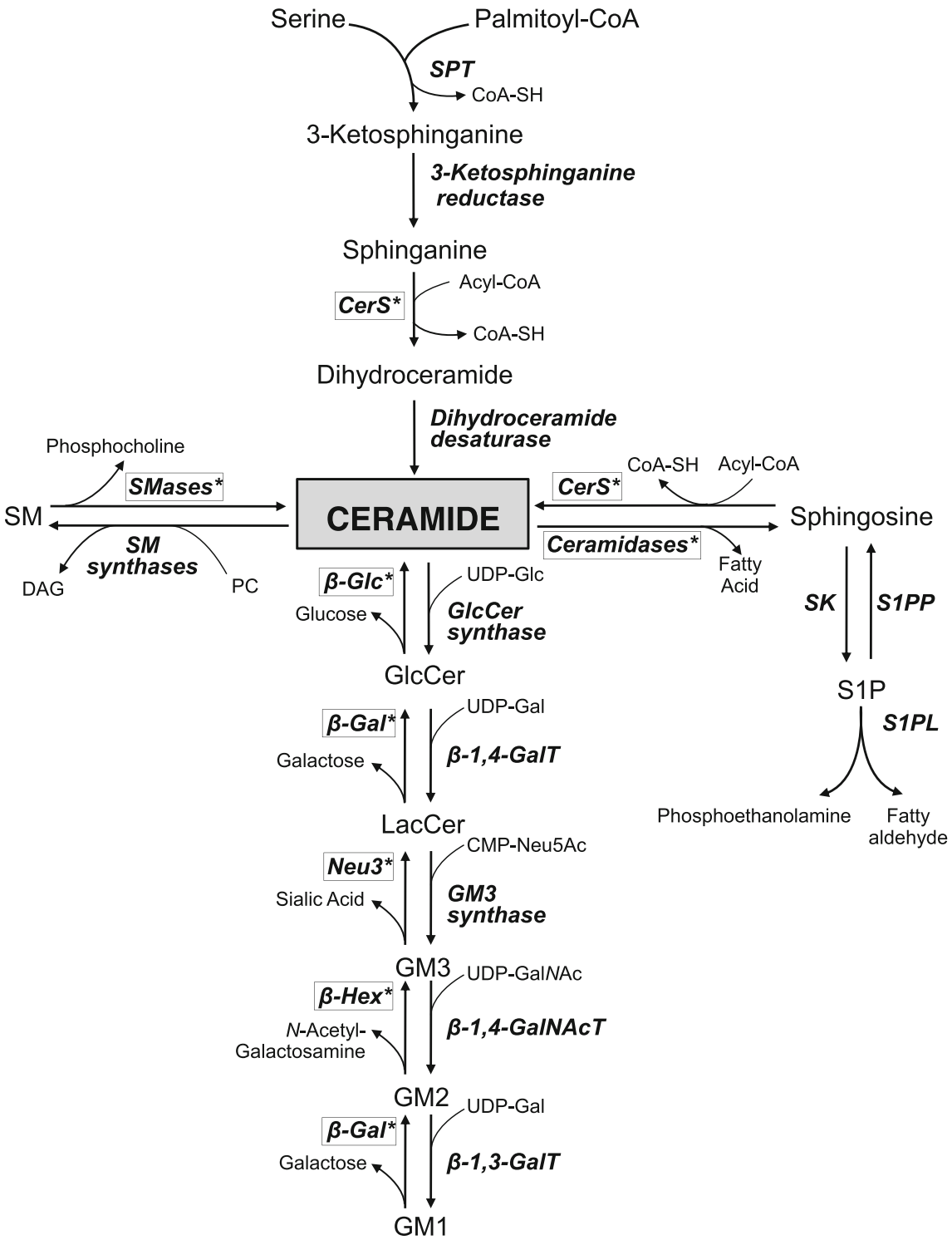
More recently, Deng and coworkers demonstrated that ceramide biogenesis is required for radiation-induced apoptosis in the germ line of C. elegans. In fact, they showed that radiations induce an increase in the concentration of ceramide localized in mitochondria and that the accumulated ceramide was required for BH3-domain of EGL-mediated displacement of CED-4 (an APAF-1–like protein) from the CED-9 (a Bcl-2 family member)/CED-4 complex. This process causes the activation of the CED-3 caspase. Relevant to these studies, radiation-induced apoptosis is regulated by two parallel pathways involving the worm homolog of the tumor suppressor p53 CEP-1, that mediates EGL-1 accumulation, and the generation of neo-synthesized ceramide via activation of CerS enzymes; both these pathways are functionally integrated at the mitochondrial membranes where the commitment step of apoptosis occurs [31].

While ceramide generation is essential for the initiation of apoptosis following radiation exposure, conversely, inhibition of its production has been correlated with radioresistance, as demonstrated in ASMase deficient human lymphoblasts and mice, which are both defective in radiation induced apoptosis [32, 33]. More recent studies indicate a new mechanism of radioresistance that is due to upregulation of the lysosomal acid ceramidase (ASAH1, 3.5.1.23) in response to radiation therapy [34]. This enzyme deacylates ceramide to yield sphingosine and a fatty acid, thus its action may prevent the accumulation of ceramide instrumental to chemotherapy and/or radiation-induced apoptosis. Moreover, sphingosine generated by acid ceramidase (AC) is rapidly phosphorylated by sphingosine kinase to the antiapoptotic and oncogenic sphingosine-1-phosphate thus further contributing to therapy resistance [35]. Most of the data supporting a role for AC as promising target in radiotherapy have been obtained in prostate cancer cells; inhibition of AC resensitizes these cancer cells to radiation-induced apoptosis by restoring ceramide accumulation [36]; in addition, other studies demonstrated a marked radiosensitization of the same tumors as a result of sphingosine kinase 1 inhibition [37]. These data underline the complexity of the pathways involved in ceramide metabolism affecting the apoptotic response to ionizing radiation.

Radiation-induced ceramide generation via sphingomyelin catabolism

Haimovitz-Friedman and colleagues [2] have first identified the relationship between the sphingomyelinase (SMase) pathway liberating ceramide from membrane sphingomyelin and radiation-induced apoptosis. In particular, this work demonstrated that radiation-induced production of apoptotic ceramide is triggered by the rapid activation of a neutral SMase. To date, several isoforms of enzymes promoting SM hydrolysis into ceramide and phosphorylcholine have been identified; SMAses (EC 3.1.4.12) can be distinguished according to their pH optima (acid, neutral and alkaline), cation dependence and subcellular location. The first and best characterized is the acid sphingomyelinase (aSMase, SMPD1), a soluble glycoprotein showing optimum activity at pH 5. aSMase is mainly localized in the lysosomes and in the outer layer of the cell plasma membrane [38]. Similarly, the secretory sphingomyelinase (sSMase) arises from the same gene encoding the acid sphingomyelinase modified via a differential protein trafficking of a common protein precursor which can be targeted either to lysosomes (aSMase) or to the Golgi secretory pathway (sSMase) depending on the different grade of mannosylation [39, 40]. Some distinct properties characterize sSMase with respect to its lysosomal counterpart; both enzymes are zinc hydrolases, but, whereas the aSMase is already firmly bound to the cation, the secreted form requires an external source of Zn^{2+} to reach the full activity [40]. In addition, sSMase exhibits a complex glycosylation pattern characterized by a low content of mannose in comparison to aSMase [40]. Moreover, the sSMAse is able to hydrolyze SM also at neutral pH [41].

