

CD47 Mediates Killing of Breast Tumor Cells via Gi-Dependent Inhibition of Protein Kinase A

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ABSTRACT

Thrombospondins (TSPs) have been implicated as antitumor and antimetastasis factors in breast cancer. Although this effect has been attributed to the antiangiogenic activity of TSPs, recent observations suggest other mechanisms may be at work. The TSP receptor CD47 (integrin-associated protein) has recently been reported to mediate a novel form of apoptosis. Here, we have studied the response of breast cancer cells to CD47 ligands TSP-1, the CD47 agonist peptide 4N1K derived from TSP-1, and the anti-CD47 monoclonal antibody 1F7. All of these ligands killed four different breast cancer cell lines. This CD47-mediated cell death did not require active caspases or Bcl-2 degradation and did not cause DNA laddering or cytochrome *c* release. Pertussis toxin (PTX) prevented CD47-mediated death, indicating the involvement of G_i. 4N1K dramatically reduced intracellular cAMP levels, an effect reversed with PTX. Forskolin, 8-bromo cAMP, and isobutylmethylxanthine (IBMX) all prevented CD47-mediated apoptosis, indicating the involvement of cAMP. H89 and protein kinase A (PKA) inhibitor peptide prevented rescue of breast cancer cells by PTX, 8-Br-cAMP, and forskolin, suggesting that the effects of cAMP are mediated via PKA-dependent phosphorylation events. Epidermal growth factor also inhibited CD47-induced apoptosis via a PKC-dependent but ERK-independent pathway. Thus, CD47-mediated killing of breast cancer cells occurs by a novel pathway involving regulation of cAMP levels by heterotrimeric G_i with subsequent effects mediated by PKA.

INTRODUCTION

Breast cancer is a major public health concern with 180,000 cases/year in the United States (1). Substantial progress has been made in the multidisciplinary management of primary breast cancer. However, despite the development of new chemotherapeutic agents like doxorubicin, paclitaxel, and tamoxifen, the battle against breast cancer is far from over (2). Chemoresponsiveness of breast cancers varies considerably, perhaps attributable to variations or defects in the response of programmed cell death pathways (3). A variety of growth factors such as insulin-like growth factor and epidermal growth factor (EGF) are thought to oppose cell death and contribute to the sustained growth of the breast cancer cells (4–6).

Over the past decade, evidence has accumulated pointing to a role for thrombospondins (TSPs) 1 and 2 as antitumor factors in breast cancer (7–18). This has been largely attributed to the demonstrated antiangiogenic effects of TSP1 (7–9, 12–14) and 2 (10, 11, 19). A primary mechanism for the angiostatic effect of the TSPs is their binding to CD36, a TSP receptor. CD36 is expressed on microvascular endothelial cells and appears to regulate the motility and proliferation of endothelial cells, activities necessary for angiogenesis to proceed (20). Additional avenues for TSP regulation of angiogenesis exist. TSP1 can directly promote endothelial apoptosis (18, 21). It can also inhibit matrix metalloproteinases such as metalloproteinase 9,

leading to a decrease in the proteolytic release and activation of vascular endothelial growth factor from matrix-bound stores (15). Recently, a nonmetastatic clone of a human breast cancer cell line was found to up-regulate TSP1 expression 15-fold (22). This was not, however, correlated with a decrease in vascularization of primary tumors of this clone, suggesting that other mechanisms besides angiogenesis might be at work.

Large vessel endothelial cells, such as human umbilical vein endothelial cells, do not express CD36 nor do they contribute to an angiogenic response (20). In human umbilical vein endothelial cells, expression of the TSP receptor CD47 leads to increased apoptosis under conditions of low or turbulent flow (23, 24). CD47, also called integrin-associated protein is a receptor for the COOH-terminal domain of TSPs (25–27). CD47/integrin-associated protein is a pentaspan membrane protein that signals via heterotrimeric G_i (26) to augment the functions of a number of integrins in processes such as cell spreading, migration, and platelet aggregation (27).

In addition to these functions clearly associated with integrin signaling, CD47 appears to function in other aspects of cellular regulation. We have reported that certain monoclonal antibodies (mAbs) versus CD47, as well as the natural CD47 ligand TSP1, and a CD47 agonist peptide called 4N1K derived from TSP1, can induce a novel form of apoptosis in transformed and activated normal T cells (28, 29), chronic lymphocytic leukemia cells (30), erythroleukemia cells and primary arterial smooth muscle cells (29). In view of the possibility that some antitumor and antimetastatic effects of TSPs might not be due to angiostatic effects, we set out to determine whether CD47, which is known to be expressed on breast cancer cells (31), might render them susceptible to apoptosis mediated by CD47 agonists.

We report here that both mAbs and native ligands that serve as CD47 agonists can induce the death of breast cancer cells derived from several isolated lines. As in T cells (29), CD47-mediated killing of breast cancer cells is mediated via heterotrimeric G_i signaling, resulting in a dramatic drop in intracellular cAMP levels. Cell death is prevented by maintaining the level of intracellular cyclic AMP, the effect of which depends on active protein kinase A (PKA). Furthermore, EGF, which can protect breast cancer cells from a variety of apoptotic insults, protects against CD47-induced cell death. This protective effect is mediated via PKC-dependent and mitogen-activated protein kinase-independent mechanisms.

