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Minireview

New advances on structural and biological functions of ceramide in apoptotic/necrotic cell death and cancer

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Abstract **Recent data on the cellular ceramide functions and its involvement in the apoptotic/necrotic cell death as well as its anticarcinogenic properties are presented. The emphasis is on the connections between the ceramide and caspase signaling pathways during the apoptotic cell death process. Notably, the experimental strategies and pharmacological tools used for establishment of the role of ceramide in triggering cell death are described. Moreover, the importance of a compartmentation of endogenous ceramide within the plasma membrane microdomains, lysosomes and mitochondria is discussed. Information on the deregulated functions of ceramide and caspase signaling pathways in several metastatic cancer types is also presented.**
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Key words: Ceramide; Caveolae/raft structure; Mitochondrial apoptotic signal; Caspase; Apoptotic/necrotic cell death; Anticarcinogenic therapy

1. Introduction

The ceramide has been recognized as an important second messager implicated in triggering apoptotic/necrotic processes in many normal and cancer cell types [1–14]. Several works have been carried out to establish the molecular mechanisms by which this endogenous sphingolipid mediates its biological effects. In particular, the subcellular localization of ceramide molecules in plasmalemmal microdomains termed caveolae or rafts, lysosomes and mitochondria represents an important factor that determines their cellular functions [11,13–20]. Moreover, the cellular ceramide generation induced by different external agents such as tumor necrosis factor-α (TNF-α), Fas ligand, interferon-γ (INF-γ) and interleukin-1β (IL-1β) through the activation of their transmembrane receptors might lead to the modulation of the activities of different protein kinases and phosphatases that regulate certain phases of apoptotic/necrotic cell death [21–30]. In addition, changes in the sphingomyelin/ceramide ratio might also result in disruption of structural integrity and permeability barrier function of cell membranes [7,10,17,19,31–34].

Importantly, the deregulated expression and/or activities of enzymes involved in the ceramide metabolism as well as the apoptotic signaling elements have also been associated with the resistance of many metastatic cancer cells to cytotoxic effects induced by ionizing radiation and chemotherapeutic agents [5,16,22,24,27,35–39]. In this context, the overexpression of several antiapoptotic growth factors including epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) and their cognate receptor tyrosine kinases on the surface of metastatic cancer cells from prostate, colon, ovary, breast, lung and skin appears to play a critical role for sustained growth and survival of invasive cancer cells by decreasing cellular ceramide levels and activating phosphoinositide 3-kinase (PI_3K)/Akt [40–43]. Of particular therapeutic interest, it has been observed that several cancer cell types are more sensitive than normal cells to cytotoxic effects induced by cellular ceramide accumulation [8,43–45].

2. Production and localization of the cellular ceramide

Natural ceramide from mammalian membranes is constituted of a long chain base and amide-linked fatty acids with acyl chain lengths varying from 16 to 24 carbon atoms (C_{16}–C_{24}). The ceramide production might result from the condensation of palmitoyl-CoA with L-serine via the de novo synthesis pathway of sphingolipids as shown in Fig. 1 [46]. The enzymes involved in the de novo synthesis of cellular ceramide seem to be principally localized within the endoplasmic reticulum and mitochondria. However, the ceramide generated within a specific site may be subsequently transported to other cellular compartments. Indeed, the ceramide might be transferred from the endoplasmic reticulum to the Golgi apparatus and mitochondria by membrane contacts between these cellular compartments [19]. Moreover, the membrane ceramide molecules might be produced partly from the sphingomyelin (SM) pathway through the SM hydrolysis catalyzed by distinct Mg^{2+}-dependent acidic sphingomyelinase (aSMase) and neutral sphingomyelinase (nSMase) and for another part from a glycosphingolipid precursor through the reaction catalyzed

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Abbreviations: AIF, apoptosis-initiating factor; aSMase, acidic sphingomyelinase; CER, ceramide; Cyt *c*, cytochrome *c*; EGF, epidermal growth factor; eNOS, endothelial nitric oxide synthase; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; INF-γ, interferon-γ; nNOS, neuronal nitric oxide synthase; NOS, nitric oxide synthase; nSMase, neutral sphingomyelinase; OE, *N*-oleoylethanolamide; oxLDL, oxidized low density lipoprotein; PDGF, platelet-derived growth factor; PKC, protein kinase C; PI_3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; SM, sphingomyelin; TNF-α, tumor necrosis factor-α; TPA, 12-*O*-tetradecanoylphorbol-13-acetate

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by glucocerebrosidase (Fig. 1) [46,47]. In addition, a secreted aSMase and a cytosolic Mg^{2+}-independent nSMase form have also been described [48,49]. On the other hand, the cellular ceramide accumulation is dependent on expression levels and activities of acidic and neutral/alkaline ceramidases whose enzymes are involved in hydrolysis of the ceramide into sphingosine which in turn might serve as a substrate for sphingosine kinase given the sphingosine-1-phosphate (Fig. 1) [20,50]. These different classes of enzymes involved in ceramide metabolism including SMases and ceramidases are characterized by their activities at optimum pH [41,51]. Notably, nSMase and alkaline/neutral ceramidase are cell membrane-associated enzymes that are active at neutral to alkaline pH while aSMase and acidic ceramidase are active only at pH 4.5–5.5 and therefore they must be localized within acidic compartments to be active. Among the acidic compartments there are caveolae or rafts and endosomes/lysosomes [15,48,52,53].