The neutral, Mg^{2+}-dependent sphingomyelinases (nSMase) are integral membrane proteins having pH optima very close to 7; all the isoforms identified so far are strictly



dependent on Mg^{2+} or Mn^{2+} for their catalytic activity. In mammals, three neutral SMases, termed nSMase1 (SMPD2), nSMase2 (SMPD3) and nSMase3 (SMPD4) have been recently identified; they are ubiquitous distributed in all tissues and particularly expressed in brain [42]. nSMase1, is a 48 KDa protein mainly localized in the microsomal fraction; nSMase2, is a membrane bound protein of 71 kDa characterized by two palmitoylation sites that are fundamental for both the enzyme stability and localization mainly at the cell PM, although the subcellular localization of nSMase2 is still unclear and needs further elucidation. The third isoform, nSMase3, a protein of 97 kDa containing an ER signal with

◀ **Fig. 1** Pathways of sphingolipid metabolism contributing to ceramide generation. A schematic representation of the main pathways of ceramide generation is shown with the indication of the enzymes whose activity has been reported to be influenced in response to ionizing radiation (included in frame with *asterisk*). Ceramide levels could be regulated by: i) *de novo* biosynthesis by the serine-palmitoyl transferase (SPT), 3-Ketosphinganine reductase and ceramide synthases (CerS); ii) sphingomyelin (SM) cycle producing ceramide through sphingomyelinases (SMases) action or the enzymatic transfer of obtained phosphocholine from the phosphatidylcholine (PC); iii) glycosphingolipid synthesis within the Golgi apparatus by the sequential addition of different monosaccharidic units by specific glycosyltransferases (UDP-glucose, UDP-Glc by glucosylceramide synthase, GlcCer synthase; UDP-galactose, UDP-Gal by β- β-1,4-Galattosyl transferase, β-1,4-GalT or by β-1,3-Galattosyl transferase, 1,3-GalT; *N*-Acetyl-Neuraminic acid, CMP-Neu5Ac by GM3 synthase; UDP-N-Acetyl-Galactosaminide, UDP-GalNac by β-1,4-N-Acetyl-Galactosaminide transferase, β-1,4-GalNAcT; iv) glycosphingolipid catabolism operated by different glycohydrolases that sequentially remove the terminal residue (β-Glucosidase, β-Glc; β-Galactosidase, β-Gal; sialidase Neu3; β-Hexosaminidase, β-Hex); v) ceramide degradation by ceramidases, which break the amidic linkage between sphingosine and fatty acid. Sphingosine can be then recycled or further degraded through the phosphorylation to sphingosine 1 phosphate (S1P), by the action of sphingosine kinases (SK) and specific lyase (S1PL). Sphingosine could be also obtained via the action of different S1P phosphatases (S1PP)

very low homology to the previously cloned nSMase1 and nSMase2, is highly expressed in striated muscle and heart muscle and localized at both the ER and Golgi level. In addition, the identification of a small group of Mg^{2+}-independent neutral sphingomyelinases mainly with cytosolic localization, has been reported. Finally, the Mg^{2+}-independent SMases also include an alkaline sphingomyelinase (bSMase, ENPP7) that is selectively expressed in the mucosa of the gastrointestinal tract and human bile [43].

The involvement of the SMase family enzymes in the production of the apoptotic Cer in response to ionizing radiation has been mostly linked to the aSMase and nSMase.

A key role of the aSMase pathway in radiotherapy has been established not only from the first studies demonstrating the rapid activation of aSMase at the plasma membrane in endothelial cells exposed to ionizing radiation, but also from the results obtained by Santana and colleagues in human lymphoblasts deficient for aSMase gene and in endothelial cells from aSMase knockout mice: both models were found to be resistant to radiation-induced apoptosis and, remarkably, the rescue of aSMase activity in these cells restored their ability to generate ceramide and to undergo apoptosis after irradiation

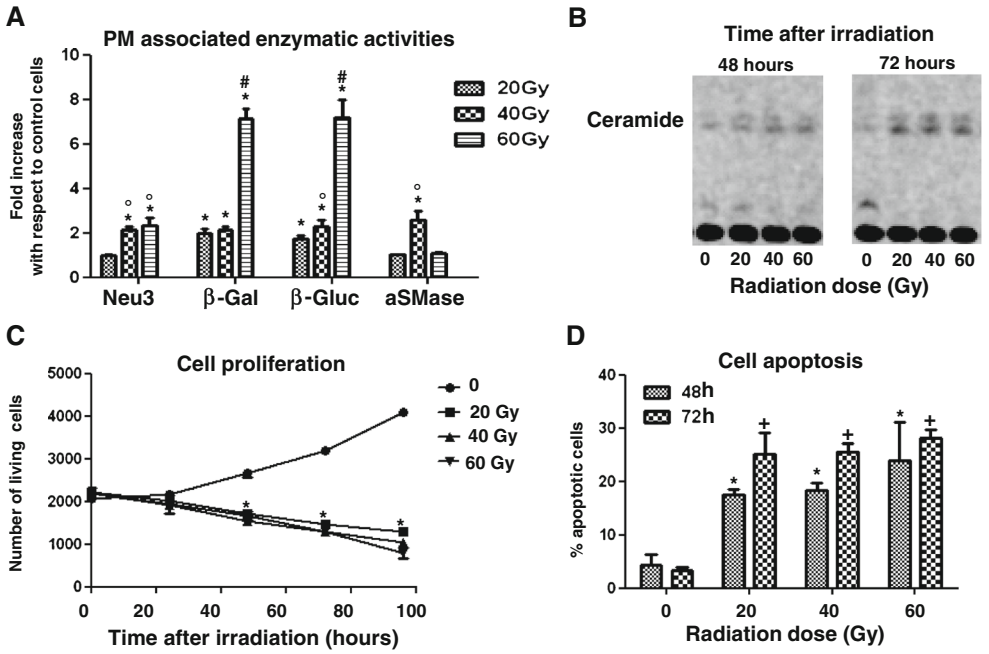


Fig. 2 Effect of ionizing radiation on the glycohydrolase activities, ceramide levels, cell proliferation and apoptosis in colon-rectal cancer cells. Colon-rectal cancer cells (CaCo2) were subjected to 20/40/60 Gy of irradiation and then analyzed at different times after exposure. **a** PM associated enzymatic activities were measured as previously described [22] after 24 h of cell exposure to 20/40/60 Gy. The enzymatic activities are expressed as fold increase with respect to unirradiated control cells whose activity expressed as pmoles/10^6 cells/h was: Neu3 3203±324; β-galactosidase (β-Gal) 3133±809; β-glucosidase (β-Glc) 1621±206; acid sphingomyelinase (aSMase) 690±79. **b** Analysis of ceramide content: cells were metabolically labeled with [1-^{3}H]sphingosine and submitted to irradiation up to 60 Gy. After 72 h post irradiation the lipids were extracted and separated by HPTLC using the solvent system hexane:chloroform:acetone:acetic acid, 20:70:20:2 by volume (optimal for ceramide separation). Radioactive lipids were detected by digital autoradiography. **c** Number of living cells determined by Trypan-Blue exclusion assay. **d** Percentage of apoptotic cells determined by the Hoechst staining. Data are the mean±SD of 3 indipendent experiments, 3 replicates for each experiment. Two Way ANOVA followed by Bonferroni post-test *$p<0.003$ vs 0 Gy; +$p<0.004$ vs 72 h; °$p<0.006$ vs 20 Gy; #$p<0.003$ vs 40 Gy

