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HPMA Copolymer-Bound Doxorubicin Induces Apoptosis in Human Ovarian Carcinoma Cells by a Fas-Independent Pathway

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Abstract: The mechanism of cell death in A2780 human ovarian carcinoma cells induced by free doxorubicin (DOX) and *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-bound DOX [P−(GFLG)−DOX] was investigated. In particular, the involvement of the Fas receptor system in drug-induced apoptosis was evaluated. P−(GFLG)−DOX was shown to effect apoptosis-induced tumor cell death as manifested by positive Annexin V−FITC staining, cleavage of procaspase 3 and its physiological substrate, poly(ADP-ribose) polymerase (PARP), and cleavage of procaspase 8. Using the fluorochrome-labeled caspase inhibitor assay, it was found that both free DOX and P−(GFLG)−DOX activated caspases 3 and 9, but both forms of DOX did not have an effect on the activity of caspase 8, when compared to untreated cells. It was shown that free DOX and P−(GFLG)−DOX upregulated Fas receptor expression at the cell membrane in a time-dependent manner. Triggering the drug-induced Fas receptor with an exogeneous soluble Fas ligand (sFasL) resulted in an increase in the extent of apoptotic cell death, indicating that the Fas signaling pathway remained functionally active. Also, antagonistic anti-Fas ZB4 antibody blocked the increase in the level of apoptosis following the application of sFasL, but did not interfere with drug-induced apoptosis. The study of the functional activity of the Fas receptor and of the activation of the most proximal effector of the caspase cascade, caspase 8, indicated that the Fas receptor pathway was not decisive in the induction of cell death by free DOX and P−(GFLG)−DOX in A2780 cells. This study suggests further investigation of the involvement of the mitochondrial pathway in A2780 cell apoptotic death, induced by free and HPMA copolymer-bound DOX.

Keywords: HPMA copolymer; doxorubicin; apoptosis; Fas receptor; ovarian carcinoma; macromolecular therapeutics

Introduction

An assortment of macromolecular therapeutics has been developed with the aim of improving the therapeutic efficacy of low-molecular weight drugs.[1,2] In particular, water-soluble polymer−anticancer drug conjugates have been studied in detail.[3−5] Their rational design, based on the physiology of normal and diseased tissues, aims to increase the solubility

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(1) Putnam, D.; Kopeček, J. Polymer Conjugates with Anticancer Activity. *Adv. Polym. Sci.* **1995**, *122*, 55−123.

(2) Duncan, R. The Dawning Era of Polymer Therapeutics. *Nat. Rev. Drug Discovery* **2003**, *2*, 347−360.

(3) Kopeček, J.; Kopečková, P.; Minko, T.; Lu, Z.-R. HPMA Copolymer-Anticancer Drug Conjugates: Design, Activity, and Mechanism of Action. *Eur. J. Pharm. Biopharm.* **2000**, *50*, 61−81.

(4) Říhová, B. Immunomodulating Activities of Soluble Synthetic Polymer-Bound Drugs. *Adv. Drug Delivery Rev.* **2002**, *54*, 653−674.

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of drugs,[6] increase the intravascular half-life,[7] optimize their pharmacokinetics and pharmacodynamics, and control their localization at cellular[8] and subcellular[9] levels.

Recently, it was revealed that macromolecular therapeutics might trigger different signaling pathways than free drugs. Indeed, differences in the gene expression profiles of human ovarian carcinoma cells after exposure to free DOX and HPMA copolymer-bound DOX [P−(GFLG)−DOX] were demonstrated in vitro[10] and in vivo.[11] Such effects may be the result of differences in their pathways of internalization and intracellular trafficking. Similarly, a different gene expression profile was observed in non-small cell lung cancer cells after exposure to free cisplatin and cisplatin-incorporated polymeric micelles.[12] Also, conjugation of a geldanamycin derivative to the HPMA copolymer resulted in stress responses in A2780 human ovarian carcinoma cells different from the responses induced by the free drug.[13]

Previous studies performed in our laboratory demonstrated that P−(GFLG)−DOX may overcome MDR1 gene-encoded resistance,[14] and activate apoptotic signaling pathways in A2780 human ovarian cancer cells in a manner different from that of free DOX.[10,11] The differences in bcl-2 gene expression have indicated the possible involvement of the mitochondrial pathway in apoptotic cell death.

Two main pathways of apoptosis in mammalian cells have been described: the mitochondrial pathway and the death receptor pathway(s).[15] The involvement of the Fas signaling pathway in chemotherapy-induced apoptosis has been extensively studied.[16] The Fas, a 48 kDa type I transmembrane receptor, is a member of the tumor necrosis factor/nerve growth factor (TNF/NGF) receptor superfamily. Binding of trimeric FasL to Fas induces trimerization of the receptor and activates the signaling pathway.[17] The adaptor protein, Fas-associated death domain (FADD),[18,19] binds to the intracellular part of Fas via the death domain, and subsequently recruits procaspase 8 to the death-inducing signaling complex (DISC).[20−22] Active caspase 8, released from the DISC, activates the caspase cascade. An anti-apoptotic factor, cellular FLICE inhibitor protein (cFLIP), can be recruited to the DISC and can block further recruitment of procaspase 8 into the complex, thereby inhibiting the activation of caspase 8.[23−25] Activated caspases finally target the cytoplasm and nuclear factors that maintain cellular architecture and are involved in DNA repair, replication, and transcription,[26]

(5) Maeda, H., Kabanov, A., Kataoka, K., Okano, T., Eds. *Polymer Drugs in the Clinical Stage: Advantages and Prospects*; Kluwer Publishers: New York, 2003.

(6) Greenwald, R. B.; Choe, Y. H.; McGuire, J.; Conover, C. D. Effective Drug Delivery by PEGylated Conjugates. *Adv. Drug Delivery Rev.* **2003**, *55*, 217−250.

(7) Shiah, J.-G.; Dvořák, M.; Kopečková, P.; Sun, Y.; Peterson, C. M.; Kopeček, J. Biodistribution and Antitumor Efficacy of Long-Circulating *N*-(2-Hydroxypropyl)methacrylamide Copolymer-Bound DOX Conjugates in Nude Mice. *Eur. J. Cancer* **2001**, *37*, 131−139.

(8) Shiah, J.-G.; Sun, Y.; Peterson, C. M.; Kopeček, J. Biodistribution of Free and *N*-(2-Hydroxypropyl)methacrylamide Copolymer-Bound Meso Chlorin e_6 and Adriamycin in Nude Mice Bearing Human Ovarian Carcinoma OVCAR-3 Xenografts. *J. Controlled Release* **1999**, *61*, 145−157.

(9) Nori, A.; Jensen, K. D.; Tijerina, M.; Kopečková, P.; Kopeček, J. Tat Conjugated Synthetic Macromolecules Facilitate Cytoplasmic Drug Delivery to Human Ovarian Carcinoma Cells. *Bioconjugate Chem.* **2003**, *14*, 44−50.