MATERIALS AND METHODS

mAbs and Chemicals. Anti-CD47 mAbs 1F7 (IgG1), 2D3 (IgG1), and B6H12 (IgG1) have been described previously (25, 26, 29, 32). FITC-labeled antihuman Bcl-2, Phycoerythrin-labeled anti-active caspase-3, purified antihuman caspase-9, mouse antihuman poly(ADP-ribose) polymerase antibody and FITC-labeled annexin V apoptosis detection kit were purchased from Phar-Mingen, San Diego, CA. Epidermal growth factor, etoposide, and doxorubicin was purchased from Sigma, St. Louis, MO. Purified mAb (SC120) to human epidermal growth factor and antihuman cytochrome *c* antibodies SC-7159 and SC-13561 was from Santa Cruz Biotechnology (Santa Cruz, CA). Mito Tracker red CMX-ROS was purchased from Molecular Probes (Eugene, OR). Forskolin (a direct activator of adenylate cyclases), 8-bromo cAMP, H-89 dihydrochloride, IBMX, wortmannin, PD98059, AG1478, PD153035, and

Received 6/11/03; revised 10/31/03; accepted 11/17/03.

Grant support: NIH Grants GM57573, GM54390, and CA65872 (to W. A. Frazier).

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pertussis toxin (PTX) were purchased from Calbiochem, San Diego, CA. PKA inhibitor peptide and Staurosporine were obtained from BIOMOL (Butler Pike, PA). Antihuman CD95 antibody CH-11 was obtained from Immunotech, (Villepinte, France) and antihuman caspase 3 antibody was obtained from Cell Signaling (Beverly, MA). Antihuman $\alpha v\beta 3$ antibody was a kind gift of Monsanto, St. Louis. The cAMP ELISA kit was purchased from Amersham/Pharmacia Biotech (Piscataway, NJ). The preparation of 4N1K peptide (KRFYVVWMKK) from the COOH-terminal domain of TSP1, the mutant 4NGG peptide (KRFYGGMWKK) and TSP-1 were described elsewhere (25).

Breast Cancer Cell Lines. The following human cell lines were purchased from American Type Culture Collection (Manassas, VA): MCF-7 (breast adenocarcinoma, pleural effusion), AU-565 (adenocarcinoma, breast), HBL-100 (breast epithelial cells with transformed morphology), and MDA-MB-231 (adenocarcinoma). The cell culture medium used was RPMI 1640 (Life Technologies, Inc., Grand Island, NE), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 units/ml penicillin and 100 μ g/ml streptomycin (Life Technologies, Inc.), and 10 μ g/ml ciprofloxacin. Henceforth, this medium will be referred to as complete medium. All of the cell lines used in the study were free from *Mycoplasma*.

Apoptosis Induction and Detection. One $\times 10^5$ breast cancer cells were treated with 10 μ g/ml 1F7, 4N1K, or 4NGG (100 μ M), etoposide or doxorubicin (10 μ g/ml each) for 24 h in 24-well plates (Nunc, Albertslund, Denmark). In some experiments, the cells were pretreated with a saturating concentration of pan caspase inhibitor (ZVAD-FMK, 100 μ M) for a period of 2 h before exposure to 1F7, 4N1K, or etoposide. After 24 h, the cells were harvested without trypsinization and were analyzed for apoptosis by the following methods. In some experiments, treated cells were incubated for 8 h, fixed with 2% paraformaldehyde, and permeabilized with *n-octyl-* α -glucopyranoside (7 mg/ml) before staining with phycoerythrin-conjugated antihuman caspase-3 antibody.

Flow Cytometric Determination of Apoptosis and Cell Death. Apoptosis was determined by monitoring changes in cell size and externalization of phosphatidylserine by flow cytometry after exposure to FITC-labeled annexin-V according to the manufacturer's instructions. The cells were harvested and stained with FITC-labeled annexin V and propidium iodide and were analyzed by flow cytometry using the CellQuest software program. A minimum of 10,000 cells were analyzed in each case with duplicate determinations.

Determination of Mitochondrial Transmembrane Potential ($\Delta \Psi_m$). The loss of mitochondrial membrane potential was studied using flow cytometry. The cells (1×10^5), treated as above, were stained with 40 μ M CMX-ROS in serum free medium and were incubated for 45 min at room temperature. The cells were washed and analyzed by fluorescence-activated cell sorting (FACS).

Quantification of Bcl-2. Intracellular staining for Bcl-2 was done according to the protocol described previously (33). In brief, cells were treated with 1F7, 4N1K, 4NGG, or etoposide at the concentrations indicated, were washed in phosphate buffer saline (PBS), and were resuspended in the same buffer. The cells were fixed with 2% paraformaldehyde (*p*-HCHO) for 5 min followed by treatment with *n-octyl-* α -glucopyranoside (7 mg/ml) for 5 min for permeabilization at room temperature. The cells were washed in PBS, stained with FITC-conjugated antihuman Bcl-2 antibody for 45 min, and analyzed by flow cytometry. Isotype-matched antibody was used as a control.

Measurement of Cyclic AMP. One $\times 10^5$ cells (MDA-MB-231 and AU-565) pretreated with or without PTX (100 ng/ml) were plated in 24-well plates in complete medium and were incubated for 24 h for evenly spreading on the surface. The cells were treated with either 4N1K or 4NGG (100 μ M each) for 24 h. The cells were harvested, and cAMP was extracted and determined using a cAMP enzyme immunoassay kit from Amersham.

