ABSTRACT

We have shown previously that the death receptor CD95 could contribute to anticancer drug-induced apoptosis of colon cancer cells. In addition, anticancer drugs cooperate with CD95 cognate ligand or agonistic antibodies to trigger cancer cell apoptosis. In the present study, we show that the anticancer drug cisplatin induces clustering of CD95 at the surface of the human colon cancer cell line HT29, an event inhibited by the inhibitor of acid sphingomyelinase (aSMase) imipramine. The cholesterol sequestering agent nystatin also prevents cisplatin-induced CD95 clustering and decreases HT29 cell sensitivity to cisplatin-induced apoptosis and the synergy between cisplatin and anti-CD95 agonistic antibodies. CD95, together with the adaptor molecule Fas-associated death domain and procaspase-8, is redistributed into cholesterol- and sphingolipid-enriched cell fractions after cisplatin treatment, suggesting plasma membrane raft involvement. Interestingly, nystatin prevents the translocation of the aSMase to the extracellular surface of plasma membrane and the production of ceramide, suggesting that these early events require raft integrity. In addition, nystatin prevents cisplatin-induced transient increase in plasma membrane fluidity that could be required for CD95 translocation. Together, these results demonstrate that cisplatin activates aSMase and induces ceramide production, which triggers the redistribution of CD95 into the plasma membrane rafts. Such redistribution contributes to cell death and sensitizes tumor cells to CD95-mediated apoptosis.

INTRODUCTION

Chemotherapeutic drugs can induce apoptotic death in a number of cultured tumor cell lines. The main death pathway activated by specific cellular damage induced by these drugs is a caspase-dependent intrinsic pathway that involves Bcl-2-related proteins and the mitochondria. Whether and how the so-called extrinsic pathway that involves the death receptor CD95 (also known as Fas or Apo-1) contributes to cytotoxic drug-induced tumor cell death is a more controversial issue (1, 2). CD95 shares with other death receptors of the tumor necrosis factor receptor superfamily an intracellular protein-interaction domain, known as the death domain, which, on engagement of the receptor by its ligand or an agonistic antibody, rapidly recruits the adaptor protein Fas-associated death domain (FADD), which, in turn, recruits the initiator caspase-8 proenzyme via a homologous death effector domain interaction (3–5). Caspase-8, which is activated in the resulting death-inducing signaling complex (DISC), either directly activates a downstream caspase cascade or cleaves the Bcl-2-related, BH3-only protein Bid to activate the caspase cascade through the mitochondria (5, 6).

Recent studies have shown that CD95-mediated cell death required the clustering of the receptor in lipid rafts (7–13). These rafts are dynamic assemblies of tightly packed proteins and lipids that float freely within the cellular membrane bilayer or cluster to form large ordered platforms. These structures, which are enriched in cholesterol, sphingomyelins, and glycosphingolipids, play a central role in many cellular processes that include membrane sorting and trafficking, cell polarization, and signal transduction. Some of the membrane proteins are constitutive raft residents, whereas other move in and out of rafts (14–16). Depending on the cell type and the raft isolation technique, it has been shown that CD95 is localized in rafts either constitutively or following interaction with its ligand (17).

In mouse thymocytes and embryonic fibroblasts, FADD and procaspase-8 are recruited to rafts on CD95 ligation, which is necessary and sufficient to initiate CD95-mediated death signaling. Disruption of rafts abolishes the initiation of CD95 death signaling in thymocytes and Jurkat cells (10, 18).

How CD95 can be redistributed to lipid rafts when interacting with its ligand has been partially depicted. The formation of initial DISCs leads to the activation of an acid sphingomyelinase (aSMase). This enzyme translocates from an intracellular compartment to the extracellular leaflet of membrane rafts where it releases extracellularly oriented ceramides. These ceramides could induce coalescence of elementary rafts and/or reorganization of these domains, thereby amplifying CD95 signaling by formation of larger complexes, further recruitment of FADD and procaspase-8, and stabilization of the DISC (7, 8, 11, 12). Transient alterations in plasma membrane fluidity, which have been identified in cells exposed to various apoptotic stimuli, also could be implicated in the recruitment of CD95 to rafts (19–21).