In general, the increase of cellular ceramide levels in acidic compartments concomitant with the activation of aSMase and/or inhibition of acidic ceramidase induced by diverse external stimuli results in the stimulation of apoptotic/necrotic signaling pathways whereas the ceramide accumulation derived from the stimulation of nSMase or inhibition of neutral ceramidase appears rather to be involved in other cellular responses [34,41]. However, certain studies have also indicated that the activation of nSMase by TNF-α might lead to ceramide generation-induced cytotoxic responses in certain cell types [27,28]. Importantly, a rise of cellular ceramide levels might further result in the activation of positive feedback loops that amplify the ceramide production.

2.1. *Role of positive autoregulative loops of SMases in the ceramide generation*

The cellular ceramide accumulation might further stimulate aSMase or nSMase-induced ceramide production via a positive feedback loop and thereby promote ceramide accumulation-mediated apoptotic death. Indeed, it has been shown that the ceramide accumulation in the lysosomal compartment induced by oxidized low density lipoproteins (oxLDLs) or exogenous C_6-ceramide (CER), leads to the upregulation of aSMase expression and enhanced ceramide-initiated apoptotic death of human macrophages and fibroblasts [9]. Conversely, it has been observed that ceramide generation in macrophages results in the stimulation of nSMase activity [54]. On the basis of these observations, it appears that ceramide accumulation within a specific cellular site might lead to an increase of expression and/or activity of aSMase and nSMase found in other cellular compartments. Hence, this autofeedback mechanism might explain how ceramide generation in the plasma membrane can modulate the activities of aSMase and nSMase and ceramide production in other compartments such as mitochondria that also serve as intracellular sites in triggering ceramide-induced apoptotic signaling cascades.

3. Localization of ceramide-induced apoptotic/necrotic cell death

The functions assumed by the ceramide in triggering apoptotic/necrotic cell death seem to be dependent on external stimuli and cell types. Notably, the ceramide molecules generated within the acidic and/or neutral compartments might cause physical perturbations in cell membranes and/or induce the activation of different response elements which can act in turn as initiators or effectors of the cell death [17,19]. However, the major challenge that must be resolved consists in establishing which acidic and/or neutral compartments serve as functional pools for ceramide-induced cell death. Indeed, although multiple investigations have been carried out to estimate the role of cellular ceramide generation in cytotoxic effects induced by diverse agents, no experimental evidence has permitted a precise answer to this question. In fact, numerous studies support the concept of a compartmentation in different cellular organelles of ceramide molecules involved in the diverse phases of apoptotic and necrotic processes. Among these subcellular compartments which have been identified by electronic microscopy and by using molecular probes acting specifically with particular cellular constituents are caveolae or rafts, lysosomes and mitochondria [15,18,51,55].

3.1. *Plasma membrane*

The caveolae and raft structures are the specialized plasmalemmal microdomains enriched in sphingolipids, cholesterol

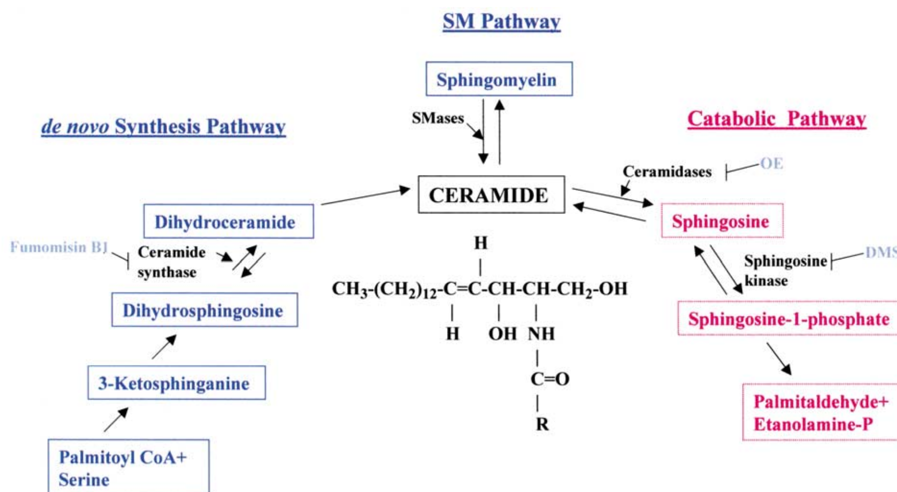

Fig. 1. Metabolic pathways involved in ceramide synthesis and degradation. The primary structure of the shown ceramide corresponds to natural brain ceramides which are a mixture of fatty acids containing the radical (R) of stearic (C_{18}), nervonic (C_{24}) and lignoceric (C_{24}) long acyl chains. Note the small polar headgroup of ceramide (–OH).