DNA Fragmentation Analysis. One $\times 10^5$ cells were treated with medium alone or treated individually with 1F7 (10 μ g/ml), 4N1K (100 μ M), 4NGG (100 μ M), etoposide, or doxorubicin for 40 h. Genomic DNA was prepared from the cells using Dneasy Tissue kit obtained from Qiagen (Valencia, CA). Approximately 5 μ g of DNA sample was electrophoretically separated on 2% agarose gel containing ethidium bromide (0.5 μ g/ml).

Western Blot Analysis. One $\times 10^6$ cells (MDA-MB-231 or AU-565) were either treated with medium alone or with 4N1K, 4NGG, 1F7, or etoposide for 8 h in 24-well plates. The cells were harvested, sonicated for 10 s, and resuspended in solubilization buffer (1% NP40, 1 mM EDTA, 50 mM Tris-HCl, and 165 mM NaCl), and then were run on 10% SDS-PAGE in NuPAGE

MOPS SDS running buffer (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes that were immunoblotted with primary antibody and peroxidase-conjugated secondary antibody. For the detection of cytochrome *c*, a membrane fraction and a cytosolic fraction were prepared after treatment. Briefly, 1×10^6 cell were collected, washed twice in ice-cold PBS and resuspended in buffer A (250 mM sucrose, 20 mM HEPES-KOH, 10 mM KCl, 1.5 mM EGTA, 1.5 mM EDTA, 1 mM MgCl₂, 1 mM DTT, and protease inhibitors). Cells were then passed through a fine-gauge needle several times and allowed to swell for 20 min on ice. The cell homogenates were centrifuged for 3000 rpm for 10 min at 4°C to clear the debris. The supernatants were further centrifuged at 40,000 rpm for 20 min. The pellet was used as a heavy membrane fraction, and the supernatant was used as a cytosolic fraction. The fractions were run on 10% SDS-PAGE as above. Detection of signals was by chemiluminescence (ECL; Amersham Biosciences).

Statistical Analysis. Statistical analysis of the data were performed using the unpaired *t* test.

RESULTS

Breast Cancer Cells Express CD47. FACS analysis indicated the presence of CD47 in breast carcinoma cells (Fig. 1). All three mAbs (1F7, B6/H12, and 2D3) used in this study stained breast cancer cells, although staining intensity varied among antibodies (Fig. 1). Staining with 1F7 was most intense in all of the breast cancer cell lines tested, whereas B6/H-12 and 2D3 had intermediate levels of staining indicating differences in epitope recognition with individual antibodies, possibly because of differences in glycosylation of CD47.

CD47 Ligation Induces Apoptosis in Breast Cancer Cell Lines. Incubation of CD47^{+/+} breast cancer cells with CD47 agonist peptide 4N1K or mAb 1F7 increased annexin V binding in a dose-dependent manner (Fig. 2). Significantly, increased annexin V binding was seen at concentrations of 4N1K as low as 12.5 μ M and as early as 8 h of treatment (Fig. 2A and Fig. 3A). On average, MCF-7, HBL-100, MDA-MB-231, and AU-565 were 40–50% positive for annexin V after 24 h of incubation with all of the CD47 ligands that caused cell death. The percentage of annexin V-positive cells was calculated after subtracting the basal level of apoptosis, which was ~10–14%. Treatment of breast cancer cells with mAb 1F7 also caused detectable levels of cell death, although significantly lower than that caused by 4N1K (Fig. 2B). B6H12, whose epitope on CD47 physically overlaps with that of 1F7,

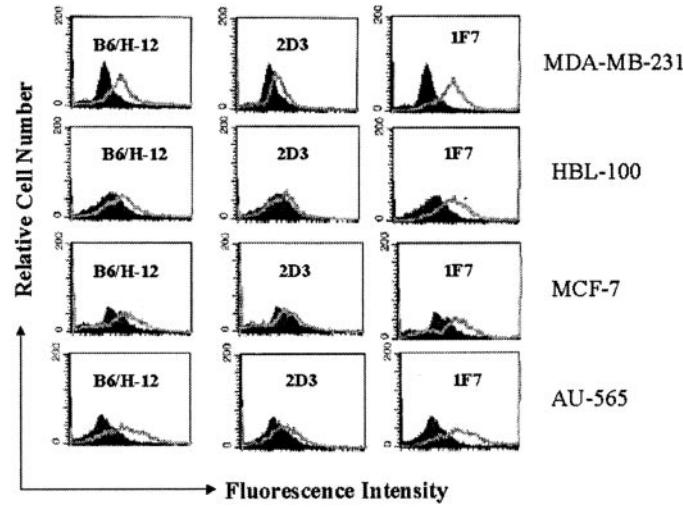


Fig. 1. Breast cancer cells express CD47. Four breast cancer cell lines (MDA-MB-231, HBL-100, MCF-7, and AU-565) were stained with monoclonal antibodies B6/H-12, 2D3, 1F7, and FITC-conjugated goat antimouse IgG. The dark histogram, the isotype control; the overlaying open histogram, surface expression of individual antigens. The experiment was repeated two times with identical results.