We have shown previously that exposure of colon carcinoma cells to various cytotoxic drugs could induce the formation of a CD95-including DISC in a ligand-independent manner. We have demonstrated the role played by this signaling complex in apoptosis induction by these drugs (22). In addition, we have shown that cytotoxic drugs could synergize with death receptor ligands to induce tumor cell death (23–25). In the present study, we show that the anticancer drug cisplatin induces CD95 receptor clustering and redistribution into lipid rafts at the surface of HT29 human colon carcinoma cells. FADD and procaspase-8 also are recruited into rafts under cisplatin treatment. CD95 colocalizes with aSMase and ceramide in cisplatin-treated cells, and imipramine, an aSMase inhibitor, prevents cisplatin-induced CD95 clustering. Thus, to involve the CD95-mediated pathway in tumor cell death, cisplatin activates an aSMase that contributes to CD95 redistribution into the lipid rafts.

MATERIALS AND METHODS

Cells, Treatment, and Apoptosis Measurement

The HT29 human colon carcinoma cell line was obtained from the American Tissue Culture Collection (Manassas, VA) and cultured in Eagle’s MEM (BioWhittaker Co., Fontenay sous Bois, France) complemented with 10% FCS (Life Technologies, Inc., Rockville, MD) and 2 mM L-glutamine (BioWhittaker Co.). Cells (7 × 10^5) were seeded in six-well flat-bottomed plates for 24 h before treatment with cisplatin (5 μg/ml; Sigma-Aldrich, St. Louis, MO), 100 ng/ml agonistic anti-CD95 antibody (CH11 clone; Immunotech, Coulter, Fullerton, CA), a combination of both, or 1 μM staurosporine (Sigma-Aldrich). When indicated, cells were pre-exposed to 10 μg/ml nystatin (Sigma-Aldrich) or to 50 μM imipramine (Sigma-Aldrich) for 1 h. Apoptosis was evidenced by staining nuclear

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chromatin with 1 μg/ml Hoechst 33342 (Aventis, Strasbourg, France) for 15 min at 37°C and then determining the percentage of cells with characteristic morphology changes by fluorescence microscopy (300 cells/point).

Immunofluorescence Microscopy

HT29 cells seeded and treated in Lab-Tek chamber slides (Nunc S/A, Polylabo, Strasbourg, France) were subsequently fixed in 2% paraformaldehyde (Sigma) for 10 min, washed twice with PBS, and then preincubated with 0.5% BSA for 15 min before incubation with a mouse anti-CD95 (ZB4, 1:100 dilution; Immunotech), a goat anti-aSMase (Santa Cruz Biotechnology, Tebu-Bio S.A., Le Perray en Yvelines, France), a mouse anti- caveolin-2 (Transduction Laboratories, Lexington, KY), or a mouse anti-ceramide (Alexis Biochemicals, Coger, Paris, France) antibody for 2 h at room temperature, washed twice in PBS, and incubated with Texas red-conjugated antinioume immunoglobulins (1:50 dilution; Jackson ImmunoResearch Laboratories), or FITC-conjugated antinioume immunoglobulins (Jackson ImmunoResearch Laboratories), or FITC-conjugated antinioume immunoglobulins (Molecular Probes Europe BV, Leiden, the Netherlands). The expression of CD95, aSMase, caveolin-2, and ceramide was analyzed using a confocal laser-scanning microscope (TCSS4d; Leica Microsystems, Wetzlar, Germany).

Cell Fractionation and Immunoblotting

Untreated and treated HT29 cells (8 × 10⁷) washed in ice-cold PBS were lysed in 1 ml buffer MBS-buffered saline (25 mM 2-(N-morpholino)ethanesulfonic acid (pH 6.5), 150 mM NaCl, and complete protease inhibitor mixture; Roche Biochemicals, Meylan, France) containing 1% Triton X-100 for 30 min at 4°C before passing them through an ice-cold cylinder cell homogenizer. Lysates were then diluted with 2 ml buffer MBS containing 80% sucrose (w/v) and placed at the bottom of a linear sucrose gradient consisting of 5 ml 5–40% sucrose (w/v) in MBS. Samples were centrifuged at 39,000 rpm for 20 h at 4°C, and 1-ml fractions were collected from the top of the gradient. To determine the location of CD95, FADD, and procaspase-8 in the cells, 60 μl of each fraction were subjected to SDS-PAGE and immunoblot analysis. After blocking for 1 h at room temperature with 8% powdered skimmed milk in Tris-buffered saline/Tween [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween 20], membranes were incubated with an anti-CD95 rabbit polyclonal antibody (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), an anti-FADD monoclonal antibody (1:1000 dilution; Transduction Laboratories), an antiprocaspase-8 monoclonal antibody (1:1000 dilution; Immunotech), an antiprocaspase-2 monoclonal antibody (1:1000 dilution; Pharmingen, San Diego, CA), or an anticaveolin-2 monoclonal antibody (1:1000; Transduction Laboratories). Membranes then were washed twice with Tris-buffered saline/Tween and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) before protein identification using an enhanced chemiluminescence detection kit (Amersham, Piscataway, NJ).