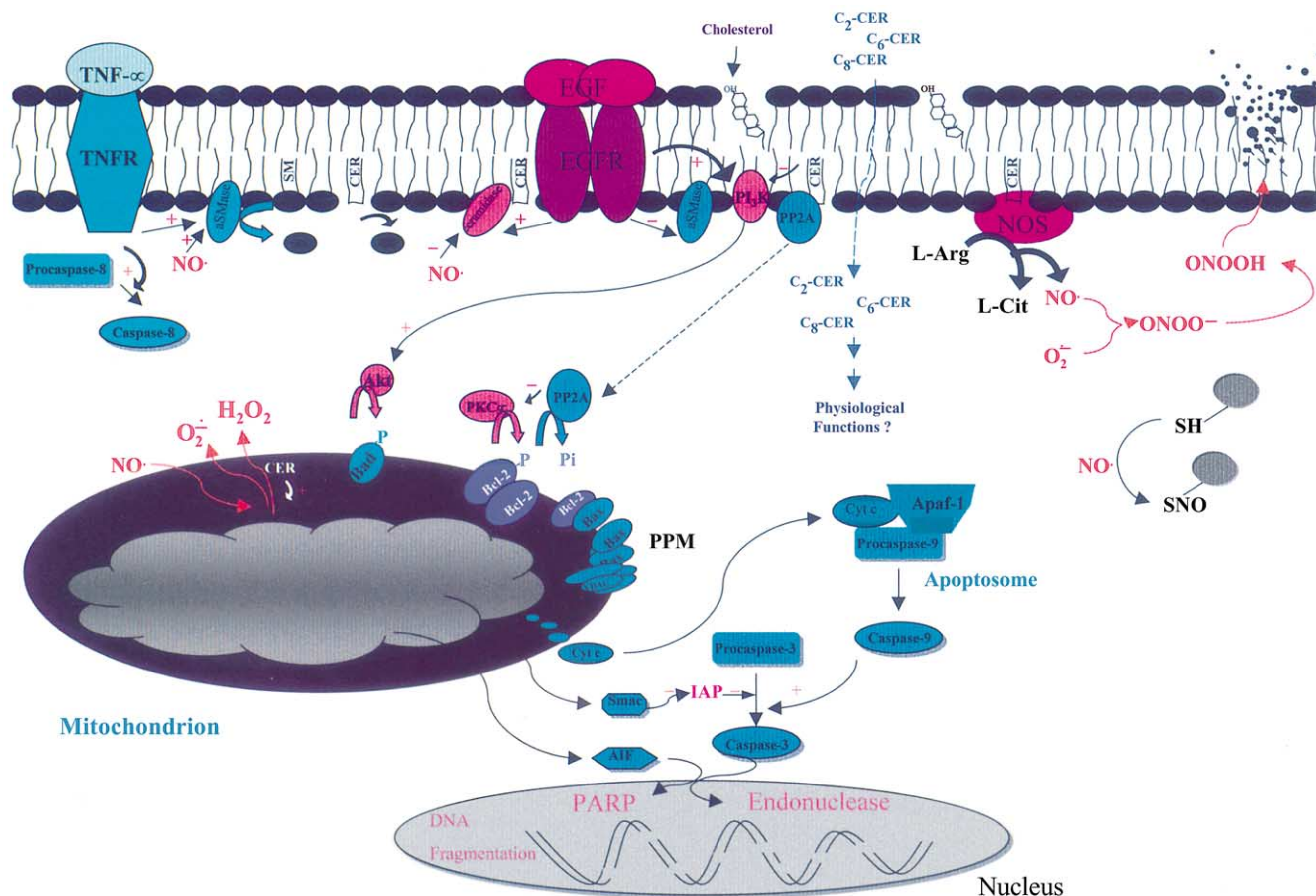

Fig. 2. Proposed cellular targets for the ceramide and NO• reactive species generated following activation of aSMase or inhibition of ceramidase activity. Note the possible inhibitory effects of the EGF-EGFR system on ceramide (CER) accumulation which might be mediated via the inhibition of aSMase activity and/or acidic ceramidase activation. Moreover, the relevance of physiological functions mediated by the intracellular short chain C_2-, C_6- and C_8-CER analogs is questioned.