[32]. Other data suggested that high level of aSMase in endothelial cells could be considered a useful parameter for the prediction of the vulnerability of the endothelium damage induced by radiotherapy [44]. Moreover, the abrogation of ceramide generation and apoptosis has been demonstrated in the endothelium of the lung and intestines and brain after whole body radiation in aSMase$^{-/-}$ mice [32, 45, 46]. These animals, however, displayed normal p53-mediated apoptosis in the thymus. In contrast, p53$^{-/-}$ mice manifested normal ceramide generation and apoptosis in the lung and intestines, while thymic apoptosis was abolished; these findings indicated that the p53 and the SM pathways of radiation-induced apoptosis are distinct and independent.

Another organ in which the abrogation of the aSMase-mediated apoptosis results in prevention of radiation induced damage and in preservation of organ function is the ovary of fertile female mice. Tilly and co-workers showed that aSMase$^{-/-}$ mice are defective in the normal apoptotic deletion of fetal oocytes during embryogenesis, leading to neonatal ovarian hyperplasia [47]. *Ex vivo*, oocytes from aSMase$^{-/-}$ mice, or wild-type oocytes treated with sphingosine-1-phosphate (S1P), a ceramide metabolite known to inhibit ceramide mediated apoptosis [48], resisted apoptosis induced by daunorubicin, confirming cell autonomy of the death defect.

Moreover, wild type mice subjected to intravenous treatment with bFGF show a dramatic reduction in the endothelial apoptotic response in the intestines, brain and lung of wild-type mice. In fact, bFGF induces an activation of the PKC and a concomitant inhibition of the SMase activity [3]. As mentioned before, the neutral SMase has also been implicated in the radiation response [49]. On the other hand, other authors reported that radiation activated nSMase in lymphoid WEHI-231 and TF-1 human myeloid leukemia cells [50]. However, the addition of bacterial nSMase was not able to induce apoptosis in Molt-4 cells, whereas its overexpression resensitize the cells to apoptosis [4]. These data support the existence of separate pools of active ceramide. Moreover, in SQ-20B human head and neck squamous cells, both nSMase and aSMase appeared involved in the radiation response [51].

Glycohydrolases activation in response to ionizing radiation

As depicted in Fig. 1, besides the action of the multiple SMases isoforms, ceramide can also be generated through the hydrolysis of glycosphingolipids operated by a set of specific glycohydrolases, mainly located in the endo-

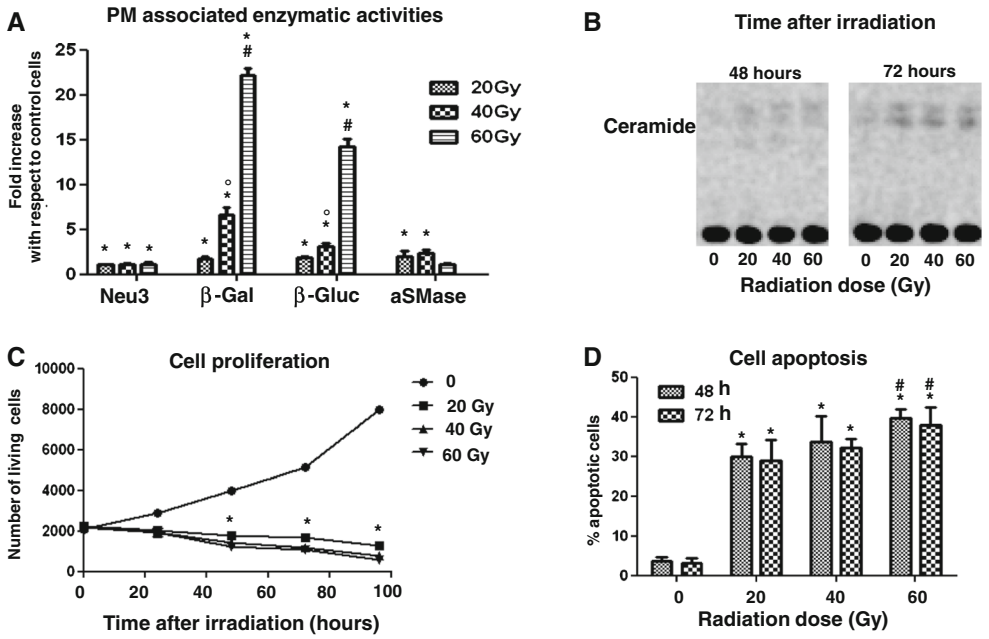


Fig. 3 Effect of ionizing radiation on the glycohydrolase activities, ceramide levels, cell proliferation and apoptosis in breast cancer cells. Breast cancer cells (T47D) were subjected to 20/40/60 Gy of irradiation and then analyzed at different times after exposure. **a** PM associated enzymatic activities were measured as previously described [22] after 24 h of cell exposure to 20/40/60 Gy. The enzymatic activities are expressed as fold increase with respect to unirradiated control cells whose activity expressed as pmoles/10^6 cells/h was: Neu3 57±4.2; β-galactosidase (β-Gal) 447±53; β-glucosidase (β-Glc) 731±120; acid sphingomyelinase (aSMase) 423±51. **b** Analysis of ceramide content: cells were metabolically labeled with [1-^{3}H]sphingosine and submitted to irradiation up to 60 Gy. After 72 h post irradiation the lipids were extracted and separated by HPTLC using the solvent system hexane:chloroform:acetone:acetic acid, 20:70:20:2 by volume (optimal for ceramide separation). Radioactive lipids were detected by digital autoradiography. **c** Number of living cells determined by Trypan-Blue exclusion assay. **d** Percentage of apoptotic cells determined by the Hoechst staining. Data are the mean±SD of 3 indipendent experiments, 3 replicates for each experiment. Two Way ANOVA followed by Bonferroni post-test *$p<0.004$ vs 0 Gy; °$p<0.005$ vs 20 Gy; #$p<0.02$ vs 40 Gy

lysosomal compartment, but also detectable at the external site of the cell surface, as demonstrated by relatively recent studies from our and other groups [52, 53]. This finding supports the functional relevance of GSL breakdown as additional mechanism of ceramide production of potential impact in cancer therapies, including radiation.