Phospholipid and Cholesterol Analysis of Lipid Raft Fractions

Lipids were extracted using the method of Folch et al. (26).

Phospholipid Analysis. An aliquot of the chloroformic phase was evaporated, and 100 μl of chloroform/methanol (4:2) were added for quantitative liquid chromatography/mass spectrometry. Phospholipid analysis was performed on a HPLC Si 2 × 200-mm column (Agilent Technologies, Palo Alto, CA) with a binary gradient of solvent A (5 mM ammonium acetate in chloroform/methanol, 4:1) and solvent B (5 mM ammonium acetate in chloroform/methanol/water, 6:3:1, v/v). Positive electrospray-mass spectrometry was performed using an MS/MS 1100 mass spectrometer (Agilent Technologies).

Cholesterol Analysis. Another aliquot of the chloroformic phase was evaporated; 60 μl of KOH 10 M and 1 ml of methanol were added; and tubes were incubated for 45 min at 56°C. After incubation, 2 ml of chloroform and 1 ml of water were added; tubes were shaken and centrifuged; and the chloroformic phase was evaporated. One hundred μl of a mixture of bis(tri-methylsilyl)trifluoroacetamide/triethylchlorosilane (4:1 v/v; Acros Organics, Fisher Scientific International, Hampton, NH) were added; samples were incubated 1 h at 80°C and evaporated; and 100 μl of hexane were added. Analysis of sterol trimethylsilyl ether was performed by gas liquid/mass spectrometry in a 6890 gas chromatograph coupled with a 7673 mass detector (Agilent Technologies). The column was a HP-5MS 30 m × 0.25 mm (Agilent Technologies), and helium was used as the carrier gas. Concentrations of phospholipids and cholesterol were determined from the ratio of the peak area of the given molecule to the peak area corresponding to the internal standard. Levels were determined by comparing this ratio with a standard curve of known amounts of cholesterol or of different species of phospholipids. Concentrations were expressed in nmol/μg of total proteins.

Metabolic Cell Labeling and Sphingolipid Quantification

Total cellular sphingomyelin and ceramide quantification was performed by labeling cells to isotopic equilibrium with 0.5 μCi/ml of [9,10-3H]palmitic acid (53.0 Ci/mmol; Amersham) for 48 h in medium complemented with 0.5% FCS and 2 mM t-glutamine. These cells then were washed and treated with cisplatin (5 μg/ml) for time course experiments. Lipids were extracted and resolved by thin-layer chromatography. Sphingomyelin and ceramide were scraped and quantitated using a liquid scintillation counter.

Flow Cytometry Analysis

The membrane expression of CD95 and the aSMase was studied by flow cytometry. HT29 cells in suspension were stained with a mouse anti-CD95 (ZB4, 1:100 dilution; Immunotech) or a goat anti-aSMase (1:100; Santa Cruz Biotechnology) in 100 μl of PBS containing 0.5% BSA and 0.1% sodium azide (Sigma-Aldrich). After 1-h incubation at 4°C and two washes in PBS, cells were incubated for 45 min at 4°C with an FITC-conjugated sheep antinoume IgG or an FITC-conjugated sheep antinoume IgG (Amersham). In all of the cases, 10,000 cells were analyzed using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Determination of Membrane Fluidity by EPR Spin-Labeling Method

The membrane fluidity of HT29 cells was determined by a spin-labeling method using electron paramagnetic resonance (EPR; Ref. 27). After treatment, cells collected in PBS were incubated with 0.5 μg/ml 12-DSA spin label (which incorporates at the inner layer of plasma membrane) for 15 min at 37°C and then were washed three times with PBS to eliminate the free spin label. The final pellet was kept on ice to prevent any spin label reduction before analyzing the EPR spectrum at room temperature (20°C) using a Bruker ESP 106 spectrometer (9.82 GHz frequency, 20 mW microwave power, 1.771 G modulation amplitude, and 100 kHz modulation frequency; Bruker Biospin, Rheinstetten, Germany). The values of inner hyperfine-splitting EPR spectra, typical for 12-DSA spin label, were used to calculate the order parameter. An increase in the order parameter reflects a decrease in membrane fluidity.