(10) Minko, T.; Kopečková, P.; Kopeček, J. Comparison of the Anticancer Effect of Free and HPMA Copolymer-Bound Adriamycin in Human Ovarian Carcinoma Cells. *Pharm. Res.* **1999**, *16*, 986−996.

(11) Minko, T.; Kopečková, P.; Kopeček, J. Efficacy of Chemotherapeutic Action of HPMA Copolymer-Bound DOX in a Solid Tumor Model of Ovarian Carcinoma. *Int. J. Cancer* **2000**, *86*, 108−117.

(12) Nishiyama, N.; Koizumi, F.; Okazaki, S.; Matsumura, Y.; Nishio, K.; Kataoka, K. Differential Gene Expression Profile Between PC-14 Cells Treated with Free Cisplatin and Cisplatin-Incorporated Polymeric Micelles. *Bioconjugate Chem.* **2003**, *14*, 449−457.

(13) Nishiyama, N.; Nori, A.; Malugin, A.; Kasuya, Y.; Kopečková, P.; Kopeček, J. Free and *N*-(2-Hydroxypropyl)methacrylamide Copolymer-Bound Geldanamycin Derivative Induce Different Stress Responses in A2780 Human Ovarian Carcinoma Cells. *Cancer Res.* **2003**, *63*, 7876−7882.

(14) Minko, T.; Kopečková, P.; Pozharov, V.; Kopeček, J. HPMA Copolymer-Bound Adriamycin Overcomes MDR1 Gene Encoded Resistance in a Human Ovarian Carcinoma Cell Line. *J. Controlled Release* **1998**, *54*, 223−233.

(15) Hengartner, M. O. The Biochemistry of Apoptosis. *Nature* **2000**, *407*, 770−776.

(16) Petak, I.; Houghton, J. A. Shared Pathways: Death Receptors and Cytotoxic Drugs in Cancer Therapy. *Pathol. Oncol. Res.* **2001**, *7*, 95−106.

(17) Solary, E.; Droin, N.; Battaieb, A.; Corcos, L.; Dimanche-Boitrel, M.-T.; Garrido, C. Positive and Negative Regulation of Apoptotic Pathways by Cytotoxic Agents in Hematological Malignancies. *Leukemia* **2000**, *14*, 1833−1849.

(18) Boldin, M. P.; Varfolomeev, E. E.; Pancer, Z.; Mett, I. L.; Camonis, J. H.; Wallach, D. A Novel Protein that Interacts with the Death Domain of Fas/APO1 Contains a Sequence Motif Related to the Death Domain. *J. Biol. Chem.* **1995**, *270*, 7795−7798.

(19) Chinnaiyan, A. M.; O'Rourke, K.; Tewari, M.; Dixit, V. M. FADD, A Novel Death Domain-Containing Protein, Interacts with the Death Domain of Fas and Initiates Apoptosis. *Cell* **1995**, *81*, 505−512.

(20) Boldin, M. P.; Goncharov, T. M.; Goltsev, Y. V.; Wallach, D. Involvement of MACH, A Novel MORT1/FADD-Interacting Protease, in Fas/APO1 and TNF Receptor-Induced Cell Death. *Cell* **1996**, *85*, 803−815.

(21) Medema, J. P.; Scaffidi, C.; Kischkel, F. C.; Shevchenko, A.; Mann, M.; Krammer, P. H.; Peter, M. E. FLICE is Activated by Association with the CD95 Death-Inducing Signaling Complex (DISC). *EMBO J.* **1997**, *16*, 2794−2804.

(22) Muzio, M.; Chinnaiyan, A. M.; Kischkel, F. C.; O'Rourke, K.; Shevchenko, A.; Ni, J.; Scaffidi, C.; Bretz, J. D.; Zhang, M.; Gentz, R.; Mann, M.; Krammer, P. H.; Peter, M. E.; Dixit, V. M. FLICE A Novel FADD-Homologous ICE/CED-3-Like Protease is Recruited to the CD95 (Fas/Apo-1) Death-Inducing Signaling Complex. *Cell* **1996**, *85*, 817−827.

(23) Irmler, M.; Thome, M.; Hahne, M.; Schneider, P.; Hofmann, K.; Steiner, V.; Bodmer, J. L.; Schroter, M.; Burns, K.; Mattmann, C.; Rimoldi, D.; French, L. E.; Tschopp, J. Inhibition of Death Receptor Signals by Cellular FLIP. *Nature* **1997**, *388*, 190−195.

and in cell death.[27] Fas-mediated and chemotherapy-induced apoptosis can converge at the level of the Fas receptor itself, FasL, DISC formation, the initiation phase of caspase 8 activation, at the level of the mitochondria, or at the level of downstream effector caspase activation.[16]

Induction of the Fas/FasL apoptotic pathway has been observed after the treatment of different tumors with various cytotoxic drugs at therapeutic concentrations.[28] It has also been reported that the exposure to cytotoxic agents as diverse as DOX, cisplatin, methotrexate, and phthalocyanine to light[29−31] may promote apoptosis by inducing the expression of Fas/FasL in some cancer cell lines. In other studies, the induction of apoptosis by cytotoxic drugs has been reported to be independent of the Fas system.[32−34] However, the final steps of Fas-independent apoptosis induced by cytotoxic drugs seem to proceed through the same downstream caspases (caspases 3 and 7) of the death receptor pathway.[32−35]

The aim of this study was to evaluate the possible involvement of the death receptor pathways in apoptotic events in A2780 human ovarian carcinoma cells after cells are exposed to free and HPMA copolymer-bound DOX. The A2780 cell line seems particularly well suited for this purpose, since it is sensitive to Fas induction following drug treatment,[36] with a low level of basal expression of Fas protein and the absence of FasL expression.[37]

Experimental Section

Chemicals. DOX was a kind gift from A. Suarato (Pfizer, Milan, Italy). Stock solutions (3 mM) were prepared in distilled water, filtered through a 0.2 μm membrane, aliquoted, and stored at −20 °C. P−(GFLG)−DOX [in which P is the HPMA copolymer backbone and GFLG (Gly-Phe-Leu-Gly) a lysosomally degradable spacer] was synthesized as previously described.[38] It contained 0.11 mmol of DOX/g of polymer (6.4 wt %); the molecular mass (M_w) = 27 kDa. P−(GFLG)−DOX stock solutions (6 mM) were prepared in distilled water, filtered through a 0.2 μm membrane, aliquoted, and stored at −20 °C. All concentrations of P−(GFLG)−DOX were expressed in DOX equivalents. Igepal CA 630, bovine pancreatic RNase A, and protease inhibitors were purchased from Sigma Chemical Co. (St. Louis, MO). All reagents for SDS−PAGE and Western blotting were purchased from Bio-Rad (Hercules, CA). Human recombinant soluble Fas ligand (sFasL) was purchased from Alexis Corp. (San Diego, CA); in every instance, sFasL was used in combination with an enhancer IgG antibody at a concentration of 1 μg/mL (Alexis Corp.). Fluorescent caspase 3, 8, and 9 inhibitors (FAM−DEVD−FMK, FAM−LETD−FMK, and FAM−LEHD−FMK, respectively) were obtained from Intergen Co. (Purchase, NY) as a component of the CaspaTag fluorescein caspase activity kits; the caspase 3, 8, and 9 nonfluorescent inhibitors (Z−DEVD−FMK, Z−LETD−FMK, and Z−LEHD−FMK, respectively) were obtained from ICN Pharmaceuticals, Inc. (Aurora, OH), dissolved in DMSO, aliquoted, and stored at −20 °C.