Among the mammalian glycohydrolases involved in glycosphingolipid metabolism whose activity/expression has been also detected at the cell surface, the β-hexosaminidase, the α-sialidase Neu3, the β-galactosidase and β-glucosidase are the better characterized [54].

The β-hexosaminidases (β-Hex, EC 3.2.1.52) are dimeric enzymes composed by two different subunits, α (HEXA) and β (HEXB). Their processing in the endoplasmic reticulum results in three isoforms: Hex A (αβ), Hex B (ββ) and Hex S (αα); the dimerization of these subunits is required for their enzymatic activity. However, only the αβ-heterodimer Hex A is able to remove the β-linked non reducing terminal N-acetyl-galactosamine (GalNAc) from ganglioside GM2 and GalNAc-GD1a, in the presence of GM2 activator protein, a specific co-factor of Hex A [55]. The presence of active β-hexosaminidase A in the external leaflet of plasma membrane has been demonstrated in cultured fibroblasts [53]. Immunological and biochemical characterization of the membrane-associated β-hexosaminidase indicated that this enzyme has the same structure of the lysosomal form. This suggests that a regulated fusion process between lysosomes and plasma membrane might represent a general mechanism of plasma membrane repairing [56].

The α-sialidase Neu3 (NEU3, EC 3.2.1.18) catalyzes the hydrolysis of the α2-3 external ketosidic linkage of sialic acid residue preferentially from gangliosides. Neu3 is the first enzymes of GSL catabolism that has been reported to uniquely localize at the cell surface with ubiquitous expression in almost all normal and pathological human tissues, such as brain [57], normal and colon rectal carcinoma tissue, hepatic tumor and kidney carcinoma [58–62]. In addition, Neu3 expression and activity were also found in both normal and pathological mammalian cell lines such as erythroid and erytroleukemic cells [63–65], fibroblasts [66], neurons, neuroblastoma cells [67], breast ductal cancer T47D cells, colon-rectal cancer cells CaCo2, colon-rectal adenocarcinoma HT29 cells, different types of ovarian cancer cells, cervix adenocarcinoma HeLa cells [62].

Nowadays, two different β-galactosidases (β-Gal) involved in the GSL metabolism have been described: β-galactocerebrosidase (GALC, EC 3.2.1.46) that is able to remove the galactose from the galactosylceramide,

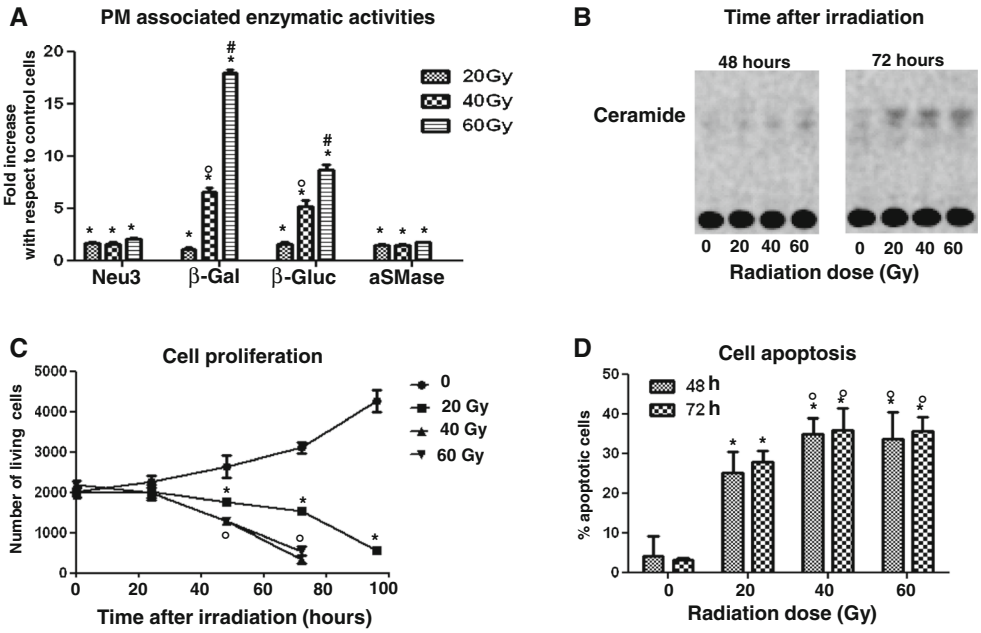


Fig. 4 Effect of ionizing radiation on the glycohydrolase activities, ceramide levels, cell proliferation and apoptosis in ovarian carcinoma cells. Ovarian carcinoma cells (A2780) were subjected to 20/40/60 Gy of irradiation and then analyzed at different times after exposure. **a** PM associated enzymatic activities were measured as previously described [22] after 24 h of cell exposure to 20/40/60 Gy. The enzymatic activities are expressed as fold increase with respect to unirradiated control cells whose activity expressed as pmoles/10^6 cells/h was: Neu3 123±30; β-galactosidase (β-Gal) 240±50; β-glucosidase (β-Glc) 462±90; acid sphingomyelinase (aSMase) 294±21. **b** Analysis of ceramide content: cells were metabolically labeled with [1-^{3}H]sphingosine and submitted to irradiation up to 60 Gy. After 72 h post irradiation the lipids were extracted and separated by HPTLC using the solvent system hexane:chloroform:acetone:acetic acid, 20:70:20:2 by volume (optimal for ceramide separation). Radioactive lipids were detected by digital autoradiography. **c** Number of living cells determined by Trypan-Blue exclusion assay. **d** Percentage of apoptotic cells determined by the Hoechst staining. Data are the mean±SD of 3 indipendent experiments, 3 replicates for each experiment. Two Way ANOVA followed by Bonferroni post-test *$p<0.005$ vs CTRL; °$p<0.002$ vs 20 Gy; #$p<0.007$ vs 40 Gy

lactosylceramide and galactosylsphingosine and the β-galactosidase (GLB1, EC 3.2.1.23) that removes the terminal non-reducing β-D-galactose residues from glycoproteins and ganglioside GM1. Besides the lysosomal enzymes, the β-galactosidase activity has also been detected at the plasma membrane level in several cell lines [52], although the identity of the protein/proteins responsible for the β-galactosidase activity is still unknown.

At least three different β-glucosidases (β-Glc) have been described: a lysosomal β-glucocerebrosidase (GBA; EC 3.2.1.45) [68], a non-lysosomal β-glucosylceramidase (GBA2; EC 3.2.1.45) [69] and a cytosolic β-glucosidase (GBA3; EC 3.2.1.21) [70]. Concerning this last enzyme, very little is known about its role; however, it has been verified that GBA3 gives only a minimal contribution to the total cell β-glucosidase activity [71]. On the other hand, GBA1 and GBA2, the two main β-glucosidase activities in almost all cell types, have been widely studied. The β-glucocerebrosidase GBA1 is the lysosomal-associated β-glucocerebrosidase that hydrolyzes the glycosidic β1-4 linkage of GlcCer and GlcSph. It is coded by the GBA gene located on the 1q21 chromosome. The enzyme is sensitive to Conduritol B Epoxide (CBE) inhibition. While GBA1 enzyme has always been described as a lysosomal-associated enzyme, some recent studies described for the first time the presence of a CBE-sensitive β-glucosidase activity also at the cell surface [52, 72].