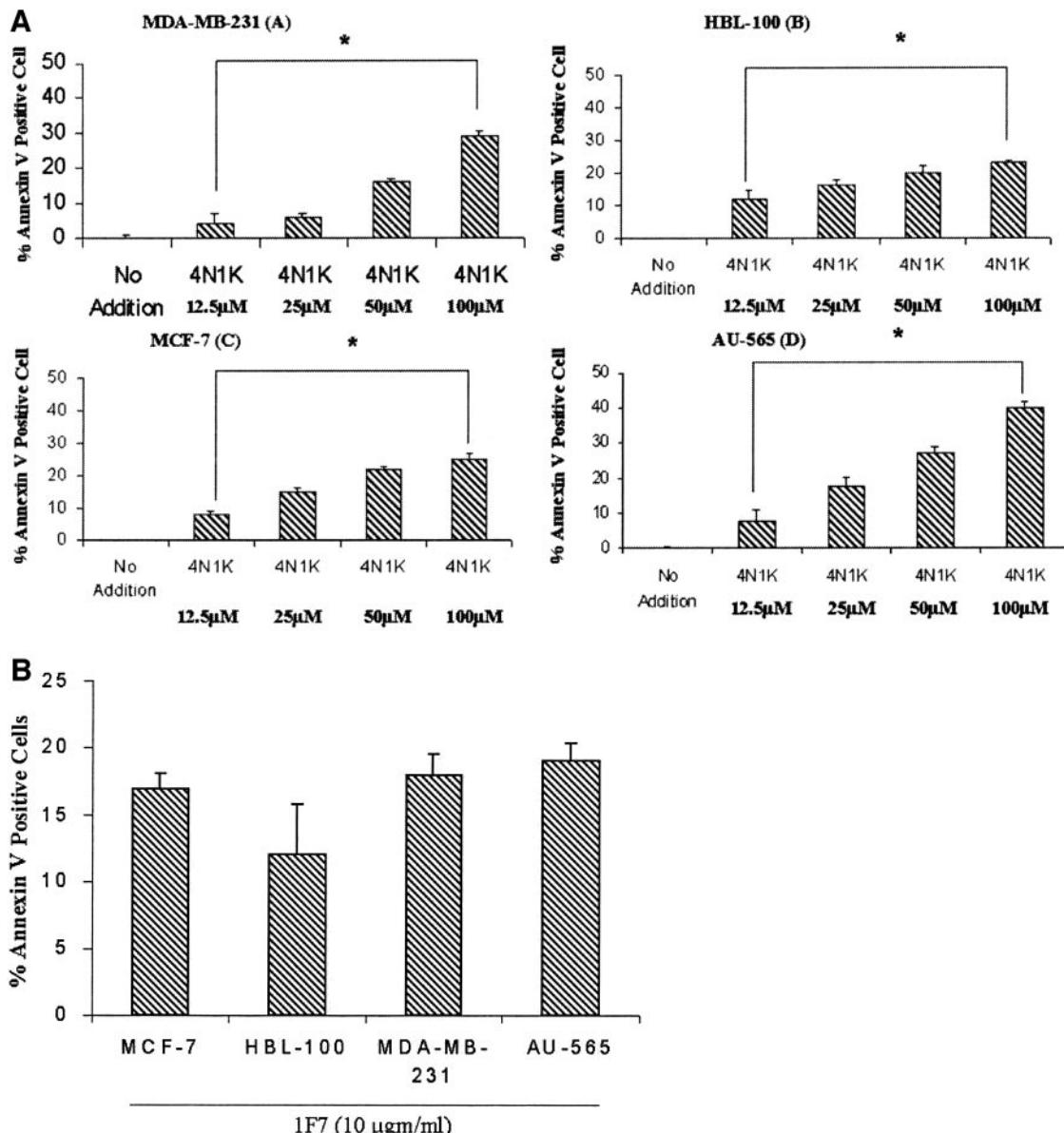


Fig. 2. Phosphatidyl serine externalization and cell killing are induced by CD47 agonist peptide 4N1K. One $\times 10^5$ breast cancer cells MDA-MB-231 (A), HBL-100 (B), MCF-7 (C), and AU-565 (D) were cultured in complete medium for 16 h and were allowed to form monolayers. The cells were challenged with different concentrations of thrombospondin (TSP)-1 COOH-terminal domain-derived agonist peptide 4N1K (A) or a fixed concentration (10 $\mu\text{g}/\text{ml}$) monoclonal antibody 1F7 (B) for another 24 h in complete medium at 37°C, 5% CO₂. The cells were harvested and stained with FITC-annexin-V and were analyzed by flow cytometry. Annexin V staining of cells alone (No Addition) generally varies between 10 and 14% and was deducted from each experimental set. The data are presented as means \pm SD of the percentage of annexin V-positive cells of individual treatments with triplicate determinations. The experiments were repeated three times with comparable results. A, *, P < 0.0002; B, *, P < 0.01; C, *, P < 0.0002; D, *, P < 0.0003 relative to highest and lowest concentration of 4N1K treatment.

induced significantly less annexin V binding than did 1F7, whereas mAb 2D3, which binds to a distant epitope on the CD47 IgV domain, had no effect (data not shown). Besides 4N1K and 1F7, platelet-derived TSP1 also induced apoptosis of breast cancer cells. A comparative study using TSP, 4N1K, and the control peptide 4NGG indicated a different degree of susceptibility of the cell lines to these agents (Fig. 3B). As expected, 4NGG did not induce apoptosis of any of the cell lines tested. In subsequent experiments, only the two most responsive cell lines, MDA-MB-231 and AU-565 were used.