Antibodies. Monoclonal anti-Fas APO-1-3 antibody, 4-1-18 anti-human caspase 3, and anti-human caspase 8 antibody were purchased from Alexis Corp. FITC-conjugated goat anti-mouse IgG was from Sigma. Mouse IgG3 isotype control

(24) Scaffidi, C.; Fulda, S.; Srinivasan, A.; Friesen, C.; Li, F.; Tomaselli, K. J.; Debatin, K. M.; Krammer, P. H.; Peter, M. E. Two CD95 APO-1/Fas Signaling Pathways. *EMBO J.* **1998**, *17*, 1675−1687.

(25) Scaffidi, C.; Schmitz, I.; Krammer, P. H.; Peter, M. E. The Role of c-FLIP in Modulation of CD95-Induced Apoptosis. *J. Biol. Chem.* **1999**, *274*, 1541−1548.

(26) Earnshaw, W. C.; Martins, M. L.; Kaufmann, S. H. Mammalian Caspases: Structure, Activation, Substrates, and Functions During Apoptosis. *Annu. Rev. Biochem.* **1999**, *68*, 383−424.

(27) Tewari, M.; Quan, L. T.; O'Rourke, K.; Desnoyers, S.; Zeng, Z.; Beidler, D. R.; Poirier, G. G.; Salvesen, G. S.; Dixit, V. M. Yama/CPP32 Beta, A Mammalian Homolog of Ced-3, is a CrmA-Inhibitable Protease that Cleaves the Death Substrate Poly(ADP-ribose) Polymerase. *Cell* **1995**, *81*, 801−809.

(28) Friesen, C.; Fulda, S.; Debatin, K.-M. Cytotoxic Drugs and the CD95 Pathway. *Leukemia* **1999**, *13*, 1854−1858.

(29) Friesen, C.; Herr, I.; Krammer, P. H.; Debatin, K. M. Involvement of the CD95 (APO-1/FAS) Receptor/Ligand System in Drug-Induced Apoptosis in Leukemia Cells. *Nat. Med.* **1996**, *2*, 574−577.

(30) Herr, I.; Wilhelm, D.; Bohler, T.; Angel, P.; Debatin, K.-M. Activation of CD95 (APO-1/Fas) Signaling by Ceramide Mediates Cancer Therapy-Induced Apoptosis. *EMBO J.* **1997**, *16*, 6200−6208.

(31) Ahmad, N.; Gupta, S.; Feyes, D. K.; Mukhtar, H. Involvement of Fas (APO-1/CD-95) During Photodynamic-Therapy-Mediated Apoptosis in Human Epidermoid Carcinoma A431 Cells. *J. Invest. Dermatol.* **2000**, *115*, 1041−1046.

(32) Eischen, C. M.; Kottke, T. J.; Martins, L. M.; Basi, G. S.; Tung, J. S.; Earnshaw, W. C.; Leibson, P. J.; Kaufmann, S. H. Comparison of Apoptosis in Wild-Type and Fas-Resistant Cells: Chemotherapy-Induced Apoptosis is Not Dependent on Fas/Fas Ligand Interactions. *Blood* **1997**, *90*, 935−943.

(33) Gamen, S.; Anel, A.; Lasierra, P.; Alava, M. A.; Martinez-Lorenzo, M. J.; Pineiro, A.; Naval, J. DOX-Induced Apoptosis in Human T Cell Leukemia is Mediated by Caspase-3 Activation in a Fas-Independent Way. *FEBS Lett.* **1997**, *417*, 360−364.

(34) Tolomeo, M.; Dusonchet, L.; Meli, M.; Grimaudo, S.; D'Alessandro, N.; Papoff, G.; Ruberti, G.; Rausa, L. The CD95/CD95 Ligand System is Not the Major Effector in Anticancer Drug-Mediated Apoptosis. *Cell Death Differ.* **1998**, *5*, 735−742.

(35) McGahon, A. J.; Costa Pereira, A. P.; Daly, L.; Cotter, T. G. Chemotherapeutic Drug-Induced Apoptosis in Human Leukaemic Cells is Independent of Fas (APO-1/CD95) Receptor/Ligand System. *Br. J. Haematol.* **1998**, *101*, 539−547.

(36) Uslu, R.; Jewett, A.; Bonavida, B. Sensitization of Human Ovarian Tumor Cells by Subtoxic CDDP to Anti-Fas Antibody-Mediated Cytotoxicity and Apoptosis. *Gynecol. Oncol.* **1996**, *62*, 282−291.

(37) Bellarosa, D.; Ciucci, A.; Bullo, A.; Nardelli, F.; Manzini, S.; Maggi, C. A.; Goso, C. Apoptotic Events in a Human Ovarian Cancer Cell Line Exposed to Anthracyclines. *J. Pharmacol. Exp. Ther.* **2001**, *296*, 276−283.

(38) Omelyanenko, V. G.; Kopečková, P.; Gentry, C.; Shiah, J.-G.; Kopeček, J. HPMA Copolymer-Anticancer Drug-OV-TL16 Antibody Conjugates. 1. Influence of the Methods of Synthesis on Binding Affinity to OVCAR-3 Ovarian Carcinoma In Vitro. *J. Drug Targeting* **1996**, *3*, 357−373.

was from PharMingen (San Diego, CA). ZB4 IgG1-blocking anti-Fas monoclonal antibody was from MBL (Watertown, MA). C210 anti-PARP antibody was from Biomol Research Laboratories Inc. (Plymouth Meeting, PA). Anti-actin antibody was from Oncogene Research Products (San Diego, CA). Goat anti-mouse and anti-rabbit horseradish peroxidase-conjugated (GAM− and GAR−HRP, respectively) antibodies were from Southern Biotechnology Associates Inc. (Birmingham, AL).

Cells. The A2780 cell line, a human ovarian carcinoma, was obtained from T. C. Hamilton (Fox Chase Cancer Center, Philadelphia, PA). Cells were grown in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (FBS) from HyClone Laboratories (Ogden, UT) and 2 mM glutamine at 37 °C in 5% CO_2 and 95% humidified air. The cells were regularly split to keep them in the logarithmic phase of growth. Cells were plated into appropriate plasticware with an initial density of 3×10^4 cells/cm^2. Experiments were initiated 48 h after cells were plated, if not otherwise stated. Cells were detached from the plastic surface with a 0.05% trypsin and 0.02% EDTA treatment (HyClone) for 1 min and processed for flow cytometry or Western blot analysis as described below.

