Fas Ligand-independent, FADD-mediated Activation of the Fas Death Pathway by Anticancer Drugs*

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Trimerization of the Fas receptor (CD95, APO-1), a membrane bound protein, triggers cell death by apoptosis. The main death pathway activated by Fas receptor involves the adaptor protein FADD (for Fas-associated death domain) that connects Fas receptor to the caspase cascade. Anticancer drugs have been shown to enhance both Fas receptor and Fas ligand expression on tumor cells. The contribution of Fas ligand-Fas receptor interactions to the cytotoxic activity of these drugs remains controversial. Here, we show that neither the antagonistic anti-Fas antibody ZB4 nor the Fas-IgG molecule inhibits drug-induced apoptosis in three different cell lines. The expression of Fas ligand on the plasma membrane, which is identified in untreated U937 human leukemic cells but remains undetectable in untreated HT29 and HCT116 human colon cancer cell lines, is not modified by exposure to various cytotoxic agents. These drugs induce the clustering of Fas receptor, as observed by confocal laser scanning microscopy, and its interaction with FADD, as demonstrated by co-immunoprecipitation. Overexpression of FADD by stable transfection sensitizes tumor cells to drug-induced cell death and cytotoxicity, whereas down-regulation of FADD by transient transfection of an antisense construct decreases tumor cell sensitivity to drug-induced apoptosis. These results were confirmed by transient transfection of constructs encoding either a FADD dominant negative mutant or MC159 or E8 viral proteins that inhibit the FADD/caspase-8 pathway. These results suggest that drug-induced cell death involves the Fas/FADD pathway in a Fas ligand-independent fashion.

Fas is a 45-kDa membrane protein that belongs to the tumor necrosis factor (TNF)/ nerve growth factor receptor family (1). Engagement of Fas by agonistic anti-Fas antibodies triggers programmed cell death in a variety of cell types (2, 3). Its natural ligand, Fas ligand (Fas-L), belongs to the TNF family (4) and can be found as a 40-kDa membrane-bound or a 26-kDa soluble cytokine (5). Similarly to agonistic anti-Fas antibodies, binding of membrane-bound or soluble Fas-L to Fas receptor can induce apoptosis in Fas-bearing cells (1, 4). The main death pathway initiated from Fas activation involves a series of death-associated molecules (6), including FADD (Fas-associated death domain-containing protein), which is an adaptor protein that is recruited to Fas receptor upon its engagement (7–9). FADD then binds to and activates procaspase-8 (also called FLICE or MACH) (8–10), which is believed to be the first step of a proteolytic cascade that triggers activation of other caspases such as caspases-7, -3, and -6 (11). Although other cell death pathways could be initiated from Fas activation (12, 13), analysis of lymphocytes from FADD −/− mice has recently demonstrated the prominent role of the FADD/procaspase-8 pathway in Fas-mediated cell death (14).

Cytotoxic drugs commonly used in cancer therapy can induce tumor cell death by apoptosis (15–17). These drugs were shown to enhance the expression of Fas (18) and Fas-L (19, 20) on the surface of certain malignant cells. It has been proposed that the molecular process leading from specific cellular damage induced by the drugs to apoptosis might involve an interaction between Fas ligand and Fas (19). However, this issue remains controversial because antagonistic anti-Fas antibodies that block Fas-L-mediated apoptosis do not always inhibit drug-induced cell death (21, 22). In the present study, we further addressed the role played by the Fas/FADD pathway in drug-induced cell death. We show that anticancer drugs can induce Fas receptor clustering and FADD recruitment to Fas receptor in a Fas ligand-independent fashion. By modulating FADD expression, we also demonstrate the role of this adaptor molecule in drug-induced apoptosis.