The non-lysosomal β-glucosylceramidase GBA2 is able to hydrolyze the GlcCer into glucose and Cer and it is specifically inhibited by nanomolar concentrations of *N*-(5-adamantane-1-yl-methoxy)pentyl-deoxynojirimycin (AMP-DNM). The cellular localization of GBA2 is still controversial, since the enzyme has been reported to be associated with the endosomal vesicles, the plasma membrane and the endoplasmic reticulum [69, 73].

All these glycohydrolases display a *trans* activity, being able to hydrolyze substrates present on the membranes of neighboring cells. The action of these enzymes modifies the GSL structure thus resulting in profound changes in plasma membrane organization and properties, which have been mainly related to different cell signaling processes [74]. As the increase in PM sphingomyelinase activity is claimed to be responsible for the formation of large ceramide-rich platforms instrumental for the cell signaling [75], the activation of the glycohydrolytic enzymes might locally increase the GSL catabolism with the subsequent production of proapoptotic Cer and the induction of cell death as demonstrated in human fibroblasts [52, 66].

The availability of a simple methodology that allows to selectively measure in living cells the activities of PM enzymes, excluding the interference of the lysosomal activities, enabled us to extend the study of these glycohydrolytic enzymes in different cell types and in different experimental conditions. In particular, we focused the attention on the possible involvement of the PM glycohydrolases in the onset of apoptosis after cell exposure to IR. These data are reported in a recent manuscript in which we provide evidence for the first time that cell surface glycohydrolases also contribute to ceramide generation and cell death induced by radiotherapy [22]. Our first experiments were carried out on human normal fibroblasts exposed to ionizing radiation, we found that the cell death was preceded not only by the activation of the sphingomyelinase, but also of the sialidase Neu3, β-glucosidase and β-galactosidase. After exposure to ionizing radiation, the activity of glycohydrolases progressively increased, reaching values 3–4 fold higher than those of control cells 96 h after treatment. On the contrary, the SMase activity remained constant along the 4 days. Very similar results were obtained in fibroblasts prepared from a Niemann-Pick Type A patient, suggesting that in these cells, the ceramide production by increased glycohydrolase activities is sufficient to compensate the lack of SMase activity. Using the same approaches, we found that, the exposure of different tumor cell lines to ionizing radiation resulted in an increased activity of plasma membrane Neu3, β-glucosidase, β-galactosidase and sphingomyelinase. Moreover, we observed that the increase in the activities in irradiated cell is cell type-dependent: i)

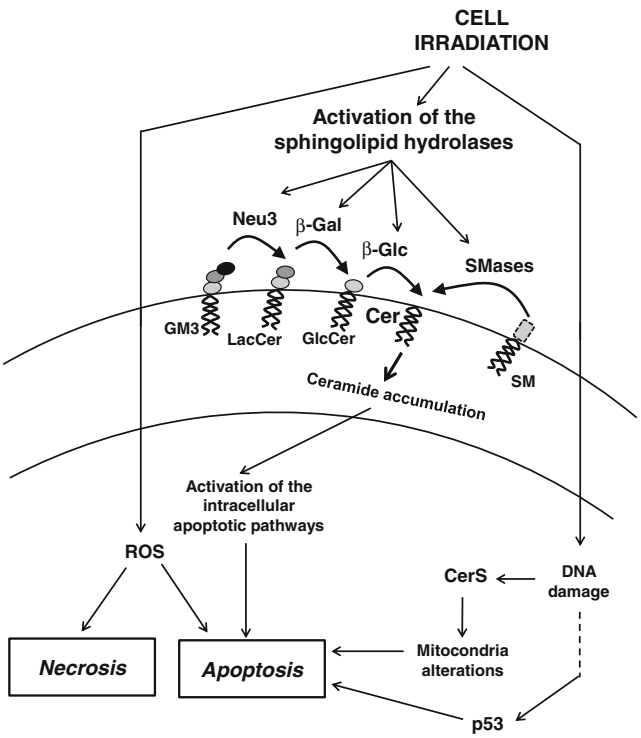


Fig. 5 Representative scheme of the cell death pathway induced by ionizing radiation. Ionizing radiation induced cell death through different pathways involving DNA damage, ROS and ceramide production. To ceramide accumulation might contribute the activation of both biosynthetic enzymes (mainly ceramide synthases family) and sphingolipid catabolic enzymes including the plasma membrane associated glycohydrolases.

Neu3 activity increased from 2 to 5 fold in neuroblastoma and ovarian cancer cells respectively, ii) β-glucosidase from 2 to 12 in breast and in ovarian cancer cells respectively, iii) β-galactosidase from 2 to 7 in colon carcinoma and neuroblastoma cells respectively. We need to elucidate the molecular events leading to the increase in the activity of PM glycohydrolases; it could be attributable to an increased expression, to an augmented PM translocation or to conformational changes of the enzyme structures. In addition, we analyzed the effect of different doses of irradiation on the glycohydrolase activities, Cer levels, cell proliferation and apoptosis in colon carcinoma cells (CaCo2, Fig. 2), ovarian cancer cells (A2780, Fig. 3) and breast cancer cells (T47D, Fig. 4). In particular, in all these tumor cells, the PM enzymatic activities were evaluated 12 h after exposure to 20, 40 and 60 Gy. As reported in the figures, we found a strong increase of the β-Gal and β-Glc activities with respect to non-irradiated cells, whereas SMase and Neu3 activities underwent a modest increase. Moreover, the increase in the enzyme activities was highly dependent on both radiation dose and time of exposure and associated with a marked reduction of cell proliferation. This phenomenon was mainly related to the induction of cell apoptosis, at least at 48 and 72 h after irradiation; indeed, at the same times we observed an increase in Cer content in all irradiated cells. We analyzed in deeper detail the effects of radiations on the T47D cells and we found that the increase in glycohydrolase activities at the plasma membrane level causes an ectopic *in-situ* production of apoptotic ceramide; these results indicate for the first time that glycosphingolipid hydrolases associated with the cell surface contributed to the trigger of the apoptotic effects of ionizing radiation via ceramide generation.

Collectively, these data demonstrated that the activation of the cell surface glycohydrolases in response to ionizing radiation is a common feature of several cancer cells. These findings strongly support a role of these enzymes in the production of apoptotic-ceramide at the plasma membrane level thus adding a new knowledge on the mechanisms of ionizing radiation-induced cell death (Fig. 5).