Cytotoxicity Assay. A2780 cells were plated in 96-well microtiter plates and cultured 48 h before being treated with various concentrations of drugs. Relative cell viability was measured by utilizing a highly water-soluble tetrazolium salt, WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt], as a component of Cell Counting Kit-8 from Dojindo Molecular Technologies, Inc. (Gaithersburg, MA). The IC_{50} dose (drug concentration which inhibits cell growth by 50% relative to nontreated control cells) was reported as a function of the drug concentration that was used.

Detection of Apoptosis by Flow Cytometry and Annexin V/PI Staining. Annexin V/PI staining was performed using the Vybrant apoptosis assay kit purchased from Molecular Probes (Eugene, OR). A2780 cells were incubated with the indicated drug concentrations for 24 h. After incubation, cells that became detached from the growth surface during apoptosis induction were collected, and later were combined with adherent cells that became detached from plastic using trypsin and EDTA. Cells were gently washed twice with cold phosphate-buffered saline containing 2% BSA (PBS−BSA 2%, pH 7.2) and then resuspended in binding buffer (HEPES supplemented with 25 mM $CaCl_2$). Cells (10^5) were incubated, protected from light, with 5 μL of fluorescein-conjugated annexin V and 1 μL of propidium iodide (PI) (100 μg/mL) for 15 min at room temperature. At the end of the incubation time, 400 μL of binding buffer was added, and the samples were analyzed on a FACScan flow cytometer using CellQuest analysis software (Becton Dickinson, Mountain View, CA). The following controls were used: unstained cells, cells stained with annexin V−FITC only, and cells stained with PI only. Singly stained cells were used to adjust electronic compensation on FL1 and FL2 channels. Also, the necessary adjustments on FL2 detectors were performed, using unstained drug-treated cells to account for DOX autofluorescence. Setting of quadrant statistics for the determination of the frequency of cells undergoing apoptosis was achieved on untreated cells stained with both annexin V−FITC and PI or on untreated cells. After being appropriately set, annexin V-positive cells were identified in the bottom right quadrant of the dot plot, whereas cells doubly stained with annexin V and PI were identified in the top right panel.[39]

Determination of the Level of Fas Expression by Flow Cytometry. After being incubated with drugs, A2780 cells (10^6) were detached from plastic using trypsin and EDTA, gently washed twice with PBS−BSA 2%, centrifuged at 300*g* for 5 min, and then incubated for 1 h at 0 °C with monoclonal anti-Fas APO-1-3 antibodies (Alexis Corp.) at a concentration of 1 μg/10^6 cells. The cells were washed twice with PBS−BSA 2% and stained for 1 h in the dark at 0 °C with FITC-conjugated goat anti-mouse IgG antibody (Sigma). Finally, cells were washed twice with PBS−BSA 2% and analyzed on a FACScan flow cytometer (Becton Dickinson). Fluorescence signals were collected in the logarithmic mode, while the relative cell numbers per channel were in the linear mode.

Western Blot. A2780 cells were washed in cold PBS and lysed in 2× sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 1 mM PMSF, and a mixture of protease inhibitors (Sigma). Proteins were separated under reducing conditions by 15% SDS−PAGE after being loaded at equal protein amounts per well and transferred to a nitrocellulose membrane. The successful transfer was controlled routinely by reversible staining of the membrane with a Pounceau S solution. After being blocked with 5% nonfat milk or 2% BSA in TBS containing 0.1% Tween 20 overnight at 4 °C, the membrane was probed with anti-human caspase 3, anti-human caspase 8, anti-PARP, or anti-actin primary antibodies. Secondary antibodies were GAR−HRP and GAM−HRP. The immunoreactive bands were visualized by incubation of the membrane with the enhanced chemiluminescence (ECL) Western blotting detection reagent from Amersham Biosciences Corp. (Piscataway, NJ).

Detection of Caspase Activation by Fluorochrome-Labeled Inhibitors. Analysis was performed using the CaspaTag Caspase Activity Kit according to the manufacturer's instructions. A2780 cells were plated in 24-well plates in 500 μL of medium. After 48 h, a new medium containing the drug at the appropriate dilutions and the caspase 3, 8, or 9 inhibitors was added and cells were incubated for 24 h at 37 °C under 5% CO_2. At the end of incubation, detached and adherent cells were collected together. The collected cells

(39) Vermes, I.; Haanen, C.; Steffens-Nakken, H.; Reutelingsperger, C. A Novel Assay for Apoptosis: Flow Cytometric Detection of Phosphatidylserine Expression on Early Apoptotic Cells Using Fluorescein Labeled Annexin V. *J. Immunol. Methods* **1995**, *184*, 39−51.

Table 1. Effect of sFASL and Anti-Fas Antibody on Apoptosis in A2780 Cells Treated with DOX and P−(GFLG)−DOX[a]

	% of apoptotic cells (annexin V−FITC positive)			
drug	no stimulation	ZB4	sFASL	sFASL and ZB4
control	8.0 ± 1.5	8.3 ± 1.5	21.8 ± 3.6[b]	13.6 ± 3.3
0.1 μM DOX	35.6 ± 6.5	35.1 ± 7.1	59.0 ± 7.8[c]	41.5 ± 7.5
15 μM P−(GFLG)−DOX	21.7 ± 2.6	19.6 ± 3.0	39.4 ± 5.8[b]	22.7 ± 3.6

[a] Cells were treated with 0.1 μM DOX or 15 μM P−(GFLG)−DOX for 24 h, and then stimulated for 4 h with 100 ng/mL sFasL alone or with the 2 mg/mL ZB4 IGg1-blocking anti-Fas monoclonal antibody. sFasL was used in combination with an enhancer IgG antibody at a concentration of 1 μg/mL. The values of the mean ± the standard deviation of three experiments are shown. Statistical comparisons of sFasL effects with and without blocking antibody ZB4 were made between respective unstimulated cells and DOX- or P−(GFLG)−DOX-treated cells. [b] $p < 0.01$. [c] $p < 0.05$.

were washed twice in PBS−BSA 2%, gently sedimented at 300*g* for 5 min, and resuspended in 300 μL of fresh medium, and 10 μL of a 30× fluorochrome-labeled inhibitors of caspase (FLICA) working dilution was added. Cells were incubated, protected from light, for 1 h at 37 °C under 5% CO_2. Labeled cells were washed, using gentle centrifugation at 300*g* for 5 min. The cell fluorescence was measured with a FACScan flow cytometer (Becton Dickinson). Unstained cells and cells stained with only carboxyfluorescein−peptide−fluoromethyl ketone stain (FAM−peptide−FMK) were used as the control.

Results

Induction of Apoptosis. As a preliminary step of our study, the cytotoxicity of both forms of DOX and their ability to induce apoptosis in A2780 cells were determined. The IC_{50} doses, determined as described in the Experimental Section, were 0.05 ± 0.01 and 7.2 ± 2.5 μM for free DOX and P−(GFLG)−DOX, respectively, after continuous drug exposure for 72 h. In this study, the induction of apoptosis was assessed at a 2 × IC_{50} dose. Flow cytometry analysis of A2780 cells stained with annexin V and PI confirmed that both free DOX and P−(GFLG)−DOX induced apoptosis in this cell line. After the cells had been exposed for 24 h, free DOX (0.1 μM) and P−(GFLG)−DOX (15 μM) induced apoptosis in 35.6 ± 6.5 and 21.7 ± 2.6% of A2780 cells, respectively (Table 1). Prolonged exposure of cells to drugs at the indicated concentrations always resulted in an increase of the percentage of the necrotic cell population (data not shown).