MATERIALS AND METHODS

Cell Lines and Culture Conditions—The HT29 and HCT116 human colon carcinoma and the U937 and Jurkat leukemic cell lines were obtained from the ATCC (Rockville, MD). HT29 and HCT116 were maintained in Eagle’s minimum essential medium supplemented with 10% fetal calf serum and 2 mM l-glutamine (Biowhittaker Co., Fontenay sous Bois, France). U937 and Jurkat cells were maintained in RPMI 1640 (Biowhittaker Co.) supplemented as above with heat-inactivated fetal calf serum.

Soluble Fas-L Production—sFas-L was collected from Neuro 2A-transfected murine cells supernatant (kindly provided by Dr. A. Fontana, Zurich, Switzerland) (23). This supernatant was obtained as described previously (18). One arbitrary unit of sFas-L was defined as 1 µl of a 100-fold concentrated supernatant of Neuro 2A cells that had been centrifuged for 48 h. An unique pool of sFas-L or mock supernatant was used throughout the study.

Cytotoxicity and Apoptosis Determination Assays—Cell viability was determined by the use of the methylene blue colorimetric assay (18) 72 h after the beginning of drug exposure, unless specified. Specific apoptosis was determined after trypanstaining cells and staining with 1 µg/ml Hoechst for 15 min at 37 °C. The percentage of apoptotic cells was determined by analyzing 300 cells. For blocking assays, cells were incubated for 1 h with 2 µg/ml of a 100-fold concentrated supernatant of Neuro 2A cells that had been centrifuged for 48 h. An unique pool of sFas-L or mock supernatant was used throughout the study.

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Fallavier, France), 50 μg/m  or 2 μg/m H29, HCT116, or U937 cells were treated with 10 μg/ml (Biovalley, Rockville, MD) plus 0.8 ng/ml vinblastine (VB; Sigma Chemical Co.), 50 ng/ml anti-Fas CH11 antibody, and apoptosis was determined as described above.

Flow Cytometric Analysis of Fas Ligand Membrane Expression— HT29, HCT116, or U937 cells were treated with 10 μg/ml cisplatin, 50 μg/ml etoposide, or 10 ng/ml vinblastine for 4 h. Jurkat cells were activated with 500 ng/ml ionomycin and 20 ng/ml phorbol 12-myristate 13-acetate (Sigma Chemical Co.) for 4 h. Fas-L expression was measured by flow cytometry by incubating cells for 45 min at +4 °C with the rat IgG2a anti-human Fas-L clone H11 (Alexis, San Diego, CA) or a rat isotype-matched control (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted at 1/500, in PBS containing 0.5% bovine serum albumin and 0.1% NaN3. After two washes in PBS, cells were incubated for 45 min with a fluorescein isothiocyanate-labeled donkey anti-rat IgG (Jackson ImmunoResearch Laboratories). The membrane was then washed twice with TPBS and incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories). The membrane was then washed twice with TBBS and revealed using an enhanced chemiluminescence detection kit (Amersham, Les Ulis, France) and autoradiography.

Expression Vectors— pCDNA3-FADD was obtained from Invitrogen (NV Leek Co., The Netherlands), pCDNA3-FADD was kindly provided by Dr. V. M. Dixit (University of Michigan Medical School, Ann Arbor, MI). Antisense construct for FADD was obtained by digestion of the full-length FADD sequence from pCDNA3-FADD with BamHI/Kpn1 and subcloning in the expression vector pBK-CMV (Stratagene, La Jolla, CA) in reverse orientation. pBK-CMV-FADD-AS construct was checked by manual sequencing (data not shown). PCI-neo was purchased from Promega (Madison, WI), PCI-MC159 and PCI-E8 were kindly provided by Dr. J. I. Cohen (NIH, Bethesda, MD). PCI-FADD-DN construct was a kind gift from Dr. C. M. Zacharchuk (NIH, Bethesda, MD).