and lipid-anchored membrane proteins but relatively depleted in phospholipids which might be characterized due to their resistance to solubilization by Triton X-100 at 4°C and by the presence of caveolin in caveolae [15]. In addition, these microdomains might contain different important signal transduction molecules including receptor tyrosine kinases (EGFR and PDGFR), TNFR, Fas, G protein-coupled receptors, initiators and effectors of intracellular pathways (G proteins, adenylate cyclase, Src, Ras, PI_3K, protein kinase C (PKC), Bad), lipid cascade elements (SM, ceramide, SMase and ceramidase) and nitric oxide synthase (NOS) [15,55–58]. It has been proposed that these membrane microdomains can permit rapid and efficient interactions between different signaling response elements. In particular, caveolae and rafts which might provide an acidic environment for aSMase have been proposed as acidic sites for ceramide accumulation-initiated cell death [18,21,52]. For instance, the Fas translocation and clustering into ceramide-rich membrane structures appears to be a prerequisite to trigger apoptotic death [56,59,60]. Moreover, the ceramide molecules generated by aSMase within caveolae and rafts might modulate the activities of other components localized in close proximity of these membrane structures such as NOS, PKC and protein phosphatases whose compounds in turn can regulate the activities of different intracellular pro-apoptotic initiators and effectors (Fig. 2) [8,21,23,61,62]. In addition, a nSMase activity has been detected within caveolae and it has been suggested that this enzyme might mediate, in conjunction with aSMase, the ceramide generation and apoptotic signals induced by TNF-α [28]. Finally, a high local concentration of ceramide in the inner leaflet of the plasma membrane appears also to lead to disorganization of raft structures and alteration of cell surface morphology concomitant with the last phases of apoptosis [17].

3.2. Lysosomal compartment

The ceramide generation within the acidic lysosomal/endosomal compartment has also been proposed to be involved in stress-induced apoptosis. Notably, it has been observed that a rise of intralysosomal pH in fibroblasts inhibits the apoptotic effect induced by oxLDL treatment [9]. Moreover, the overexpression of acidic ceramidase has been observed to prevent this oxLDL-induced apoptosis. In fact, the oxLDL molecules are constituted of oxysterols and fatty acid peroxides that might induce the apoptosis. In addition, oxLDL, TNF-α, Fas ligand and INF-γ have been reported to induce the release of endosomal/lysosomal cathepsin D protease into the cytosol whose enzyme may initiate a proteolytic cascade leading to apoptosis [63]. Hence, cathepsin D might represent an important apoptotic signaling element induced by ceramide accumulation in the acidic lysosomal compartment.

3.3. Mitochondrial compartment

Certain works have revealed that the components of sphingolipid signaling pathways such as SM, ceramide, ceramide synthase, SMase and ceramidase are present within the mitochondrial membranes [11,19,50]. However, whether the mitochondrial sphingolipids are also regrouped within the raft-like structures has not been established precisely. Moreover, the role assumed by mitochondria in ceramide-induced cell death has only been considered recently due to the fact that the mitochondria appear generally to exhibit a weak increase of ceramide levels in response to tested anticarcinogenic agents. Therefore, it is difficult to show a direct relationship between the mitochondrial ceramide content and the rate of cell death. Importantly, it has been observed that the transfection of fusion proteins consisting of bacterial nSMase combined with specific targeting signals to different cellular compartments including plasma membrane, cytoplasm, mitochondria, Golgi apparatus, endoplasmic reticulum or nucleus resulted in an increase of ceramide levels in all these compartments while only the mitochondrial ceramide accumulation caused the apoptotic death of MCF7 breast cancer cells [11]. Furthermore, several anticarcinogenic agents acting as stimulators of ceramide generation have been reported to activate a mitochondria-dependent apoptotic pathway [10,24,26,64–66]. Thus, the ceramide production within the mitochondrial membrane induced by different external stimuli might trigger certain events during apoptotic/necrotic cell death [11,41].