RESULTS

Cisplatin Induces CD95 Receptor Clustering at the Surface of HT29 Cells. We and others have shown previously that anticancer drugs could induce Fas clustering at the cell surface of various human cancer cell lines (6, 22). We confirmed this observation in HT29 cells by using confocal fluorescence microscopy (Fig. 1). Untreated HT29 cells demonstrated a weak and diffuse CD95 staining at their surface. Exposure to 5 μg/ml cisplatin for 4 h induced a redistribution of the protein with a dense, patchy CD95 staining, suggesting clustering of the death receptor at the surface of tumor cells. Interestingly, 1-h pretreatment with 50 μg/ml nystatin, a polyene antifungic agent that complexes and sequesters cholesterol, dramatically reduced CD95 clustering (Fig. 1). This observation suggested that cholesterol could play a role in the plasma membrane clustering of the death receptor. Methyl-β-cyclodextrin, a cholesterol-depleting agent that also disrupts rafts, was too toxic toward cells when tested alone to detect any effect on CD95 clustering induced by cisplatin treatment (data not shown). Likewise, 1-h pretreatment with 50 μM imipramine, an inhibitor of aSMase, also reduced cisplatin-induced CD95 clustering (Fig. 1), suggesting the involvement of the aSMase in this process.

Nystatin Decreases Cisplatin-Induced Apoptosis in HT29 Cells. Because CD95 has been suggested to play a role in cytotoxic drug-induced apoptosis in a variety of tumor cells, we analyzed...
cisplatin-induced apoptosis in HT29 cells (Fig. 2A). Cisplatin for 4 h before staining with an anti-CD95 antibody and analysis by fluorescence laser confocal microscopy. One representative of three independent experiments is shown.

whether nystatin-induced inhibition of CD95 clustering could interfere with death triggered by cisplatin (Fig. 2A; Refs. 2, 22). A 1-h pretreatment with 10 μg/ml nystatin was observed to partially prevent cisplatin-induced apoptosis in HT29 cells (Fig. 2A). Conversely, nystatin did not interfere with apoptosis induced by staurosporine, a compound that induces apoptosis through a CD95-independent pathway (Fig. 2B). Nystatin alone triggered a small amount (~10%) of cell death 72 h after cell treatment. At this time point, nystatin still negatively interfered with cisplatin-induced apoptosis (Fig. 2A). Tumor cell exposure to cytotoxic drugs also has been shown to sensitize these cells to death receptor-mediated cell death (23–25). We show here that nystatin prevents cisplatin-mediated sensitization of HT29 cells to apoptosis induced by the CH11 agonistic anti-Fas antibody (Fig. 2C). These results suggested that CD95 clustering at the surface of tumor cells could be required for cisplatin-induced apoptosis and synergy between cisplatin and CD95 agonists.