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

References

1. Zelefsky, M.J., Fuks, Z.V.I., Hunt, M., Lee, H.J., Lombardi, D., Ling, C.C., Reuter, V.E., Venkatraman, E.S., Leibel, S.A.: High dose radiation delivered by intensity modulated conformal radiotherapy improves the outcome of localized prostate cancer. J Urol **166**, 876–881 (2001)
2. Haimovitz-Friedman, A., Kan, C.C., Ehleiter, D., Persaud, R.S., McLoughlin, M., Fuks, Z., Kolesnick, R.N.: Ionizing radiation acts on cellular membranes to generate ceramide and initiate apoptosis. J Exp Med **180**, 525–535 (1994)
3. Kolesnick, R., Fuks, Z.: Radiation and ceramide-induced apoptosis. Oncogene **22**, 5897–5906 (2003)
4. Zhang, Y., Yao, B., Delikat, S., Bayoumy, S., Lin, X.H., Basu, S., McGinley, M., Chan-Hui, P.Y., Lichenstein, H., Kolesnick, R.: Kinase suppressor of Ras is ceramide-activated protein kinase. Cell **89**, 63–72 (1997)
5. Chalfant, C.E., Rathman, K., Pinkerman, R.L., Wood, R.E., Obeid, L.M., Ogretmen, B., Hannun, Y.A.: De novo ceramide regulates the alternative splicing of caspase 9 and Bcl-x in A549 lung adenocarcinoma cells. Dependence on protein phosphatase-1. J Biol Chem **277**, 12587–12595 (2002)
6. Kashiwagi, K., Shirai, Y., Kuriyama, M., Sakai, N., Saito, N.: Importance of C1B domain for lipid messenger-induced targeting of protein kinase C. J Biol Chem **277**, 18037–18045 (2002)
7. Huwiler, A., Brunner, J., Hummel, R., Vervoordeldonk, M., Stabel, S., van den Bosch, H., Pfeilschifter, J.: Ceramide-binding and activation defines protein kinase c-Raf as a ceramide-activated protein kinase. Proc Natl Acad Sci U S A **93**, 6959–6963 (1996)
8. Gulbins, E., Bissonnette, R., Mahboubi, A., Martin, S., Nishioka, W., Brunner, T., Baier, G., Baier-Bitterlich, G., Byrd, C., Lang, F., et al.: FAS-induced apoptosis is mediated via a ceramide-initiated RAS signaling pathway. Immunity **2**, 341–351 (1995)
9. Gulbins, E., Grassme, H.: Ceramide and cell death receptor clustering. Biochim Biophys Acta **1585**, 139–145 (2002)
10. Huwiler, A., Johansen, B., Skarstad, A., Pfeilschifter, J.: Ceramide binds to the CaLB domain of cytosolic phospholipase A2 and facilitates its membrane docking and arachidonic acid release. FASEB J **15**, 7–9 (2001)
11. Deiss, L.P., Galinka, H., Berissi, H., Cohen, O., Kimchi, A.: Cathepsin D protease mediates programmed cell death induced by interferon-gamma, Fas/APO-1 and TNF-alpha. EMBO J **15**, 3861–3870 (1996)
12. Gulbins, E., Szabo, I., Baltzer, K., Lang, F.: Ceramide-induced inhibition of T lymphocyte voltage-gated potassium channel is mediated by tyrosine kinases. Proc Natl Acad Sci U S A **94**, 7661–7666 (1997)
13. Lepple-Wienhues, A., Belka, C., Laun, T., Jekle, A., Walter, B., Wieland, U., Welz, M., Heil, L., Kun, J., Busch, G., Weller, M., Bamberg, M., Gulbins, E., Lang, F.: Stimulation of CD95 (Fas) blocks T lymphocyte calcium channels through sphingomyelinase and sphingolipids. Proc Natl Acad Sci U S A **96**, 13795–13800 (1999)
14. Westwick, J.K., Bielawska, A.E., Dbaibo, G., Hannun, Y.A., Brenner, D.A.: Ceramide activates the stress-activated protein kinases. J Biol Chem **270**, 22689–22692 (1995)
15. Basu, S., Bayoumy, S., Zhang, Y., Lozano, J., Kolesnick, R.: BAD enables ceramide to signal apoptosis via Ras and Raf-1. J Biol Chem **273**, 30419–30426 (1998)
16. Cremesti, A.E., Goni, F.M., Kolesnick, R.: Role of sphingomyelinase and ceramide in modulating rafts: do biophysical properties determine biologic outcome? FEBS Lett **531**, 47–53 (2002)
17. Cremesti, A., Paris, F., Grassme, H., Holler, N., Tschopp, J., Fuks, Z., Gulbins, E., Kolesnick, R.: Ceramide enables Fas to cap and kill. J Biol Chem **276**, 23954–23961 (2001)
18. Kolesnick, R.N., Goni, F.M., Alonso, A.: Compartmentalization of ceramide signaling: physical foundations and biological effects. J Cell Physiol **184**, 285–300 (2000)
19. Hueber, A.O., Bernard, A.M., Herincs, Z., Couzinet, A., He, H.T.: An essential role for membrane rafts in the initiation of Fas/CD95-triggered cell death in mouse thymocytes. EMBO Rep **3**, 190–196 (2002)
20. Zundel, W., Giaccia, A.: Inhibition of the anti-apoptotic PI(3)K/Akt/Bad pathway by stress. Genes Dev **12**, 1941–1946 (1998)