Absence of DNA Fragmentation and Poly(ADP-Ribose) Polymerase Cleavage in CD47-Induced Apoptosis. DNA fragmentation is a common feature of apoptotic cell death. However, treatment of the two breast cancer cell lines with either 1F7 or 4N1K failed to induce DNA fragmentation (Fig. 4A). In contrast, treatment with etoposide or

doxorubicin caused the characteristic nucleosomal fragmentation of cellular DNA (Fig. 4A). CD47-induced cell death also does not result in cleavage of the nuclear protein poly(ADP-ribose) polymerase, a downstream substrate of activated caspases (4B).

CD47-Dependent Killing Is Caspase Independent. Thus, we next investigated the involvement of caspases in CD47-induced cell death. Despite high surface expression of FAS/CD95, breast cancer cells were resistant to apoptosis induced by the cross-linking of FAS with mAb CH-11 (data not shown). Caspase activation is known to be critically important for the enactment of classical apoptotic pathways (34, 35). Caspase 3 along with caspases 6 and 7 are believed to be activated after disruption of the mitochondrial membrane and release of intermembrane cytochrome c (36). Once activated, these caspases promote further mitochondria membrane permeabilization causing further caspase

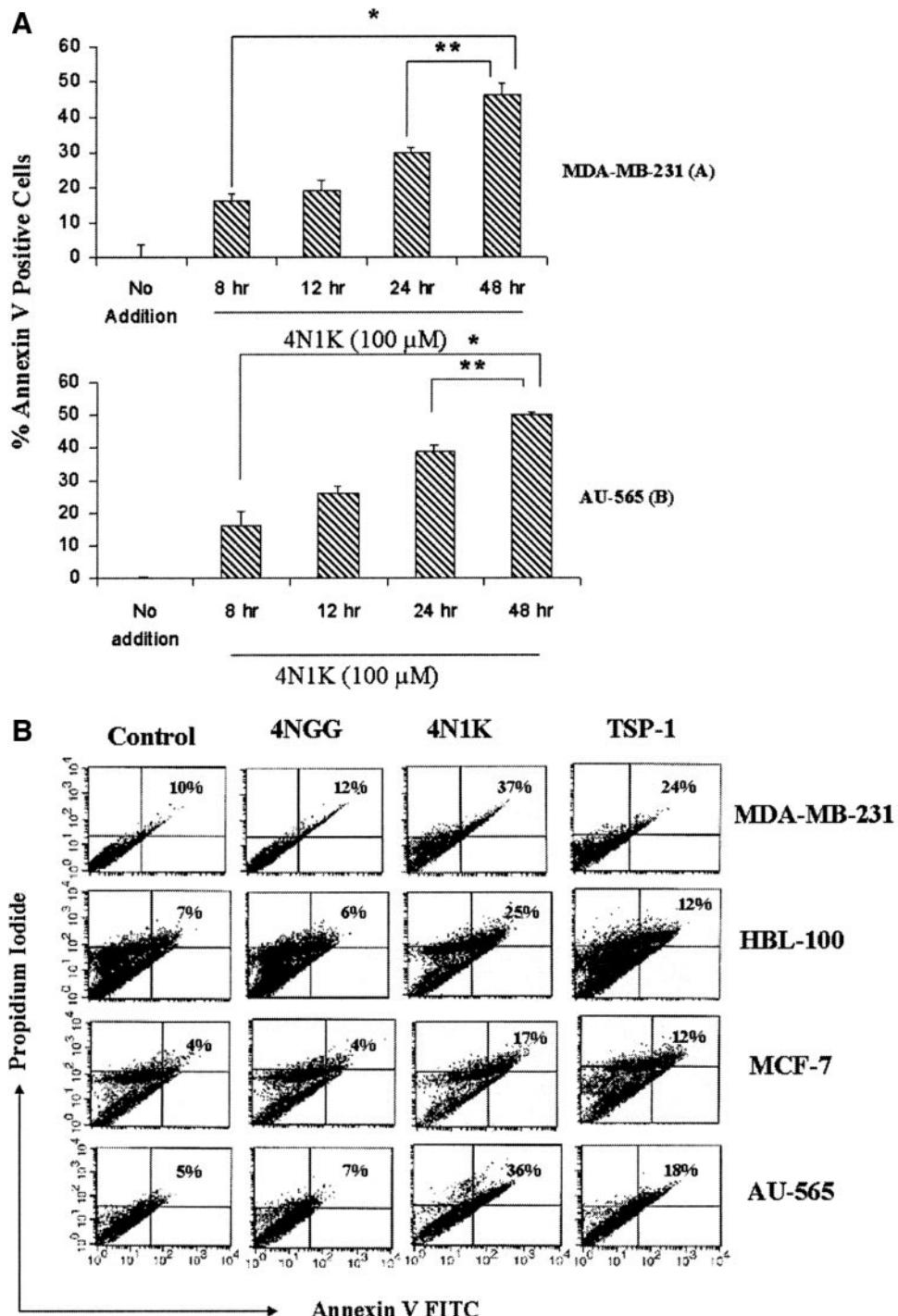


Fig. 3. Prolonged exposure to 4N1K augments 4N1K-induced apoptosis in breast cancer cells. Both MDA-MB-231 (A) and AU-565 (B) cells were treated as stated in Fig. 2 and were incubated from 8 to 48 h. The cells were harvested and stained with FITC-conjugated annexin V and were analyzed by flow cytometry. The data are presented as means \pm SD of percentage of annexin V-positive cells for each treatment with triplicate determinations (A). A, *, $P < 0.001$; **, $P < 0.0002$; B, *, $P < 0.006$; **, $P < 0.0003$. The experiment was repeated three times with similar results. MDA-MB-231, HBL-100, MCF-7, and AU-565 were cultured in the presence of CD47 agonist peptide 4N1K, the inactive control peptide 4NGG (100 μ M each), or thrombospondin-1 (TSP-1; 10 μ g/ml) for 24 h. Apoptosis was determined by FITC-annexin-V staining and the uptake of propidium iodide with flow cytometry. Within each dot plot, the percentage of annexin-V-positive cells (B). The experiment was repeated three times.