Western Blotting— For immunoblotting, cells were washed in PBS, lysed in the boiling buffer [1% SDS, 10 mM Tris, pH 7.4] for 10 min at 4 °C, and boiled for 5 min. Proteins (30 μg) were separated on a polyacrylamide SDS containing gel and transferred onto polyvinylidene difluoride membranes (Bio-Rad, Ivry sur Seine, France). After blocking nonspecific binding sites overnight by 5% nonfat milk in TBBS (PBS with 0.1% Tween 20), the membrane was incubated for 2 h at room temperature with anti-human FADD (Transduction Laboratories, Lexington, KY) or the loading control anti-human Hsp70 (StressGen Biotechnologies, Victoria, Canada). The membrane was then washed twice with TBBS and incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories). The membrane was then washed twice with TBBS and revealed using an enhanced chemiluminescence detection kit (Amersham, Les Ulis, France) and autoradiography.

Stable Transfections— HT29 cells were seeded for 24 h and transfected with pCDNA3-AU1-FADD containing full-length human FADD cDNA or an empty vector construct pCDNA3 by the use of the lipofection reagent Dac-30, according to the manufacturer’s instructions (Eurogentec Co., Seraing, Belgium). Single clones were picked 2–3 weeks after transfection and selected in medium containing 1 μg/ml geneticin.

Transient Transfections— HT29, HCT116, and U937 cells were seeded 24 h before transfection and transfected with 1 μg of PCI-neo, PCI-MC159, PCI-E8, pBK-CMV, pBK-CMV-FADD-AS, PCI-FADD-DN, or a combination of different plasmids using LipofectAMINE Plus (Life Technologies Co., Gaithersburg, MD), except for U937 cells, for which transfection were performed using Superfect (Qiagen, Courtaboeuf, France), according to the manufacturer’s instructions. HT29 and HCT116 cells were treated or not 16 h after transfection with 10 μg/ml...
Cells were either receptor clustering.

**Fig. 2.** Flow cytometric analysis of Fas ligand expression on the plasma membrane of tumor cells. HT29, HCT116, and U937 cells were treated at doses indicated in the legend to Fig. 1 and analyzed 4 h after the beginning of drug treatment for Fas-L expression by flow cytometry using the rat anti-human Fas-L antibody H11. Untreated Jurkat cells and Jurkat cells activated with 500 ng/ml ionomycin plus 20 ng/ml phorbol 12-myristate 13-acetate were used as negative and positive controls, respectively. Gray, control antibody; white, specific antibody. Results are representative of four independent experiments.

**Fig. 3.** Anticancer drugs induce Fas receptor clustering. Cells were either left untreated (NT) or treated with 10 ng/ml TNF-α, 10 μM CDDP, 50 μM VP16, 10 ng/ml VB, or 2 AU/ml sFas-L. 4 h later, cells were fixed and labeled for confocal laser scanning microscopy as described under “Materials and Methods.” Cells were viewed and photographed using a confocal microscope with a ×40 objective. Bar, 10 μm.

**RESULTS**

**Fas Ligand Is Not Involved in Anticancer Drug-induced Apoptosis**—We have shown recently that anticancer drugs could increase Fas receptor membrane expression and sensitize tumor cells to Fas apoptotic pathway (18). To determine whether drug-induced cell death could involve an interaction between Fas receptor and Fas-L, we used the antagonistic ZB4 anti-Fas antibody and the Fas-IgG blocking molecule. HT29 and HCT116 colon carcinoma cells were treated for 72 h with 5 or 10 μM CDDP, 10 μM doxorubicin, or 2 AU/ml recombinant sFas-L. U937 human leukemic cells were treated with either 50 μM etoposide for 4 h or 10 ng/ml vinblastine for 24 h or 50 ng/ml CH11 anti-Fas agonistic antibody in the presence of 0.8 μg/ml cycloheximide for 24 h. All these treatments were performed in the absence or in the presence of either 2 μg/ml ZB4 anti-Fas blocking antibody or 2 μg/ml Fas-IgG molecule added to the culture medium 1 h before drug exposure. In these conditions, both ZB4 antibody and Fas-IgG reagent inhibited apoptosis induced by sFas-L and CH11 antibody in colon cancer cell lines and U937 cells, respectively (Fig. 1). By contrast, neither ZB4 antibody nor Fas-IgG reagent influenced drug-induced cell death in the tested cell lines (Fig. 1). These results suggested that Fas-Fas-L interaction might not play a central role in drug-induced cell death.