21. Rehemtulla, A., Hamilton, C.A., Chinnaiyan, A.M., Dixit, V.M.: Ultraviolet radiation-induced apoptosis is mediated by activation of CD-95 (Fas/APO-1). J Biol Chem **272**, 25783–25786 (1997)
22. Aureli, M., Bassi, R., Prinetti, A., Chiricozzi, E., Pappalardi, B., Chigorno, V., Di Muzio, N., Loberto, N., Sonnino, S.: Ionizing radiations increase the activity of the cell surface glycohydrolases and the plasma membrane ceramide content. Glycoconj J **29**, 585 (2012)
23. Spiegel, S., Foster, D., Kolesnick, R.: Signal transduction through lipid second messengers. Curr Opin Cell Biol **8**, 159–167 (1996)
24. Hannun, Y.A., Luberto, C., Argraves, K.M.: Enzymes of sphingolipid metabolism: from modular to integrative signaling. Biochemistry **40**, 4893–4903 (2001)
25. Merrill Jr., A.H., Schmelz, E.M., Dillehay, D.L., Spiegel, S., Shayman, J.A., Schroeder, J.J., Riley, R.T., Voss, K.A., Wang, E.: Sphingolipids–the enigmatic lipid class: biochemistry, physiology, and pathophysiology. Toxicol Appl Pharmacol **142**, 208–225 (1997)
26. Mandon, E.C., Ehses, I., Rother, J., van Echten, G., Sandhoff, K.: Subcellular localization and membrane topology of serine palmitoyltransferase, 3-dehydrosphinganine reductase, and sphinganine N-acyltransferase in mouse liver. J Biol Chem **267**, 11144–11148 (1992)
27. White-Gilbertson, S., Mullen, T., Senkal, C., Lu, P., Ogretmen, B., Obeid, L., Voelkel-Johnson, C.: Ceramide synthase 6 modulates TRAIL sensitivity and nuclear translocation of active caspase-3 in colon cancer cells. Oncogene **28**, 1132–1141 (2009)
28. Liao, W.C., Haimovitz-Friedman, A., Persaud, R.S., McLoughlin, M., Ehleiter, D., Zhang, N., Gatei, M., Lavin, M., Kolesnick, R., Fuks, Z.: Ataxia telangiectasia-mutated gene product inhibits DNA damage-induced apoptosis via ceramide synthase. J Biol Chem **274**, 17908–17917 (1999)
29. Mesicek, J., Lee, H., Feldman, T., Jiang, X., Skobeleva, A., Berdyshev, E.V., Haimovitz-Friedman, A., Fuks, Z., Kolesnick, R.: Ceramide synthases 2, 5, and 6 confer distinct roles in radiation-induced apoptosis in HeLa cells. Cell Signal **22**, 1300–1307 (2010)
30. Vit, J.P., Rosselli, F.: Role of the ceramide-signaling pathways in ionizing radiation-induced apoptosis. Oncogene **22**, 8645–8652 (2003)
31. Deng, X., Yin, X., Allan, R., Lu, D.D., Maurer, C.W., Haimovitz-Friedman, A., Fuks, Z., Shaham, S., Kolesnick, R.: Ceramide biogenesis is required for radiation-induced apoptosis in the germ line of C. elegans. Science **322**, 110–115 (2008)
32. Santana, P., Pena, L.A., Haimovitz-Friedman, A., Martin, S., Green, D., McLoughlin, M., Cordon-Cardo, C., Schuchman, E.H., Fuks, Z., Kolesnick, R.: Acid sphingomyelinase-deficient human lymphoblasts and mice are defective in radiation-induced apoptosis. Cell **86**, 189–199 (1996)
33. Alphonse, G., Aloy, M.T., Broquet, P., Gerard, J.P., Louisot, P., Rousson, R., Rodriguez-Lafrasse, C.: Ceramide induces activation of the mitochondrial/caspases pathway in Jurkat and SCC61 cells sensitive to gamma-radiation but activation of this sequence is defective in radioresistant SQ20B cells. Int J Radiat Biol **78**, 821–835 (2002)
34. Mahdy, A.E., Cheng, J.C., Li, J., Elojeimy, S., Meacham, W.D., Turner, L.S., Bai, A., Gault, C.R., McPherson, A.S., Garcia, N., Beckham, T.H., Saad, A., Bielawska, A., Bielawski, J., Hannun, Y.A., Keane, T.E., Taha, M.I., Hammouda, H.M., Norris, J.S., Liu, X.: Acid ceramidase upregulation in prostate cancer cells confers resistance to radiation: AC inhibition, a potential radiosensitizer. Mol Ther **17**, 430–438 (2009)
35. Beckham, T.H., Lu, P., Cheng, J.C., Zhao, D., Turner, L.S., Zhang, X., Hoffman, S., Armeson, K.E., Liu, A., Marrison, T., Hannun, Y.A., Liu, X.: Acid ceramidase-mediated production of sphingosine 1-phosphate promotes prostate cancer invasion through upregulation of cathepsin B. Int J Cancer **131**, 2034–2043 (2012)
36. Cheng, J.C., Bai, A., Beckham, T.H., Marrison, S.T., Yount, C.L., Young, K., Lu, P., Bartlett, A.M., Wu, B.X., Keane, B.J., Armeson, K.E., Marshall, D.T., Keane, T.E., Smith, M.T., Jones, E.E., Drake Jr., R.R., Bielawska, A., Norris, J.S., Liu, X.: Radiation-induced acid ceramidase confers prostate cancer resistance and tumor relapse. J Clin Invest **123**, 4344–4358 (2013)
37. Sinha, U.K., Schorn, V.J., Hochstim, C., Chinn, S.B., Zhu, S., Masood, R.: Increased radiation sensitivity of head and neck squamous cell carcinoma with sphingosine kinase 1 inhibition. Head Neck **33**, 178–188 (2011)
38. Goni, F.M., Alonso, A.: Sphingomyelinases: enzymology and membrane activity. FEBS Lett **531**, 38–46 (2002)
39. Schissel, S.L., Jiang, X., Tweedie-Hardman, J., Jeong, T., Camejo, E.H., Najib, J., Rapp, J.H., Williams, K.J., Tabas, I.: Secretory sphingomyelinase, a product of the acid sphingomyelinase gene, can hydrolyze atherogenic lipoproteins at neutral pH. Implications for atherosclerotic lesion development. J Biol Chem **273**, 2738–2746 (1998)
40. Schissel, S.L., Keesler, G.A., Schuchman, E.H., Williams, K.J., Tabas, I.: The cellular trafficking and zinc dependence of secretory and lysosomal sphingomyelinase, two products of the acid sphingomyelinase gene. J Biol Chem **273**, 18250–18259 (1998)
41. Milhas, D., Clarke, C.J., Hannun, Y.A.: Sphingomyelin metabolism at the plasma membrane: implications for bioactive sphingolipids. FEBS Lett **584**, 1887–1894 (2010)
42. Horres, C.R., Hannun, Y.A.: The roles of neutral sphingomyelinases in neurological pathologies. Neurochem Res **37**, 1137–1149 (2012)
43. Nilsson, A., Duan, R.D.: Alkaline sphingomyelinases and ceramidases of the gastrointestinal tract. Chem Phys Lipids **102**, 97–105 (1999)
44. Marathe, S., Schissel, S.L., Yellin, M.J., Beatini, N., Mintzer, R., Williams, K.J., Tabas, I.: Human vascular endothelial cells are a rich and regulatable source of secretory sphingomyelinase. Implications for early atherogenesis and ceramide-mediated cell signaling. J Biol Chem **273**, 4081–4088 (1998)
45. Pena, L.A., Fuks, Z., Kolesnick, R.N.: Radiation-induced apoptosis of endothelial cells in the murine central nervous system: protection by fibroblast growth factor and sphingomyelinase deficiency. Cancer Res **60**, 321–327 (2000)
46. Paris, F., Fuks, Z., Kang, A., Capodieci, P., Juan, G., Ehleiter, D., Haimovitz-Friedman, A., Cordon-Cardo, C., Kolesnick, R.: Endothelial apoptosis as the primary lesion initiating intestinal radiation damage in mice. Science **293**, 293–297 (2001)
47. Morita, Y., Perez, G.I., Paris, F., Miranda, S.R., Ehleiter, D., Haimovitz-Friedman, A., Fuks, Z., Xie, Z., Reed, J.C., Schuchman, E.H., Kolesnick, R.N., Tilly, J.L.: Oocyte apoptosis is suppressed by disruption of the acid sphingomyelinase gene or by sphingosine-1-phosphate therapy. Nat Med **6**, 1109–1114 (2000)
48. Kolesnick, R.: The therapeutic potential of modulating the ceramide/sphingomyelin pathway. J Clin Invest **110**, 3–8 (2002)
49. Rodriguez-Lafrasse, C., Alphonse, G., Aloy, M.T., Ardail, D., Gerard, J.P., Louisot, P., Rousson, R.: Increasing endogenous ceramide using inhibitors of sphingolipid metabolism maximizes ionizing radiation-induced mitochondrial injury and apoptotic cell killing. Int J Cancer **101**, 589–598 (2002)
50. Bruno, A.P., Laurent, G., Averbeck, D., Demur, C., Bonnet, J., Bettaieb, A., Levade, T., Jaffrezou, J.P.: Lack of ceramide generation in TF-1 human myeloid leukemic cells resistant to ionizing radiation. Cell Death Differ **5**, 172–182 (1998)
51. Chmura, S.J., Nodzenski, E., Beckett, M.A., Kufe, D.W., Quintans, J., Weichselbaum, R.R.: Loss of ceramide production confers resistance to radiation-induced apoptosis. Cancer Res **57**, 1270–1275 (1997)
52. Aureli, M., Masilamani, A.P., Illuzzi, G., Loberto, N., Scandroglio, F., Prinetti, A., Chigorno, V., Sonnino, S.: Activity of plasma membrane beta-galactosidase and beta-glucosidase. FEBS Lett **583**, 2469–2473 (2009)
53. Mencarelli, S., Cavalieri, C., Magini, A., Tancini, B., Basso, L., Lemansky, P., Hasilik, A., Li, Y.T., Chigorno, V., Orlacchio, A.,