Activation of Caspases. The activation of caspases in A2780 cells was assessed by Western blots and flow cytometry. The exposure of A2780 cells to free DOX and P−(GFLG)−DOX resulted in the activation of caspase 3 and the formation of a 17 kDa cleaved domain of caspase 3 (Figure 2). In addition, the cleavage of the physiological substrate of caspase 3, PARP, resulted in the formation of an 85 kDa fragment (Figure 3). The activation of caspase 8 during the incubation of A2780 cells with drugs was also detected (Figure 4). It resulted in the formation of the 45/43 cleaved fragments of caspase 8. The formation of the 18 kDa fragment was observed only when A2780 cells were treated with sFasL. Because of the critical role of caspase 8 in the activation of the caspase cascade in the Fas-mediated pathway, a caspase activity assay was used to confirm the ability of free DOX and P−(GFLG)−DOX to induce caspase 8 activation.

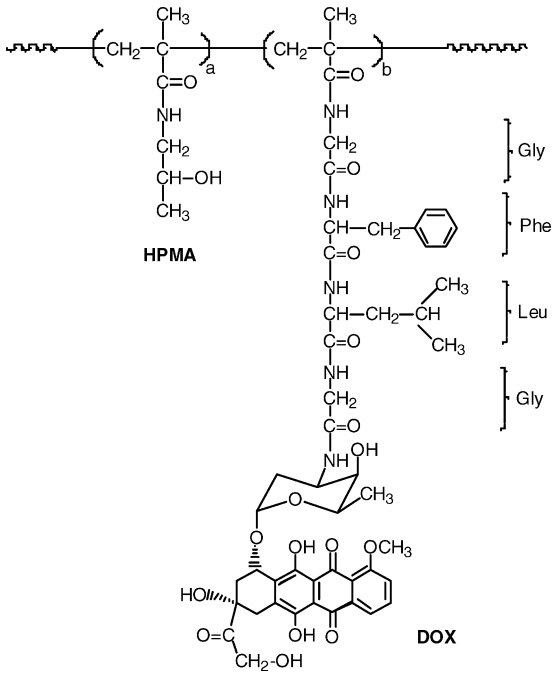

Figure 1. Structure of the HPMA copolymer−doxorubicin conjugate [P−(GFLG)−DOX]. P is the HPMA copolymer backbone and GFLG (Gly-Phe-Leu-Gly) a lysosomally degradable spacer. The conjugate contained 0.11 mmol of doxorubicin/g of polymer (6.4 wt %); the molecular mass (M_w) = 27 kDa.

The flow cytometry analysis was carried out using a 15 mW argon ion laser at 488 nm. Fluorescein was assessed on the FL1 channel. A histogram of the log FL1 (*X*-axis) versus the number of cells (*Y*-axis) was generated. Two populations of cells appeared on the histogram. The majority of cells were caspase-negative and were represented in the first peak within the second log decade of the FL1 (*X*-axis), whereas the caspase-positive population of cells appeared as a second peak or shoulder of the first peak showing an increased fluorescence intensity. The mean fluorescence of a population of cells was determined (Figure 5), and the relative caspase activity (as a percentage of the control) was calculated. On the basis of these results, P−(GFLG)−DOX slightly increased the level of activation of caspase 3 and caspase 9 (1.7- and 1.17-fold, respectively), while the free

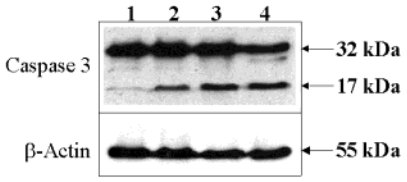

Figure 2. Activation of procaspase 3 after exposure of A2780 cells to DOX and P−(GFLG)−DOX. A2780 cells (1×10^6) were treated with 0.1 μM DOX (lane 2) or 15 μM P−(GFLG)−DOX (lane 3) for 24 h, or 100 ng/mL sFASL (lane 4) for 4 h. Lane 1 contained untreated cells. Cell lysate was prepared as described in the Experimental Section. Cellular proteins were separated by 15% SDS−PAGE and then transferred to the nitrocellulose membrane. The nitrocellulose membrane was probed with anti-human caspase 3 monoclonal antibodies at a 1/1000 dilution. The secondary antibody was GAM−HRP diluted 1/5000, and the blot was developed with ESL. The arrows indicate the position of 32 kDa procaspase 3 and the 17 kDa active subunit of caspase 3. The blot was reprobed with an anti-actin antibody to evaluate the loading of the cellular extracts. Only a section of the immunoblots indicating the protein isoforms is shown.

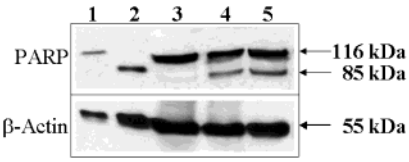

Figure 3. Cleavage of the caspase 3 specific substrate PARP in A2780 cells exposed to DOX and P−(GFLG)−DOX. A2780 cells (1×10^6) were treated with 0.1 μM DOX (lane 4) or 15 μM P−(GFLG)−DOX (lane 5) for 24 h. Lane 3 contained untreated A2780 cells: lane 1, PARP enzyme (SW-111, Biomol); and lane 2, HL60 cells induced to undergo apoptosis using etoposide (SW-102, Biomol). Cell lysate was prepared as described in the Experimental Section. Cellular proteins were separated by 15% SDS−PAGE and transferred to the nitrocellulose membrane. The membrane was probed with anti-human PARP polyclonal antibodies at a 1/500 dilution. The secondary antibody was GAR−HRP diluted 1/5000, and the blot was developed with ESL. The arrows indicate the position of the 116 kDa uncleaved and 85 kDa cleaved isoforms of PARP. The blot was reprobed with an anti-actin antibody to evaluate the loading of the cellular extracts. Only a section of the immunoblots indicating the protein isoforms is shown.

DOX significantly increased the activity of caspase 3 and caspase 9 (3.6- and 3.3-fold, respectively), when compared to that of untreated cells. Both forms of DOX had no apparent effect on the activity of caspase 8 in A2780 cells under the experimental conditions that were used (Figure 6).