4. Estimation of the role of ceramide in triggering apoptotic/necrotic processes

4.1. Characterization of ceramide functions by using exogenous ceramides

Some studies performed using exogenous and cell-permeable ceramide analogs such as short chain C_2-, C_6- and C_8-CER as well as exogenous and natural long chain C_{16}- to C_{24}-CER revealed that the ceramide might participate in the apoptotic/necrotic death of several normal and cancer cell types [1,2,41,45,64]. However, the physiological relevance of observations obtained with exogenous short chain ceramide molecules must be questioned because these derivatives which are able to reach the cytosol are not necessarily delivered and incorporated in appropriate intracellular compartments (Fig. 2). Indeed, the exogenous long chain ceramide derivatives and natural ceramides appear rather to accumulate on the cell surface and are not delivered into the cytosol [11,18,41,56]. In fact, this is not surprising since the high degree of hydrophobicity of C_{16}- to C_{24}-acyl chains (*R*) and small polar headgroup (–OH) of natural ceramide molecules might do harm to their delivery to cytosol (Fig. 1). In this context, it has also been observed that induction of endogenous ceramide generation within the lysosomal compartment in fibroblasts from a patient with Farber disease, which is characterized by inborn lysosomal ceramidase deficiency, was accompanied by ceramide accumulation in lysosomes but not by their delivery to cytosol [67]. However, it has been reported that certain exogenous long chain ceramide molecules are able to reach the active signaling pool on the inner leaflet of the plasma membrane by a trans-bilayer transport mechanism designated flip-flop in several normal and cancer cells. Interestingly, use of natural bovine brain ceramides prepared by dispersion in a solvent mixture consisting of ethanol and dodecane has notably permitted the demonstration of a direct relationship between ceramide accumulation in the plasma membrane of U937 cells and the rate of apoptotic cell death [1]. Moreover, it has been observed that use of natural ceramide makes it possible to restore the sensitivity of aSMase−/− hepatocytes to cytotoxic effects of Fas receptor ligand [68]. Altogether, these findings strongly suggest that the natural long chain ceramide molecules are not delivered in cytosol but rather accumulate within the cellular compartment where they are generated such as cell membranes. Thus, the results obtained with the exogenous and cell-permeable short chain ceramide analogs which might be delivered in the cytosol and subsequently to other intracellular compartments must be analyzed with caution because the structural and biological functions mediated by these ceramide derivatives might differ from those of endogenous long chain ceramides.

4.2. Characterization of ceramide functions by using activators and inhibitors of endogenous ceramide accumulation

The use of exogenous enzymes implicated in generating cellular ceramide including aSMase from human placenta and bacterial nSMase as well as acidic and alkaline/neutral ceramidase inhibitors, such as *N*-oleoylethanolamide (OE) and (1*S*,2*R*)-D-erythro-2-(*N*-myristoylamino)-1-phenyl-1-propanol respectively, has made it possible to perceive that the ceramide accumulation in distinct acidic and neutral compartments might lead to the cell death of normal and cancer cells [11,27,40,41,69]. For instance, the induction of aSMase and ceramide synthase or inhibition of acidic ceramidase activity by different agents such as TNF-α, Fas ligand, IL-1β, IFN-γ, nerve growth factor, Δ^9-tetrahydrocannabinol, the endocannabinoid anandamide, 25-dihydroxyvitamin D_3, NO donors, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), OE and ionizing radiation has been observed to result in a massive ceramide-initiated cell death [5,8,25,27,31,41,42,48,56]. In particular, the ceramide generation induced by aSMase appears to be one of the principal response elements involved in the cell death mediated via stress [9]. In support of this, it has been observed that Niemann–Pick lymphoblasts with an aSMase deficiency and thymocytes from aSMase knockout mice did not undergo apoptosis in response to ionizing radiation [33]. Moreover, the ceramide generation within an acidic compartment induced by exogenous aSMase or TNF-α has been proposed to play a critical role in triggering the apoptosis mediated by these agents in human leukemic L-929 and U937 cells and prostate cancer LNCaP cells [26,27]. In addition, inhibition of de novo ceramide synthesis by using a specific inhibitor of ceramide synthase, fumonisin B1, has indicated that this cascade is involved in the apoptotic response induced by many cytotoxic agents (Fig. 1) [5,10,42].

5. Characterization of different modes of ceramide-induced cell death

5.1. Apoptosis

The normal and cancer cells undergo programmed cell death in response to specific signals which are accompanied by activating common cascade elements such as procaspases. The mitochondria have a central function in regulating apoptosis signaling cascades because this cellular compartment, which is evolutionarily conserved, contains several antiapoptotic and proapoptotic effectors that act in concert during

the cell life to determine if a cell must live or die [70–73]. Among the factors involved in the mitochondrial phase of the apoptotic process, there is the Bcl-2 protein family that plays a key role in normal and cancer cells [72,73]. Notably, several proapoptotic members of this family such as the proteins Bax, Bak, Bad and Bid have been shown to change location from raft structure or cytoplasm to mitochondria during apoptosis where they induce the mitochondrial inner and outer membrane disruption and matrix swelling [74–77]. In fact, these proapoptotic proteins might participate in the formation of mitochondrial permeability transition pores which might lead to the release of several mitochondrial factors such as cytochrome *c* (Cyt *c*), apoptosis protease activator factor-1, apoptosis-initiating factor (AIF), and second-generation mitochondrial activator (Smac) into the cytosol where they synergistically activate the different procaspases (Fig. 2) [72,78,79]. In contrast, the antiapoptotic members of the Bcl-2 family such as Bcl-2 and Bcl-x_L proteins, which are localized on the mitochondrial surface at contact sites where inner and outer membrane are in close proximity, might counteract the actions of pore-forming proteins such as Bax by forming heterodimers Bax/Bcl-2 or Bax/Bcl-x_L and thereby prevent the release of Cyt *c* (Fig. 2) [4,11,72,80,81].