Emiliani, C., Sonnino, S.: Identification of plasma membrane associated mature beta-hexosaminidase A, active towards GM2 ganglioside, in human fibroblasts. FEBS Lett **579**, 5501–5506 (2005)
54. Aureli, M., Loberto, N., Chigorno, V., Prinetti, A., Sonnino, S.: Remodeling of sphingolipids by plasma membrane associated enzymes. Neurochem Res **36**, 1636–1644 (2011)
55. Kolter, T., Sandhoff, K.: Sphingolipid metabolism diseases. Biochim Biophys Acta **1758**, 2057–2079 (2006)
56. Reddy, A., Caler, E.V., Andrews, N.W.: Plasma membrane repair is mediated by Ca(2+)-regulated exocytosis of lysosomes. Cell **106**, 157–169 (2001)
57. Fanzani, A., Colombo, F., Giuliani, R., Preti, A., Marchesini, S.: Cytosolic sialidase Neu2 upregulation during PC12 cells differentiation. FEBS Lett **566**, 178–182 (2004)
58. Kopitz, J., von Reitzenstein, C., Muhl, C., Cantz, M.: Role of plasma membrane ganglioside sialidase of human neuroblastoma cells in growth control and differentiation. Biochem Biophys Res Commun **199**, 1188–1193 (1994)
59. Monti, E., Preti, A., Venerando, B., Borsani, G.: Recent development in mammalian sialidase molecular biology. Neurochem Res **27**, 649–663 (2002)
60. Miyagi, T., Wada, T., Yamaguchi, K.: Roles of plasma membrane-associated sialidase NEU3 in human cancers. Biochim Biophys Acta **1780**, 532–537 (2008)
61. Miyagi, T., Wada, T., Yamaguchi, K., Hata, K., Shiozaki, K.: Plasma membrane-associated sialidase as a crucial regulator of transmembrane signalling. J Biochem **144**, 279–285 (2008)
62. Ueno, S., Saito, S., Wada, T., Yamaguchi, K., Satoh, M., Arai, Y., Miyagi, T.: Plasma membrane-associated sialidase is up-regulated in renal cell carcinoma and promotes interleukin-6-induced apoptosis suppression and cell motility. J Biol Chem **281**, 7756–7764 (2006)
63. Kakugawa, Y., Wada, T., Yamaguchi, K., Yamanami, H., Ouchi, K., Sato, I., Miyagi, T.: Up-regulation of plasma membrane-associated ganglioside sialidase (Neu3) in human colon cancer and its involvement in apoptosis suppression. Proc Natl Acad Sci U S A **99**, 10718–10723 (2002)
64. Venerando, B., Fiorilli, A., Croci, G., Tringali, C., Goi, G., Mazzanti, L., Curatola, G., Segalini, G., Massaccesi, L., Lombardo, A., Tettamanti, G.: Acidic and neutral sialidase in the erythrocyte membrane of type 2 diabetic patients. Blood **99**, 1064–1070 (2002)
65. Tringali, C., Anastasia, L., Papini, N., Bianchi, A., Ronzoni, L., Cappellini, M.D., Monti, E., Tettamanti, G., Venerando, B.: Modification of sialidase levels and sialoglycoconjugate pattern during erythroid and erytroleukemic cell differentiation. Glycoconj J **24**, 67–79 (2007)
66. Valaperta, R., Chigorno, V., Basso, L., Prinetti, A., Bresciani, R., Preti, A., Miyagi, T., Sonnino, S.: Plasma membrane production of ceramide from ganglioside GM3 in human fibroblasts. FASEB J **20**, 1227–1229 (2006)
67. Chigorno, V., Cardace, G., Pitto, M., Sonnino, S., Ghidoni, R., Tettamanti, G.: A radiometric assay for ganglioside sialidase applied to the determination of the enzyme subcellular location in cultured human fibroblasts. Anal Biochem **153**, 283–294 (1986)
68. Huang, Q., Shur, B.D., Begovac, P.C.: Overexpressing cell surface beta 1.4-galactosyltransferase in PC12 cells increases neurite outgrowth on laminin. J Cell Sci **108**(Pt 2), 839–847 (1995)
69. Neufeld, E.B., Cooney, A.M., Pitha, J., Dawidowicz, E.A., Dwyer, N.K., Pentchev, P.G., Blanchette-Mackie, E.J.: Intracellular trafficking of cholesterol monitored with a cyclodextrin. J Biol Chem **271**, 21604–21613 (1996)
70. van Weely, S., Brandsma, M., Strijland, A., Tager, J.M., Aerts, J.M.: Demonstration of the existence of a second, non-lysosomal glucocerebrosidase that is not deficient in Gaucher disease. Biochim Biophys Acta **1181**, 55–62 (1993)
71. Daniels, L.B., Coyle, P.J., Chiao, Y.B., Glew, R.H., Labow, R.S.: Purification and characterization of a cytosolic broad specificity beta-glucosidase from human liver. J Biol Chem **256**, 13004–13013 (1981)
72. Yap, T.L., Velayati, A., Sidransky, E., Lee, J.C.: Membrane-bound alpha-synuclein interacts with glucocerebrosidase and inhibits enzyme activity. Mol Genet Metab **108**, 56–64 (2013)
73. Korschen, H.G., Yildiz, Y., Raju, D.N., Schonauer, S., Bonigk, W., Jansen, V., Kremmer, E., Kaupp, U.B., Wachten, D.: The non-lysosomal beta-glucosidase GBA2 is a non-integral membrane-associated protein at the endoplasmic reticulum (ER) and Golgi. J Biol Chem **288**, 3381–3393 (2012)
74. Yu, R.K., Macala, L.J., Taki, T., Weinfield, H.M., Yu, F.S.: Developmental changes in ganglioside composition and synthesis in embryonic rat brain. J Neurochem **50**, 1825–1829 (1988)
75. Miyagi, T., Tsuiki, S.: Evidence for sialidase hydrolyzing gangliosides GM2 and GM1 in rat liver plasma membrane. FEBS Lett **206**, 223–228 (1986)