activation. Interestingly, we observed that pretreatment of MDA-MB-231 or AU-565 with the broad spectrum caspase inhibitor ZVAD-FMK (pan caspase inhibitor, PCI) did not prevent phosphatidylserine exposure induced by 4N1K or 1F7 (Fig. 5, A and B). On the other hand, apoptosis initiated by etoposide was significantly reduced in the presence of PCI (Fig. 5, A and B). The lack of caspase activation by 4N1K and 1F7 was further documented using intracellular staining and FACS analysis with an antibody that specifically detects activated (cleaved) caspase 3 (Fig. 5C). In contrast, substantial caspase 3 cleavage was detected in cells treated with either etoposide or staurosporine (Fig. 5C). The absence of caspase 3 and 9 cleavage in cells treated with either 4N1K

or 1F7 was also documented by Western blot analysis, whereas etoposide treatment resulted in cleavage of caspase 3 and 9 (Fig. 5, D and E). These data indicate that CD47-induced apoptosis of breast cancer cells is independent of caspase activation, just as observed in lymphocytes (28–30).

CD47-Mediated Cell Death Does Not Result in Degradation of Bcl-2 or Release of Cytochrome c. The Bcl-2 family of proteins, including its pro- and antiapoptotic members, regulates apoptosis induced by a variety of agents (37). Present evidence suggests that Bcl-2 acts upstream of caspase 3 activation, at the level of cytochrome *c* release, to prevent apoptosis (38, 39). However, our results indicate that CD47 engagement does not alter the intracellular Bcl-2 protein level. As seen in

Fig. 4. Effect of CD47-mediated apoptosis on DNA laddering. One $\times 10^5$ AU-565 breast cancer cells were treated with 4N1K or 4NGG (100 μM each), 1F7 (10 $\mu\text{g}/\text{ml}$), etoposide (*ETO*) or doxorubicin (*DOX*; 10 $\mu\text{g}/\text{ml}$ each), for 40 h. The DNA was harvested from cells using the Dneasy Tissue kit obtained from Qiagen (Valencia, CA). Lane M contains marker (A). One $\times 10^6$ cells treated as above were incubated for 12 h. The total cell lysates were prepared and run on 10% SDS-PAGE and were blotted for nuclear protein poly(ADP-ribose) polymerase as described in "Materials and Methods" (B). The experiment was repeated three times with similar observation. Lane C, control; cells treated with medium alone. *kDa*, M_r in thousands.

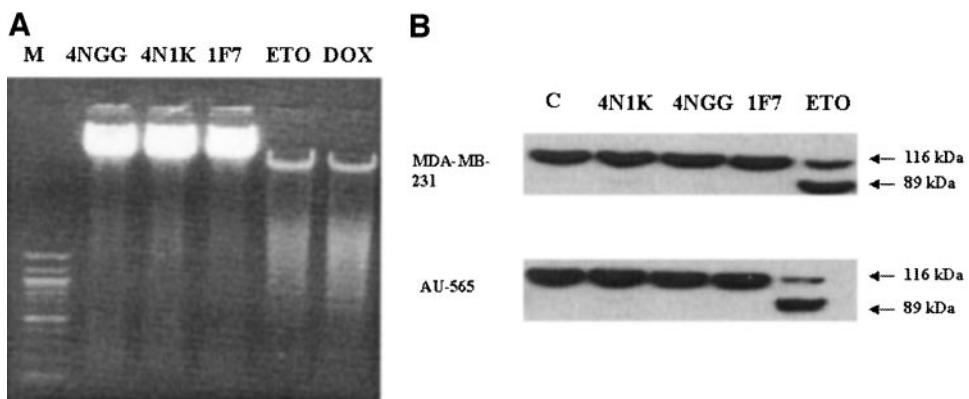


Table 1, neither the peptide agonist 4N1K nor the death-inducing mAb 1F7 caused a reduction in the cellular content of Bcl-2 in MDA-MB-231 cells or AU-565 cells. Furthermore, CD47-induced apoptosis failed to induce cytochrome *c* release from mitochondria as shown by Western

blotting of particulate and soluble fractions of cells treated with 4N1K or 1F7. Cells treated with etoposide or staurosporine displayed readily detectable amounts of cytochrome *c* in the cytosol (Fig. 6, A and B). Immunofluorescent detection of cytochrome *c* by confocal microscopy