The ceramide accumulation seems also to be involved in activation of caspase cascades in certain cell types but the common effector where these two apoptotic signaling pathways intersect has not been identified precisely [4,13,14,17,24,82]. In general, the changes in endogenous ceramide levels induced by diverse agents including TNF-α and Fas receptor ligand seem to occur after activation of procaspase-8 but prior to activation of procaspases-9 and -3 that act as important effectors in the apoptotic cell death [24,26,72]. Moreover, certain cellular targets have been proposed as intracellular mediators for ceramide-induced apoptosis via the mitochondria including reactive oxygen species (ROS), the ceramide-activated protein kinases and phosphatases [8,21,23,29,30,55,83–86]. In particular, the ceramide accumulation within the mitochondria might induce changes in the electron transport chain leading to generation of ROS such as hydrogen peroxide concomitant with the membrane permeability transition whose mitochondrial events seem to be involved in triggering ceramide-mediated apoptotic/necrotic responses (Fig. 2) [32,65,66,87,88]. For instance, it has been reported that the treatment of rat liver cells with TNF-α results in an increase of the mitochondrial ceramide amount concomitant with an overproduction of hydrogen peroxide [32]. Moreover, it has been noticed that the ceramide might potentiate the increase of mitochondrial membrane permeability transition induced by pore-forming Bax protein [21,88]. Importantly, this mitochondrial permeability transition mediated by ceramide-induced Bax activation also resulted in a rise of Cyt *c* release, caspase-3 activation and DNA fragmentation in prostatic cancer DU145 cells [12]. In addition, the stress-induced ceramide accumulation in neuroblastoma cells might lead to release of mitochondrial AIF [78]. Interestingly, the AIF translocation from mitochondria to the nucleus induced by the ceramide has also been associated with a partial chromatin condensation which is manifest without activation of caspase cascades while peripheral chromatin condensation and formation of nuclear bodies seem rather to implicate the cytosolic Cyt *c* and caspases.

On the other hand, the cellular ceramide accumulation might also counteract the antiapoptotic effect of Bcl-2 in several cell types. Indeed, recent works have shown that C_2-CER can activate a cytosolic heterotrimeric protein phosphatase 2A (PP2A) by releasing its Bα subunit which is translated from the cytoplasm to mitochondria where it induces rapid dephosphorylation of Bcl-2 and thereby its inaction (Fig. 2) [30,84,89]. Furthermore, it has been reported that C_2- and C_6-CER might cause an inhibition of PKC-α by activating a protein phosphatase and this leads to inhibition of Bcl-2 protein activity in certain cell types [21,84,90]. Hence, these mitochondrial effects of the ceramide concomitant with caspase activation might lead to a number of distinctive biochemical and morphological changes such as chromosomal condensation, pH change, internucleosomal DNA fragmentation and shrinking of the cells which are regarded as the points of no return resulting ultimately in cell death by secondary necrosis and disruption of apoptotic bodies [42].

5.2. Necrosis

Several works have indicated that another type of cell death which seems to be more necrotic than apoptotic might be manifest in certain normal and cancer cell types under specific stimulatory conditions. This necrotic pathway of cell death appears to be mediated by bifurcation of apoptotic pathways above described through the activation of other specific death factors which might act independently or in cooperation with the caspases [6,7,37,45,82,87,91–93]. In this context, the cellular ceramide accumulation appears to result in activation of distinct apoptotic and necrotic signaling pathways in numerous normal and cancer cell types. Indeed, it has been observed that the broad caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (2-VAD-fmK) markedly decreases the rate of DNA fragmentation and apoptotic cell death but does not inhibit the necrotic cell death induced by C_2-CER in normal and malignant human lymphocytes and prostatic cancer cells LNCaP, DU145 and PC3 [6,7,93]. Moreover, it has been observed that the natural ceramide mainly induced the necrotic death of RINm5F insulin-producing cells [94]. Interestingly, the treatment of rat liver cells with C_2-CER or TNF-α has also been observed to result in an increase of the mitochondrial ceramide levels [59,95]. In fact, the mitochondrial ceramide molecules might directly cause disruption of the mitochondrial electron transport chain by inducing the production of oxygen species such as superoxide radical ($O_2^{\bullet}$) and hydrogen peroxide (H_2O_2) whose reactive products can induce lipid peroxidation (Fig. 2) [64]. In this context, the ceramide might trigger, in a concentration- and time-dependent manner, the necrotic death of hepatocytes by disrupting mitochondrial membranes [79]. Altogether, these observations suggest that apoptotic and non-apoptotic mechanisms of cell death might be induced by ceramide and high concentrations of ceramide might favor cell death by a necrotic pathway.