Cisplatin Induces CD95 Redistribution into the Membrane Lipid Rafts. The recent demonstration that membrane rafts played an essential role in the initiation of CD95-induced cell death in various cell types led us to investigate whether cisplatin could redistribute CD95 into cholesterol- and sphingolipid-rich microdomains of HT29 cell plasma membrane (7–13). A typical feature of these domains, identified in all of the eukaryotic cells, is their resistance to the nonionic detergent Triton X-100 at 4°C (28). Thus, untreated and cisplatin-treated cells, without or with a pretreatment with nystatin, were lysed in a Triton X-100 detergent-containing buffer at low temperature. These lysates were subjected to ultracentrifugation onto a linear sucrose gradient to collect 11 fractions whose lipid content, including cholesterol and sphingomyelin, was determined by mass spectrometry. Under all of the tested conditions, four of these fractions (3, 4, 5, and 6) were enriched in cholesterol and sphingomyelin and expressed caveolin-2, a raft-associated protein involved in the formation of small surface invaginations named caveolae (Fig. 3). Each fraction from this density gradient was analyzed using immunoblots for the presence of CD95. In untreated cells, CD95 could not be detected in fractions enriched into cholesterol and sphingomyelin, suggesting that CD95 was not constitutively expressed in the raft microdomains in these cells. Cisplatin exposure induced a redistribution of CD95 that partially partitioned to cholesterol- and sphingomyelin-enriched fractions, and this redistribution was prevented by nystatin pretreatment (Fig. 3). In addition, the adaptor molecule FADD and procaspase-8 also were observed to partially partition with cholesterol- and sphingomyelin-enriched fractions under cisplatin exposure, whereas procaspase-2 was not redistributed. Again, this effect was prevented by pre-exposure to nystatin (Fig. 3). Confocal laser-scanning microscopy analysis indicated that CD95 and caveolin-2 colocalized in cisplatin-treated HT29 cells, not in untreated cells (Fig. 4). This observation suggested that the Triton X-100-insoluble fractions were the membrane domains in which CD95 had clustered during cisplatin exposure.

Cisplatin Induces Ceramide Generation and Colocalization of Clustered CD95 with an aSMase. It has been suggested recently that CD95 clustering on ligation could depend on surface ceramide generation through translocation of an aSMase onto the extracellular leaflet of the cell membrane (7, 8, 12). We observed that exposure of HT29 cells to cisplatin induced a rapid and transient generation of ceramide (between 15 min and 1 h after cisplatin treatment) that correlated with hydrolysis of sphingomyelin, two effects that were prevented by cell pretreatment with nystatin (Fig. 5A). Confocal microscopy analysis indicated that ceramide colocalized with clustered CD95 at the surface of cisplatin-treated HT29 cells (Fig. 5B). Cisplatin also induced the rapid appearance of the aSMase at the surface of tumor cells, as detected by fluorescence confocal microscopy as soon as 15 min after the beginning of cell treatment, whereas the enzyme was hardly detected in untreated nonpermeabilized cells (Fig. 5C). Interestingly, the aSMase colocalized with clustered CD95

**Fig. 1.** Cisplatin induces CD95 receptor clustering. HT29 cells were either exposed to 10 μg/ml nystatin or 50 μM imipramine for 1 h, then left untreated or treated with 5 μg/ml cisplatin for 4 h before staining with an anti-CD95 antibody and analysis by fluorescence laser confocal microscopy. One representative of three independent experiments is shown.

**Fig. 2.** Nystatin decreases cisplatin-induced cell death and sensitization to CD95-mediated apoptosis: A, HT29 cells were either exposed to 10 μg/ml nystatin for 1 h or not, then left untreated or treated with 5 μg/ml cisplatin for indicated times before identifying apoptotic cells by staining nuclear chromatin with Hoechst 33342. B, HT29 cells were either exposed to 10 μg/ml nystatin for 1 h or not, then left untreated or treated with 5 μg/ml cisplatin or 1 μM staurosporine for 24 h. Apoptosis was determined as in A. C, HT29 cells were either exposed to 10 μg/ml nystatin for 1 h or not, then left untreated or treated with 5 μg/ml cisplatin or 100 ng/ml agonistic CH11 anti-CD95 antibody or the combination of both for 24 h. Apoptosis was determined as in A. Results, mean ± SD of three independent experiments (300 cells/point).
Fig. 3. Cisplatin induces CD95 translocation into membrane lipid rafts. Top, HT29 cells were either exposed to 10 μg/ml nystatin for 1 h or not, then left untreated or treated with 5 μg/ml cisplatin for 4 h, then lysed in 1% Triton X-100, and fractionated on a linear sucrose density gradient centrifugation. Cholesterol (gray bars) and sphingomyelin (black bars) analyses were performed in each fraction by mass spectrometry. Bottom, an equal volume of each collected fraction was submitted to SDS-PAGE before analysis of CD95, Fas-associated death domain (FADD), procaspase-8 (C8), procaspase-2 (C2), and caveolin-2 expression by Western blot analysis. One representative of three independent experiments is shown.