Then we used flow cytometry to analyze the expression of Fas-L on the plasma membrane of the studied cell lines. Untreated Jurkat cells and Jurkat cells exposed to phorbol 12-myristate 13-acetate + ionomycin were used as negative and positive controls, respectively. Whereas Fas-L was expressed on the plasma membrane of untreated U937 cells, it remained undetectable on the membrane of HT29 and HCT116 cells (Fig. 2). Exposure of the three cell lines to cytotoxic drugs did not modulate Fas-L expression on their plasma membrane (Fig. 2).
These results further argued against a role for a Fas-Fas-L interaction in drug-induced cell death.

**Anticancer Drugs Induce Fas Receptor Clustering**—We used confocal laser scanning microscopy and ZB4 anti-Fas antibody to analyze the effect of anticancer drugs on Fas receptor expression at the surface of tumor cells. Exposure of tumor cells to recombinant sFas-L, which induces the clustering of Fas receptor at the surface of tumor cells, was used as a positive control, whereas cell exposure to 10 ng/ml TNF-α, which does not modify the pattern of Fas expression, was used as a negative control (Fig. 3). All the cytotoxic drug/cell line combinations induced the clustering of Fas receptor on tumor cell plasma membrane as soon as 4 h after the beginning of cell treatment (Fig. 3).

**Anticancer Drugs Induce the Recruitment of FADD to Fas Receptor**—To test the possibility that anticancer drugs could also induce the recruitment of the adaptor protein FADD to Fas receptor, co-immunoprecipitation studies were performed. HT29 cells were either left untreated or treated with 10 μM cisplatin, 2 AU/ml sFas-L, or 10 μM doxorubicin for 4 h and analyzed for co-immunoprecipitation using an antibody directed against Fas receptor. Western blot analysis using an anti-FADD antibody revealed that cisplatin and doxorubicin, similar to soluble recombinant Fas-L, induced FADD recruitment to Fas receptor (Fig. 4). Similar results were obtained with HCT116 cells after treatment with cisplatin and doxorubicin (not shown).

**Overexpression of FADD Sensitizes HT29 Cells to Cisplatin-induced Cytotoxicity**—To determine whether FADD association to Fas receptor after clustering could be relevant for anticancer drug-induced apoptosis, we performed a series of experiments aiming to modulate FADD expression. HT29 cells were stably transfected with a construct encoding FADD full-length cDNA in pCDNA3 plasmid. After geneticin treatment, we selected two clones, namely FD2 and FD5, that expressed nearly two times more FADD messenger RNA (not shown) and protein (Fig. 5A, inset) as compared with the CO4 clone transfected with an empty construct. Both FADD-overexpressing clones were more sensitive to soluble recombinant Fas-L (sFas-L) (not shown) and cisplatin-induced cytotoxicity when compared with control mock transfected cells in a 72-h methylene blue colorimetric assay (Fig. 5A). The FD5 clone demonstrated also significantly higher sensitivity to sFas-L (not shown) and cisplatin-induced apoptosis (Fig. 5B) when compared with CO4 cells.

**Transient Expression of FADD Antisense, FADD-DN, or MC159 and/or E8 Constructs Prevents Cisplatin-induced Cytotoxicity**—To further confirm the role of FADD in the cytotoxic activity of anticancer drugs, we transiently transfected HT29 cells with constructs encoding either antisense FADD or sense FADD-DN, MC159, or E8 proteins. FADD-DN is a dominant negative construct that is capable of blocking Fas signal transduction (25). MC159 and E8 are two viral proteins that inhibit apoptosis at the level of FADD and procaspase-8, respectively (26). Transient overexpression of both FADD antisense construct (Fig. 6A) and FADD-DN (Fig. 6B) prevented cisplatin-induced cytotoxicity in HT29 cells, as measured by a methylene blue colorimetric assay. Transient overexpression of either MC159 and/or E8 proteins demonstrated effects similar to those of FADD antisense (Fig. 6C) and protected cells from cisplatin-induced cytotoxicity.