6. Role of $NO^{\bullet}$ generation in ceramide-induced apoptotic/necrotic cell death

Some observations indicate that functional relationships exist between the cellular levels of ceramide and free radical gas, nitric oxide ($NO^{\bullet}$, nitrogen monoxide) during activation of apoptotic/necrotic cascades in several normal and transformed cells. In support of this, it has been reported that the ceramide precursors and enzymes modulating its metabolism are colo-

calized within the caveolae or rafts with NOS isoforms such as inductive type iNOS or constitutively expressed types eNOS and nNOS which catalyze the formation of NO• and L-citrulline from L-arginine (Fig. 2) [15,62]. Then, this suggests that these membrane microdomains might serve as functional sites for the ceramide and NO• generation whose products might participate alone or in synergy to trigger the apoptotic/necrotic signaling cascades. As a matter of fact, the ceramide and NO• accumulation within caveolae and rafts seems to lead to cell membrane damage whose injuries are associated with the apoptotic/necrotic cell death [32]. In fact, NO• molecules can react with the superoxide anions ($O_2^{\bullet}$) to generate the highly cytotoxic peroxynitrite anion ($^{-}$OONO) or its protonated form (HOONO). These oxidant products might subsequently initiate the membrane lipid peroxidation and thereby be responsible in part for necrotic features observed for numerous cancer cells at high concentrations of NO• and/or ceramide (Fig. 2).

Although the molecular mechanisms involved in the relationships between the ceramide and NO•-induced cell death have not been identified precisely, certain cellular signaling connections have been proposed. In particular, NO• generation via iNOS activation might result in a massive ceramide accumulation by activating aSMase/nSMase and inhibiting acidic ceramidase by NO• concomitant with an increase of apoptotic/necrotic cell death [85,96]. Moreover, the cytotoxic effects induced by the cellular ceramide accumulation might be potentiated in the presence of different pharmacological sources of NO•-donating compounds such as spermine-NO•, (*Z*)-1-{*N*-methyl-*N*-[6-(*N*-methylammoniohexyl)-amino]}diazen-1-ium-1,2-diolate, *S*-nitroso-glutathione and sodium nitroprusside in diverse normal and cancer cell types such as renal, mesangial and glomerular endothelial cells and promyelocytic leukemia HL-60 cells [85]. Interestingly, the ceramide has also been proposed to act as a mediator of nitrosative and oxidative stress-induced cell death mediated by different NO• donors and superoxide-generating substances [97].

In addition, NO• and its derivatives generated within caveolae or rafts might act as mediators to translate the external stimuli induced by ceramide at the surface of cell membrane in different intracellular signaling events. Indeed, the high diffusibility and hydrophobicity of NO• and its rapid conversion in vitro and in vivo to very unstable and reactive nitrogen species which can interact with several target macromolecules containing NO•-reactive Cys residues and enzymes such as lipoxygenases, peroxidases and prostaglandin H synthase might lead to diverse perturbations in cellular compartments including the mitochondria that are detrimental to cells (Fig. 2) [98]. Interestingly, eNOS activation by C_2-CER has been observed by quantitative immunofluorescence techniques to result in its translocation from endothelial cell membrane to intracellular sites [62]. In addition, NO• and its derivatives might also induce important damage to mitochondrial membranes and thereby initiate the activation of caspase cascades and apoptotic death of numerous cells [96]. Notably, NO• and ROS such as peroxynitrite, which can interact at the binding site of oxygen on the mitochondrial Cyt *c* oxidase, seem to act as potent inhibitors of the mitochondrial respiratory chain [26,99]. Thus, on the basis of these observations, it appears that cellular NO• might act in concert with the ceramide to induce a greater degree of apoptotic/necrotic death via the damage caused to cellular membranes.

7. Antiapoptotic effects of growth factors induced by decreasing ceramide level

A variety of external stimuli induced by numerous growth factors and neuropeptides are generally necessary to permit cell survival by inhibiting apoptotic signals. Among these, certain growth factors such as EGF and PDGF have been reported to induce in part their antiapoptotic effects in many normal and cancer cells in interacting with their cognate receptor tyrosine kinases EGFR and PDGFR which are localized within caveolae or rafts [15,18,21,40,41,52,57,58]. In particular, the antiapoptotic signals mediated by the EGF–EGFR system might be induced in part by lowering ceramide levels via an inhibition of aSMase activity and/or by activating the acidic ceramidase [41]. Hence, EGF appears able to inhibit TNF-α, IFN-γ, aSMase and natural C_{16}-CER-induced apoptosis in trophoblasts by decreasing cellular ceramide levels [41]. Moreover, it has been observed that the inhibition of acidic ceramidase activity by specific inhibitors such as OE potentiates apoptotic responses induced by diverse cytotoxic agents in the presence of EGF [41,42]. Thus, since the acidic ceramidase is overexpressed in certain cancer cell types including prostatic cancer cells [100], it is likely that the inhibitory effect of EGF on intrinsic activity of this enzyme might contribute to cancer cell survival.