Expression of the Fas Receptor and Functional Activity. The expression of Fas protein in A2780 cells was assessed by flow cytometry. Both forms of DOX upregulated Fas receptor expression on the cell membrane. The peak of the extent of the Fas protein expression on the A2780 cell membrane was observed at 16−24 h with free DOX and at

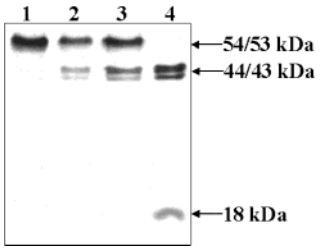

Figure 4. Activation of procaspase 8 after exposure of A2780 cells to DOX and P−(GFLG)−DOX. Cells (1×10^6) were treated with either 0.1 μM DOX (lane 2) or 15 μM P−(GFLG)−DOX (lane 3) for 24 h, or 100 ng/mL sFASL (lane 4) for 4 h. Lane 1 contained untreated cells. Cell lysate was prepared as described in the Experimental Section. Cellular proteins were separated by 15% SDS−PAGE and transferred to the nitrocellulose membrane. The membrane was probed with anti-human caspase 8 monoclonal antibodies at a 1/200 dilution. The secondary antibody was GAM−HRP diluted 1/1000, and the blot was developed with ESL. The arrows indicate the position of 54/53 kDa procaspase 8, the 45/43 kDa intermediate, and the 17 kDa active subunit of caspase 8. Only a section of the immunoblots indicating the protein isoforms is shown.

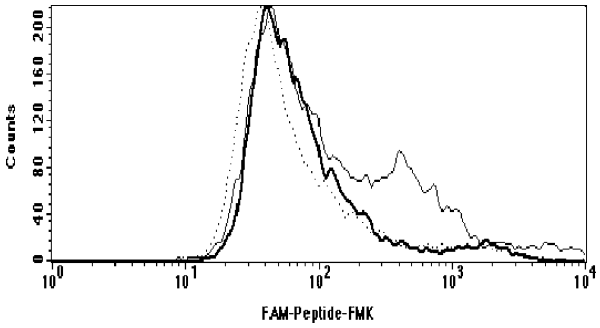

Figure 5. Single-color analysis of caspase 3 activity in A2780 cells treated with DOX. A2780 cells were treated for 24 h with 0.1 μM free DOX with (thick line) or without (thin line) the caspase 3 inhibitor Z−DEVD−FMK. The dotted line represents data for untreated cells. Cells were labeled with FAM−DEVD−FMK, washed, and analyzed on a FACScan flow cytometer as described in the Experimental Section. The frequency histogram of the number of events (*Y*-axis) vs fluorescein intensity (*X*-axis) shows two different cell populations. Caspase-negative cells lie within the second log decade of the *X*-axis, whereas caspase-positive cells lie within the third and fourth log decades as a second peak or a shoulder of the first peak. Histograms were used to determine median cell fluorescence. Results are representative of one of three separate experiments.

36−48 h with P−(GFLG)−DOX (Figure 7). When a drug-induced Fas receptor was triggered by exogenous sFasL (100 ng/mL), an increase in the extent of apoptotic cell death occurred, indicating that the Fas signaling pathway was functionally active. To assess whether the activation of the Fas receptor could be involved in drug-induced cell death in this cell line, we further analyzed the effect of an antagonist ZB4 antibody on drug-induced apoptosis. An-

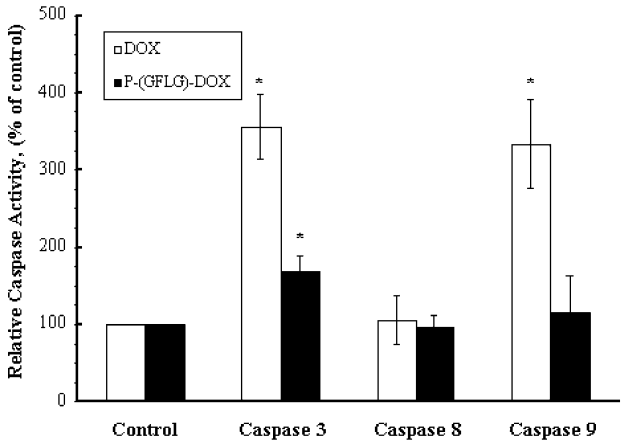

Figure 6. Relative activity of caspases 3, 8, and 9 in A2780 cells treated with 0.1 μM DOX (white columns) and 15 μM P−(GFLG)−DOX (black columns). After being incubated with drug for 24 h, cells were labeled with the fluorescent caspase inhibitor (FAM−peptide−FMK), washed, and analyzed on a FACScan flow cytometer. The median cell fluorescence was determined, and the relative activity of each caspase was calculated as the percentage of untreated cells. Data are representative of one of three separate experiments. Statistical comparisons were made between drug-treated and untreated cells.

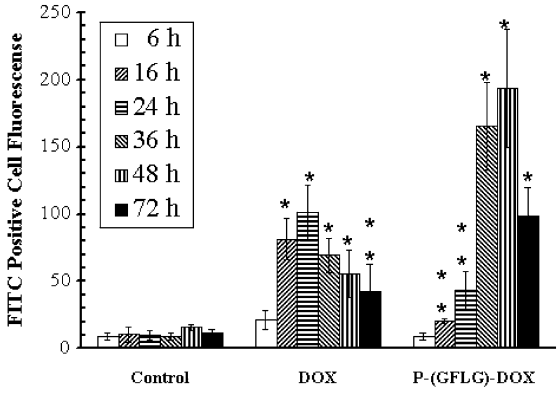

Figure 7. FAS receptor expression on A2780 cells after exposure to DOX and P−(GFLG)−DOX. A2780 cells were treated for 6, 16, 24, 36, 48, and 72 h with 0.05 μM free doxorubicin or 7 μM P−(GFLG)−DOX. After being incubated with drug, cells (10^6) sequentially were washed, incubated with monoclonal anti-Fas APO-1-3, washed, and stained with an FITC-conjugated goat anti-mouse IgG antibody. Cell analysis was performed on a FACScan flow cytometer as described in the Experimental Section. Data are presented as the mean $\pm$ the standard deviation. Statistical comparisons were made between cells treated with DOX and P−(GFLG)−DOX and untreated cells at respective time points (one asterisk indicates a p of <0.01, and two asterisks indicate a p of <0.05).

tagonistic anti-Fas ZB4 antibody blocked the increase in the level of apoptosis following application of sFasL, but it did not interfere with drug-induced apoptosis (Table 1). Cell death inhibition through the anti-Fas ZB4 antibody confirmed the specificity of sFasL-induced cell death.

Discussion

Covalent attachment of low-molecular weight anticancer drugs to polymeric carriers results in drug delivery systems (macromolecular therapeutics) with numerous advantages. Improvements in solubility, pharmacokinetic profiles, biodistribution, accumulation in solid tumors, targetability, and therapeutic efficacy have been observed when compared to those of low-molecular weight drugs.[1−5] Results of preclinical evaluations were validated in clinical trials.[40,41] However, it was recently demonstrated in vitro[10,12,13,42] and in vivo[11] that macromolecular therapeutics can trigger signaling pathways different from those with free drugs. In particular, P−(GFLG)−DOX overcame existing pumps and downregulated the MDR1 gene, induced apoptosis and lipid peroxidation, and inhibited DNA repair, replication, and biosynthesis in sensitive (A2780) and DOX-resistant (A2780/AD) human ovarian carcinoma cells[10] and xenografts in nude mice[11] when compared to free DOX. In a preliminary study, it was found that P−(GFLG)−DOX induced apoptosis by the activation of caspase signaling pathways different from that induced by free DOX. In addition to the upregulation of caspases 3, 7, and 9 induced by free DOX, P−(GFLG)−DOX also upregulated caspases 6 and 8 at the level of gene expression and, possibly, at the level of protease activation.[42] Caspase 8 activation is a critical intracellular event, reflecting the activation of the Fas receptor signaling pathway. In this study, we investigated the possible convergence of Fas-mediated and chemotherapy-induced apoptosis at the level of the Fas receptor, and at the level of effector and executioner caspase activation.