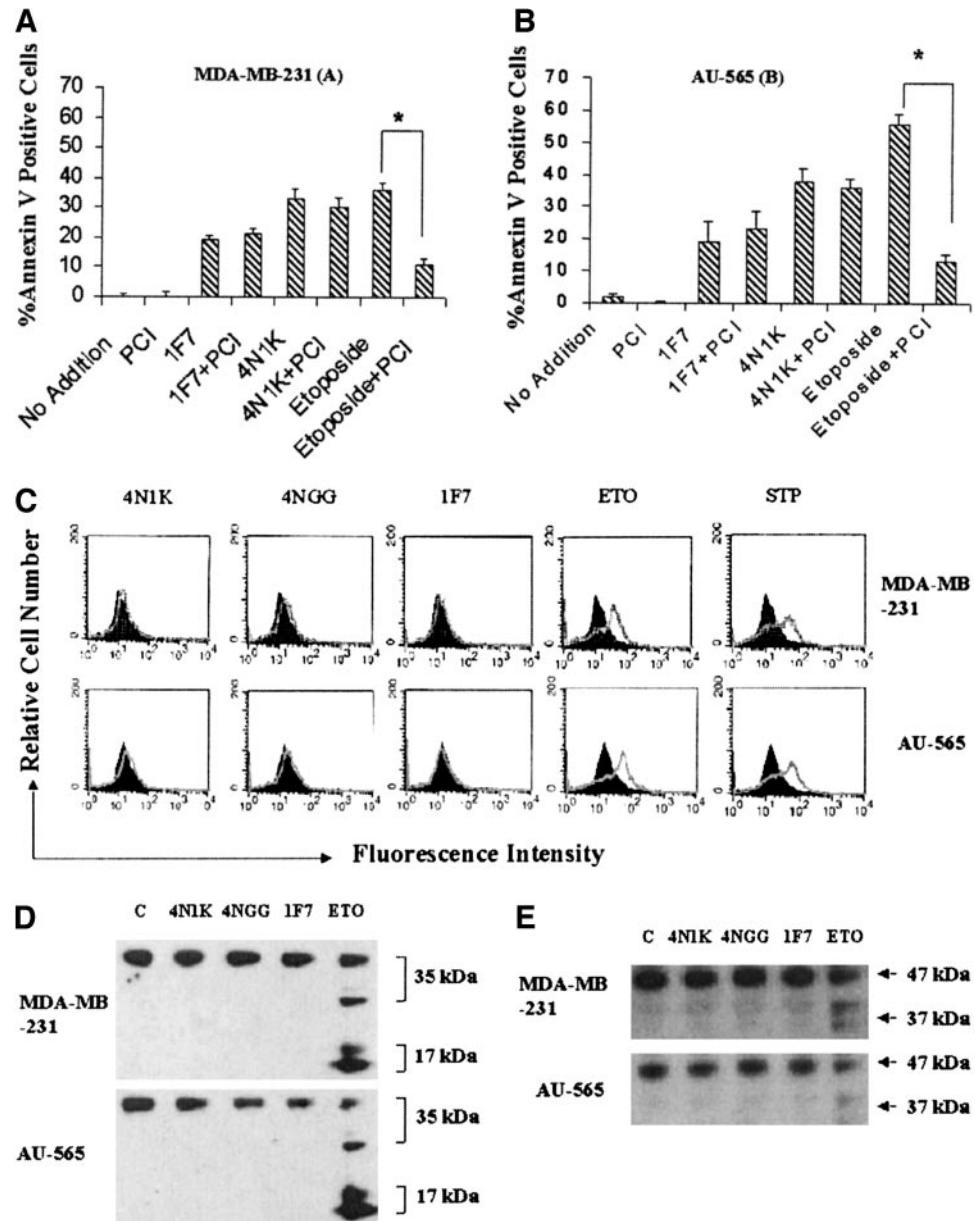


Fig. 5. CD47-Induced apoptosis is caspase independent. One $\times 10^5$ MDA-MB-231 (A) or AU-565 (B) cells were cultured in complete medium for 16 h and were allowed to form monolayers. The cells were treated with the broad spectrum caspase inhibitor ZVAD-FMK (PCI), 100 μM for 2 h before being challenged with 4N1K (100 μM each) or etoposide (*ETO*; 10 $\mu\text{g}/\text{ml}$) and were incubated for another 24 h. The cells were harvested and stained with FITC-conjugated annexin V and were analyzed by flow cytometry. The data are presented as means \pm SD of percentage of annexin V-positive cells for each treatment with triplicate determinations. *, $P < 0.0001$ in A and B. The experiment was repeated three times with similar results (A, B). For intracellular caspase 3 staining, the treated cells were fixed and permeabilized and stained with phycoerythrin-conjugated antihuman caspase 3 (PCI) and were analyzed by fluorescence-activated cell sorting. STP, staurosporine. The dark histogram, the caspase 3 staining in untreated cells; the light histogram, the corresponding staining in treated cells (C). For Western blotting, cell lysates were prepared from 1×10^6 untreated (Lane C, Control) and treated cells; they were run on 10% SDS-PAGE and were blotted with rabbit antihuman caspase 3 (D) and rabbit antihuman caspase 9 (E). The experiment was repeated three times with similar results. *kDa*, M_r in thousands.

Table 1 CD47-induced apoptosis does not alter Bcl-2 levels

One $\times 10^5$ MDA-MB-231 and AU-565 tumor cells were plated in 24-well plates in triplicate and were cultured in complete medium for 24 h. The cells were challenged with 4N1K, 4NGG (100 μ M), 1F7 (10 μ g/ml), or etoposide (2 μ g/ml) for another 24 h. The cells were fixed with 1% paraformaldehyde followed by permeabilization with *n*-octylglucopyranoside (7 mg/ml). The cells were stained with FITC-conjugated antihuman Bcl-2 and were analyzed by flow cytometry. Isotype matched control antibody was used as a control. The data are represented as means \pm SD of percentage positive cells as well as mean fluorescence intensity with triplicate determinations. The experiment was repeated three times with comparable results.