Fig. 4. Cisplatin induces CD95 colocalization with caveolin-2. HT29 cells were left untreated or treated with 5 μg/ml cisplatin for 4 h, then stained with a mouse anti-CD95 and a mouse antivealolin-2 antibody, and analyzed by confocal laser microscopy. One representative of three independent experiments is shown.

onto the outer leaflet of the plasma membrane in cisplatin-treated cells (Fig. 5C). A 1-h pretreatment with 10 μg/ml nystatin partially prevented CD95 clustering and aSMase externalization induced by cisplatin (Fig. 5C). Accordingly, cisplatin-induced aSMase membrane expression was inhibited by 1-h pretreatment with nystatin (Fig. 5D).

**Cisplatin Increases Membrane Fluidity.** To further understand how CD95 could be redistributed to membrane rafts, we analyzed the effect of cisplatin on plasma membrane fluidity in cultured HT29 cells by using stearic acid spin label 12-DSA followed by EPR spectrometry. The EPR signals allowed calculation of the membrane order parameter, which has been demonstrated to be inversely related to membrane fluidity. Fig. 6A shows that cisplatin induces a rapid decrease in the membrane order parameter. This decrease was identified as soon as 15 min after the beginning of cell treatment with 5 μg/ml cisplatin and persisted 4 h later. The membrane fluidity returned to normal values 24 h after initiation of cisplatin exposure (Fig. 6A) and was prevented by 1-h pretreatment with nystatin (Fig. 6B).

**DISCUSSION**

The present study indicates that exposure of a cancer cell line to a cytotoxic drug can induce the formation of large CD95 aggregates and the redistribution of CD95, together with the DISC-forming molecules FADD and procaspase-8, into plasma membrane rafts. The cholesterol-sequestering drug nystatin, which negatively interferes with these events, also decreases cisplatin-induced apoptosis and the synergistic cytotoxic effect of cisplatin with an agonistic anti-CD95 antibody (29). Interestingly, nystatin also prevents aSMase activation, ceramide generation, and the transient increase in plasma membrane fluidity induced by the cytotoxic drug. Together, these observations suggest that plasma membrane raft integrity may be required for optimal cytotoxicity of cisplatin and synergy with death receptor agonists.

It was suggested initially that engagement of CD95 led to receptor trimerization that preceded the formation of the DISC (30). It then was proposed that CD95 could exist in a trimerized state before ligand interaction (31) and that CD95-L induced the multimerization of trimerized proteins, generating aggregates of activated receptors to produce high local concentration of DISC (17, 32). Clustering of a cell surface receptor on binding to its specific ligand has been described for a variety of other receptors, such as immunoglobulin and T-cell receptors (33, 34). In several of these systems, patches of aggregated receptors were shown to migrate toward one pole of the cell to form a cap via an energy-dependent process involving cytoskeleton reorganization, thus facilitating signal transduction by the local assembly of the various signaling elements.

We have shown previously that anticancer drugs could enhance the expression of CD95 or induce clustering of the receptor at the surface of a variety of tumor cells, which was associated with the ligand-independent formation of the DISC. This signaling pathway was shown to contribute to drug-induced apoptosis in some cancer cells and to sensitize these cells to apoptosis induced by CD95-L (22, 23, 35). By using cisplatin and the human colon carcinoma cell line HT29, we show here that these effects are associated with the redistribution of CD95 into the membrane rafts. These microdomains were isolated by the virtue of their detergent insolubility and further characterized by identifying their high concentration in cholesterol and sphingomyelin. CD95 colocalization with raft-associated proteins has been associated previously with engagement by its specific ligand or agonistic antibodies (7–13). Recent studies have suggested a central role of membrane rafts for clustering or aggregation of a variety of other receptor molecules (36–40). The mechanisms trapping receptor molecules in membrane rafts could involve hydrophobic modifications, such as myristoylation, palmitoylation, or double acetylation, interaction with a binding partner that itself associates with raft lipids, or structure of a membrane-spanning domain that mediates a preferential localization in ceramide-enriched domains (41). Clustering of molecules with modest individual raft affinity also could increase their affinity for these membrane domains. How CD95 is redistributed to rafts under interaction with its cognate ligand or on exposure to a cytotoxic drug requires additional investigation.