We found that P−(GFLG)−DOX, as well as free DOX, induced apoptosis in A2780 cells. After cells had been incubated for 24 h, free DOX or P−(GFLG)−DOX, at $2IC_{50}$ concentrations, induced apoptosis in 36% and 22% of A2780 cells, respectively. Immunoblot analysis of A2780 cells demonstrated caspase 3 activation. The cleaved caspase 3 active domain was found in cells treated with both forms of DOX. In addition, the physiological substrate for caspase 3, PARP, was cleaved. It is interesting to note that the PARP cleavage was time-dependent and was similar to the kinetics of the Fas receptor expression (data not shown). Caspase 3 activation was confirmed using fluorochrome-labeled inhibi-

(40) Vasey, P. A.; Kaye, S. B.; Morrison, R.; Twelves, C.; Wilson, P.; Duncan, R.; Thomson, A. H.; Murray, L. S.; Hilditch, T. E.; Murray, T.; Burtles, S.; Fraier, D.; Frigerio, E.; Cassidy, J. Phase I Clinical and Pharmacokinetic Study of PK1 [*N*-(2-Hydroxypropyl)methacrylamide Copolymer Doxorubicin]: First Member of a New Class of Chemotherapeutic Agents, Drug-Polymer Conjugates. *Clin. Cancer Res.* **1999**, *5*, 83−94.

(41) Seymour, L. W.; Ferry, D. R.; Anderson, D.; Hesslewood, S.; Julyan, P. J.; Poyner, R.; Doran, J.; Young, A. M.; Burtless, S.; Kerr, D. J. Hepatic Drug Targeting: Phase I Evaluation of Polymer-Bound Doxorubicin. *J. Clin. Oncol.* **2002**, *20*, 1668−1676.

(42) Minko, T.; Kopečková, P.; Kopeček, J. Preliminary Evaluation of Caspase-Dependent Apoptosis Signaling Pathways of Free and HPMA Copolymer-Bound DOX in Human Ovarian Carcinoma Cells. *J. Controlled Release* **2001**, *71*, 227−237.

tors of caspases (FLICA). The FLICA ligands are carboxyfluorescein (FAM)-labeled peptide fluoromethyl ketones (FMK) that specifically, with 1:1 stoichiometry, covalently bind to active centers of caspases.[43] The amino acid sequence of the peptide moiety of the reagents provides selectivity vis-à-vis the respective caspase. The irreversible binding of the enzymes to the active centers of the caspases through the FMK moiety ensures that only the cells with the activated enzymes become labeled.[43] In our study, we found that both forms of DOX induced cell fluorescence increases of 3.6 times (DOX) and 3.4 times [P−(GFLG)−DOX], indicating caspase 3 activation.

Caspase 3 is the central executioner of the apoptotic program of mammalian cells, and many apoptotic events may be finally linked to this enzyme. In contrast, caspase 8 principally participates in only the Fas-mediated apoptotic pathway, as described above. We found that the treatment of A2780 cells with either form of DOX resulted in caspase 8 cleavage and formation of the intermediate products of caspase 8 proteolysis: 45/43 and 38/36 kDa proteins. However, the formation of the 20/18 kDa cleaved domain was observed only when cells were exposed to exogenous sFasL, indicating the activation of the most proximal caspase of the Fas-mediated apoptotic pathway. Incidentally, caspase 8 activation was not confirmed using the fluorochrome-labeled caspase 8 inhibitor. In flow cytometry studies, caspase 8 activity remained on the same level when cells were treated with either form of DOX. Possibly, this reflected the fact that A2780 cells treated with DOX induced very small amounts of active caspase 8.

Currently, two different models of death receptor apoptosis pathways have been described (*24*). In type I cells, caspase 8 is recruited to the death-inducing signaling complex (DISC), resulting in the release of active caspase 8 in quantities that are sufficient to directly activate caspase 3.[44] Despite the similar expression levels of surface Fas and signaling molecules, in type II cells the formation of the DISC is inefficient, producing very small quantities of active caspase 8 at the cell surface. The amount of caspase 8 is insufficient to induce the activation of caspase 3, but sufficient to cleave the Bid protein,[45,46] resulting in the apoptogenic activation of mitochondria. Recently, a number of transgenic and knockout studies have provided evidence for the existence of the two pathways in vivo.[47−50] In both cases, Fas apoptosis execution of thymocytes and peripheral T cells was independent of the mitochondria, identifying them as type I cells, whereas the liver was found to be a type II tissue that requires the mitochondrial branch of the Fas pathway to apoptosis.

In contrast to the role of caspase 8 in apoptosis, in which the role of the mitochondria may vary between different cell types,[24] we observed that both forms of DOX induced an activation of caspase 9, which is clearly mitochondria-dependent. Caspase 9 activity showed a relevant increase when cells were treated with free DOX, but P−(GFLG)−DOX induced only slight, and statistically insignificant, caspase 9 activation. We assume that such differences in drug effects may depend on the intracellular free DOX concentration caused by the rate of drug release via oligopeptide spacer cleavage in the lysosome.

Another possible level of convergence for Fas-mediated and drug-induced apoptosis is the Fas receptor itself. We found that both forms of DOX upregulate the expression of the Fas receptor on the surface of A2780 cells in a time-dependent manner. Because of the difference in the mechanism of cell entry and intracellular distribution, P−(GFLG)−DOX induced maximal Fas receptor expression with a delay of more than 12 h, when compared with free DOX (Figure 7). This suggests that one of the reasons that resistance to chemotherapy may be overcome, and the successful clinical use of HPMA copolymer-bound DOX, is the upregulation of Fas receptors on a cell surface. By increasing the number of Fas receptors on the plasma membrane, tumor cells may become sensitive to the attacking cells of the immune system, preventing further development and growth of the cancer.[4,51,52]

(43) Smolewski, P.; Bedner, E.; Du, L.; Hsieh, T. C.; Wu, J. M.; Phelps, D. J.; Darzynkiewicz, Z. Detection of Caspases Activation by Fluorochrome-Labeled Inhibitors: Multiparameter Analysis by Laser Scanning Cytometry. *Cytometry* **2001**, *44*, 73−82.

(44) Stennicke, H. R.; Jürgensmeier, J. M.; Shin, H.; Deveraux, Q.; Wolf, B. B.; Yang, X.; Zhou, Q.; Ellerby, H. M.; Ellerby, L. M.; Bredesen, D.; Green, D. R.; Reed, J. C.; Froelich, C. J.; Salvesen, G. S. Pro-caspase-3 is a major physiologic target of caspase-8. *J. Biol. Chem.* **1998**, *273*, 27084−27090.