Importantly, the activation of the PI_3K/Akt/Bad survival signaling pathway mediated by growth factors such as EGF, PDGF and insulin-like growth factor 1 through their cognate transmembrane receptors appears to be required for the prevention of apoptosis in many normal and cancer cell types [18,54,101–103]. Indeed, dysfunction of the PI_3K/Akt signaling pathway has been associated with the transformation and tumor progression of many human cancer types [102]. The phosphorylation of mitochondrial apoptotic Bad factor by Akt serine/threonine kinase seems to be responsible in part of this cell survival signal (Fig. 2) [53]. In this context, it is interesting to notice that IL-4 deprivation of the cells has been observed to lead to the segregation of Bad from lipid rafts to mitochondria suggesting the possibility of complex cross-talks between these two subcellular compartments [104]. On the other hand, it has also been reported that the proapoptotic stress response elements are able to downregulate the PI_3K/Akt survival signaling cascade by activating aSMase and ceramide generation. In fact, the ceramide seems to act as second messenger by recruiting caveolin within caveolae whose protein in turn might inhibit PI_3K activity and survival signals (Fig. 2) [18].

In addition, the caveolae and raft sphingolipid-enriched microdomains contain cholesterol and they are characterized by a highly packed structure which might be associated with a high degree in acyl chain order of ceramide and hydrophobicity of cholesterol core (Fig. 2) [5,34,105]. Then, since the ceramide generation within these microdomains leads to apoptosis in numerous cells, it is likely that changes in the lipid composition of caveolae and rafts such as a decrease of the sphingomyelin/ceramide ratio or cholesterol amount might alter their structural characteristics. Indeed, lipid changes in caveolae and rafts might influence the apoptotic signal elements which are localized within these microdomains and are involved in strict control of cell survival [18,33,34,52]. As a matter of fact, several observations have indicated that the cholesterol content in these membrane microdomains is a

determinant factor that modulates the activity of different signaling elements [53,105,106]. Cholesterol can play a critical role for integrity of these ordered lipid structures by intercalating under sphingolipid headgroups and thereby promoting a tighter lipid packing (Fig. 2) [105,107]. For instance, the caveolin-negative human prostate cancer LNCaP cells contain cholesterol-rich lipid rafts that mediate in part the antiapoptotic signaling of EGF through the activation of the PI_3K/Akt signaling pathway [53]. Strikingly, the binding of EGF or PDGF to their cognate receptors concentrated in caveolin-negative raft structures might also lead to heterologous desensitization of the other receptor by translocation and sequestration in caveolae [108]. Moreover, disruption of caveolae by cholesterol depletion has been observed to inhibit EGFR activity and subsequent Akt activation induced by angiotensin II [109]. On the basis of these observations, it appears that the changes in ceramide, cholesterol and growth factor receptor levels within lipid rafts and caveolae might represent a dynamic process that modulates the activity of multiple signaling transduction elements.

8. Prospects for the development of new anticancer therapies

To improve the efficacy of anticancer therapies against invasive cancers, it is necessary to identify the molecular mechanisms responsible for the resistance of metastatic cancer cells to current treatments. In this context, the resistance of metastatic cancer cells from prostate, colon, ovary, breast, lung and skin to radiotherapy and chemotherapy has notably been associated with the aberrant response elements in ceramide and/or caspase signaling cascades [5,16,22,36–38,44]. As a matter of fact, it has been observed that the decrease of endogenous ceramide levels by overexpression of glucosylceramide synthase results in a multidrug resistance phenotype in human cancer cells [44]. Of therapeutic interest, the cellular ceramide generation induced by different agents such as TNF-α and TPA has been reported to sensitize the metastatic cancer cells to the apoptotic effect of ionizing radiation [5,37,110]. Moreover, cellular ceramide accumulation has been observed to cause the death of cancer cells from prostate and cervical tumors while normal cells were less sensitive to cytotoxic effects induced by ceramide [8,44]. Therefore, the mixed use of distinct agents acting as activators of ceramide accumulation such as EGFR or PDGFR inhibitors and $NO^{\bullet}$ donors and caspase cascades might represent a promising combinatory strategy to enhance the sensitivity of metastatic cancers to conventional anticarcinogenic therapies.

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