The demonstration that CD95 is redistributed or enriched in raft fraction under ligand or cytotoxic drug exposure suggests a role for rafts in CD95 function. The observation that FADD and procaspase-8, which both contribute to the DISC formation, are simultaneously...
redistributed to the rafts enforces this hypothesis. The ability of nystatin to prevent redistribution of the DISC-forming molecules into the rafts and to decrease apoptosis in cancer cells exposed to cisplatin also suggests a link between these events. How clustering of CD95 receptors within rafts contributes to CD95 signaling and cytotoxic drug-induced apoptosis remains a matter of speculation. This event may favor the formation of CD95 macroaggregates, stabilize the ligand/receptor interaction, or facilitate trans-activation of intracellular signaling molecules.

One of the signaling molecules whose synthesis is induced by exposure to cytotoxic drugs and engagement of CD95 receptor is ceramide. Cellular ceramide can be generated either by hydrolysis of sphingomyelin or de novo synthesis. We show here that the generation of ceramide in cisplatin-treated HT29 cells is associated with the rapid and transient activation of a sphingomyelinase. Hydrolysis of sphingomyelin can be catalyzed by one of the three known sphingomyelinases (acid, neutral, and alkaline). Exposure to cisplatin induces relocalization of an aSMase isoform to the extracellular leaflet of plasma membrane and a colocalization with clustered CD95. Observations in lymphocytes have suggested a pathway through activation and translocation of an aSMase to the extracellular leaflet of membrane rafts, where the enzyme releases extracellularly oriented ceramides, was required for the formation of CD95 clusters at the cell surface on ligation and subsequent amplification of CD95 signaling (8, 12). Our data corroborate these observations because clustered CD95 colocalizes with ceramide at the cell surface of cisplatin-treated HT29 cells, and imipramine, an inhibitor of aSMase, prevented cisplatin-induced CD95 clustering. Thus, the ability of nystatin to prevent sphingomyelinase activation and ceramide generation in colon

Fig. 5. Cisplatin induces colocalization of clustered CD95 with an acid sphingomyelinase (aSMase). A, analysis of ceramide (top) and sphingomyelin (bottom) contents was performed in HT29 cells labeled to isotopic equilibrium with [9,10-3H] palmitic acid and then exposed to 5 μg/ml cisplatin for indicated times (●). The same experiment was performed in cells exposed to 10 μg/ml nystatin for 1 h before cisplatin (●). Results are given as percentages of control ± SD of three independent experiments. B, HT29 cells were left untreated or treated with 5 μg/ml cisplatin for 1 h, stained with a mouse anti-CD95 and a mouse anticeramide antibody, and analyzed by confocal laser microscopy. One representative of three independent experiments is shown. C, HT29 cells were left untreated or treated with 5 μg/ml cisplatin for indicated times (min) without or with a pre-exposure to 10 μg/ml nystatin for 1 h. Cells were stained with a mouse anti-CD95 and a goat anti-aSMase antibody and analyzed by confocal laser microscopy. One representative of three independent experiments is shown. D, HT29 cells were left untreated or treated with 5 μg/ml cisplatin for 4 h without or with a pre-exposure to 10 μg/ml nystatin for 1 h. Cells were stained with a mouse anti-CD95 and a goat anti-aSMase antibody and analyzed by flow cytometry. One representative of three independent experiments is shown.
cancer cells exposed to cisplatin could indicate that nystatin somehow prevents interaction of acidic sphingomyelinase with its substrate.

Transient alterations in plasma membrane fluidity have been identified in cells exposed to various apoptotic stimuli (19–21). This event may result in enhanced lateral mobility of plasma membrane constituents and reorganization of membrane molecule partitioning. We show here that exposure of HT29 cells to cisplatin induces a transient increase in plasma membrane fluidity that is prevented by nystatin. How membrane fluidity contributes to the redistribution of CD95 at the surface of cisplatin-treated tumor cells remains to be investigated.

The ether lipedelfosin and the chemopreventive agent resveratrol, two molecules with chemotherapeutic potential, have been shown recently to induce clustering and relocalization of CD95 into membrane rafts in Jurkat cells and colon cancer cells, respectively, indicating that the observed effects are not specific of cisplatin (18, 42).

We have shown previously that anticancer drugs could sensitize colon cancer cells to death receptor ligands other than CD95-L, such as tumor necrosis factor-related apoptosis-inducing ligand-R2, to rafts in colon cancer cells (24, 25). Together, these results indicate that anticancer drugs can induce the redistribution of a series of plasma membrane-associated proteins into rafts, which could either contribute to their cytotoxic activity or sensitize cells to other extracellular insults.

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