(45) Li, H.; Zhu, H.; Xu, C. J.; Yuan, J. Cleavage of BID by Caspase 8 Mediates the Mitochondrial Damage in the FAS Pathway of Apoptosis. *Cell* **1998**, *94*, 491−501.

(46) Luo, X.; Budihardjo, I.; Zou, H.; Slaughter, C.; Wang, X. Bid, a Bcl2 Interacting Protein, Mediates Cytochrome *c* Release from Mitochondria in Response to Activation of Cell Surface Death Receptors. *Cell* **1998**, *94*, 481−490.

(47) Lacronique, V.; Mignon, A.; Fabre, M.; Viollet, B.; Rouquet, N.; Molina, T.; Porteu, A.; Henrion, A.; Bouscary, D.; Varlet, P.; Joulin, V.; Kahn, A. Bcl-2 Protects from Lethal Hepatic Apoptosis Induced by an Anti-Fas Antibody in Mice. *Nat. Med.* **1996**, *2*, 80−86.

(48) Rodriguez, I.; Matsuura, K.; Khatib, K.; Reed, J. C.; Nagata, S.; Vassalli, P. A bcl-2 Transgene Expressed in Hepatocytes Protects Mice from Fulminant Liver Destruction but not from Rapid Death Induced by Anti-Fas Antibody Injection. *J. Exp. Med.* **1996**, *183*, 1031−1036.

(49) Wei, M. C.; Zong, W.-X.; Cheng, E. H.-Y.; Lindsten, T.; Panoutsakopoulou, V.; Ross, A. J.; Roth, K. A.; MacGregor, G. R.; Thompson, C. B.; Korsmeyer, S. J. Proapoptotic BAX and BAK: A Requisite Gateway to Mitochondrial Dysfunction and Death. *Science* **2001**, *292*, 727−730.

(50) Yin, X. M.; Wang, K.; Gross, A.; Zhao, Y.; Zinkel, S.; Klocke, B.; Roth, K. A.; Korsmeyer, S. J. Bid-Deficient Mice are Resistant to Fas-Induced Hepatocellular Apoptosis. *Nature* **1999**, *400*, 886−891.

(51) Maggi, C. A. Therapeutic Opportunities from the Pharmacological Manipulation of the Fas System. *Pharmacol. Res.* **1998**, *38*, 1−34.

(52) Říhová, B.; Strohalm, J.; Hoste, K.; Jelínková, M.; Hovorka, O.; Kovář, M.; Plocová, D.; Širová, M.; St'astný, M.; Schacht, E.; Ulbrich, K. Immunoprotective Therapy with Targeted Anticancer Drugs. *Macromol. Symp.* **2001**, *172*, 21−28.

Because of the inability of A2780 cells to induce mRNA and protein expression of FasL,[37] the triggering of Fas with FasL does not occur in those cells. Accordingly, apoptosis can be induced by only the induction of the downstream steps of the corresponding signaling pathway. In our experiments, both forms of DOX were able to induce apoptosis in A2780 cells, confirming the recent observation that chemotherapeutic drugs may induce apoptosis even in the absence of Fas−FasL interaction.[53] Also, it was reported that Fas-mediated apoptosis occurs in the presence of RNA and protein synthesis inhibitors,[28] or enucleated cells,[54] suggesting that all of the components necessary for signal transduction are present and the Fas activation simply sets in action preexisting apoptotic machinery. When we added exogenous sFasL to A2780 cells treated with polymer-bound DOX, a significant increase in the extent of apoptotic cell death occurred (Table 1). Antagonistic blocking ZB4 antibodies almost completely prevented an increase in the percentage of apoptotic cells induced with sFasL, but did not change the percentage of apoptotic cells induced with chemotherapeutic agents alone. This allows us to conclude that the Fas receptor pathway remains functionally active during the treatment of A2780 cells with free DOX and P−(GFLG)−DOX.

A2780 cells, despite the expression of the surface Fas receptor, are not able to express signaling Fas ligand molecules.[37] One may hypothesize that the formation of the DISC is inefficient, and as a result, only very small quantities of active caspase 8 are generated. If so, the apoptosis occurring in A2780 cells induced with P−(GFLG)−DOX may be the result of caspase 3 activation, enhanced by mitochondrial proteins. Nevertheless, all the data presented above, and the fact that antagonistic Fas antibodies did not change the drug's effect on apoptotic cell death, allow us to conclude that apoptosis induced in A2780 cells with P−(GFLG)−DOX is not related to the activation of the Fas receptor apoptotic pathway.

Future studies should determine the possible formation of truncated Bid by a small amount of activated caspase 8; truncated Bid may cross over from the death receptor pathway to the mitochondrial pathway. In addition, evaluating the possible activation of procaspase 8 through the postmitochondrial cascade may be interesting. From that point of view, it would be necessary to investigate the effects of the Bcl-2 family of proteins on the induction of apoptosis in ovarian carcinoma cells exposed to free and HPMA copolymer-bound DOX.

Abbreviations Used

Bid, Bcl-2 interacting domain; cFLIP, cellular FLICE inhibitor protein; D, aspartic acid; DISC, death-inducing signaling complex; DOX, doxorubicin; E, glutamic acid; ECL, enhanced chemiluminescence; FADD, Fas-associated death domain; GFLG, glycylphenylalanylleucylglycine; FAM, carboxyfluorescein; FasL, Fas ligand; FLICA, fluorochrome-labeled inhibitor of caspases; FMK, fluoromethyl ketone; GAM−HRP, goat anti-mouse antibody−horseradish peroxidase conjugate; GAR−HRP, goat anti-rabbit antibody−horseradish peroxidase conjugate; HPMA, *N*-(2-hydroxypropyl)methacrylamide; H, histidine; L, leucine; MDR, multidrug resistance; P−(GFLG)−DOX, DOX bound to HPMA copolymer via a GFLG spacer; P, HPMA copolymer backbone; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; PMSF, phenylmethanesulfonyl fluoride; sFasL, recombinant soluble FasL; T, threonine; TNF/NGF, tumor necrosis factor/nerve growth factor; V, valine; Z, benzyloxycarbonyl; Z−DEVD−FMK, caspase 3 inhibitor Z−D(OCH_3)-E(OCH_3)-V-D(OCH_3)−FMK; Z−LETD−FMK, caspase 8 inhibitor Z−L-E(OCH_3)-T-D(OCH_3)−FMK; Z−LEHD−FMK, caspase 9 inhibitor Z−L-E(OCH_3)-H-D(OCH_3)−FMK.

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(53) Wesselborg, S.; Engels, I. H.; Rossmann, E.; Los, M.; Schulze-Osthoff, K. Anticancer Drugs Induce Caspase-8/FLICE Activation and Apoptosis in the Absence of CD95 Receptor/Ligand Interaction. *Blood* **1999**, *93*, 3053−3063.

(54) Schulze-Osthoff, K.; Walczak, H.; Droge, W.; Krammer, P. H. Cell Nucleus and DNA Fragmentation are Not Required for Apoptosis. *J. Cell Biol.* **1994**, *127*, 15−20.