**Antisense FADD or FADD-DN Prevent Anticancer Drug-induced Apoptosis**—To determine whether the reduced cytotoxicity observed after cisplatin treatment in HT29 cells tran-
siently transfected by a FADD antisense construct was related to a decreased induction of apoptosis. HT29 cells were transiently transfected for 16 h with indicated vectors: control vectors (pBK and pC1), pBK-FADD-AS antisense vector (AS), pC1-FADD-DN (FADD-DN), pC1-E8 (E8), and/or pC1-MC159 (MC159). Cells were then treated with 10 μM cisplatin for 72 h, and cell viability was determined by the methylene blue colorimetric assay. Results are expressed as the means ± S.D. of three independent experiments.

**DISCUSSION**

The molecular mechanisms involved during the course of apoptosis upon exposure to anticancer drugs are still poorly understood but are of major importance for the understanding of tumor cell killing. Induction of apoptosis occurs through multiple pathways and depends on the stimulus. We have previously shown that anticancer agents could increase Fas receptor at the tumor cell surface by enhancing Fas gene expression. The increase in Fas receptor was functional, and drug-treated tumor cells were rendered more sensitive to Fas-mediated apoptosis (18). Whether interaction of Fas receptor with its ligand plays a role in anticancer drug-mediated cytotoxicity remains a controversial issue (21). Here, we show that the antagonistic anti-Fas antibody ZB4 and the Fas-IgG molecule, used at a concentration that inhibits Fas-mediated and radiation-induced apoptosis (27), have no effect on anticancer drug-induced apoptosis in three different cell lines. Moreover, we failed to detect any modification of Fas-L expression on the plasma membrane of tumor cells exposed to cytotoxic agents (27, 28). In accordance with previously reported observations in other cell systems (21, 22, 29), these results argue against a role for a Fas receptor-Fas-L interaction in drug-induced cell death.

The FADD-DN construct was also capable of preventing HCT116 cells from cisplatin-induced apoptosis (Fig. 7).

**FIG. 6.** Inhibition of cisplatin-induced cytotoxicity by FADD antisense, FADD-DN, or viral MC159 and/or E8 constructs. HT29 cells were transiently transfected for 16 h with indicated vectors: control vectors (pBK and pC1), pBK-FADD-AS antisense vector (AS), pC1-FADD-DN (FADD-DN), pC1-E8 (E8), and/or pC1-MC159 (MC159). Cells were then treated with 10 μM cisplatin for 72 h, and cell viability was determined by the methylene blue colorimetric assay. Results are expressed as the means ± S.D. of three independent experiments.

**FIG. 7.** Inhibition of anticancer drug-induced apoptosis by FADD antisense or FADD-DN constructs. HT29, HCT116, or U937 cells were transiently transfected for 16 h with indicated vectors: control vectors (pBK and pC1), pBK-FADD-AS antisense vector (AS), or pC1-FADD-DN and treated as indicated with 10 μM CDDP or 50 μM VP16 for 72 h. U937 cells were treated with 50 μM etoposide for 4 h or with 10 ng/ml VB for 24 h. Apoptosis was determined by Hoechst staining. Each point is the mean of 300 cells counted. Results are expressed as the means ± S.D. of three independent experiments.
toxicity of cytotoxic agents in HT29 cells in which FADD expression was reduced by transient expression of an antisense construct. Similar results were obtained by inhibiting the FADD/procaspase-8 pathway at different levels using transient expression of viral proteins (26) and FADD-DN dominant negative construct (25). Altogether our data indicate that anticancer drugs can induce Fas receptor clustering in a Fas-L-independent fashion and recruit FADD to Fas receptor in the cytotoxic process leading to apoptosis.

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