	MDA-MB-231		AU-565	
Conditions	% positive cells	Mean fluorescence intensity	% positive cells	Mean fluorescence intensity
Isotype control	6.3 \pm 1.58	11.7 \pm 0.69	8.47 \pm 0.87	18.98 \pm 1.36
Cell only	45 \pm 1.69	96.33 \pm 1.23	53.4 \pm 2.12	96 \pm 2.96
4NGG	47.2 \pm 3.3	98.96 \pm 2.24	55.5 \pm 1.1	93.26 \pm 4.54
4N1K	46 \pm 2.9	100.88 \pm 4.4	55.7 \pm 2.5	91.9 \pm 3.3
1F7	45.9 \pm 1.16	87.0 \pm 5.56	55.9 \pm 3.7	94.33 \pm 5.3
Etoposide	20.7 \pm 2	28.61 \pm 1.57	23.59 \pm 4.6	29.39 \pm 2.7

also revealed a redistribution of cytochrome *c* to the cytoplasm induced by etoposide, but not by 4N1K (Fig. 6C).

CD47-Dependent Killing of Breast Cancer Cells Is Mediated via Gi. We have reported that the effects of CD47 related to integrin signaling or regulation are mediated by heterotrimeric Gi (25–27). In addition, CD47 killing of lymphocytes is largely Gi dependent as well (29). We, thus, wanted to determine whether heterotrimeric Gi was involved in the CD47-mediated killing of breast cancer cells. Pretreatment of these cells with PTX alone at the highest concentration used (100 ng/ml, 24 h) did not increase the level of annexin V binding (Fig. 7A). Furthermore, PTX had no effect on the apoptosis induced by etoposide (Fig. 7A). In contrast, PTX inhibited 4N1K-induced apoptosis in a dose-dependent manner, resulting in a 70–80% reduction in apoptosis at a concentration of 100 ng/ml in two different cell lines (Fig. 7A).

CD47 Induces a PTX-Sensitive Loss of Mitochondrial Transmembrane Potential, $\Delta\Psi_m$. One of the hallmarks of both apoptotic and programmed necrotic cell death is a loss of potential across the mitochondrial membrane, termed $\Delta\Psi_m$, that drives the synthesis of ATP (40, 41). We thus investigated the effect of 4N1K on $\Delta\Psi_m$. 4N1K treatment of MDA-MB-231 or AU-565 cells resulted in a decrease in $\Delta\Psi_m$ to nonviable levels in 32 and 44% of the cells, compared with 51 and 62% of the cells with etoposide treatment, respectively (Fig. 7B). The effect of 4N1K on cell death as judged by annexin V positivity (Fig. 7A) is virtually eliminated by treatment of the cells with PTX. The same PTX treatment protocol resulted in the same degree of inhibition (70–80%) of the decrease in $\Delta\Psi_m$ (Fig. 7B). Thus, loss of $\Delta\Psi_m$ is prevented by PTX treatment of the cells

and is, therefore, downstream of a heterotrimeric G protein of the Gi family (42).

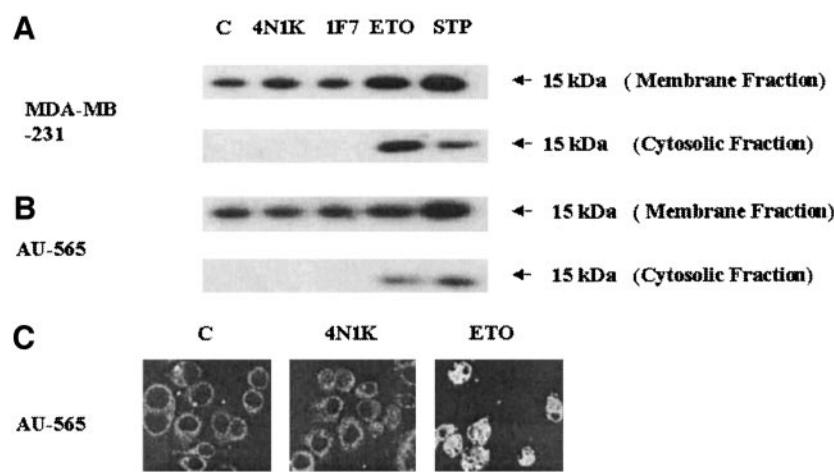
Elevated cAMP Levels Inhibit CD47-Mediated Apoptosis via PKA. Because CD47 signaling is mediated via activation of Gi, the α subunit of Gi could inhibit adenylate cyclase activity, resulting in lower intracellular levels of cAMP (42–44). Breast cancer cells treated with 4N1K showed a dramatic reduction of intracellular cAMP levels, which were largely restored on pretreatment with PTX (Fig. 8A and B).

To determine whether preventing the drop in cAMP level would spare the cells from apoptosis, we used three means of elevating intracellular cAMP. Cells were treated with 8-Br-cAMP (a cell-permeable cAMP derivative), forskolin (a direct activator of adenylate cyclase), or IBMX (a cAMP phosphodiesterase inhibitor). As shown in Fig. 8C, all three agents strongly inhibited 4N1K-induced apoptosis. 8-Br-cAMP, forskolin, and IBMX also inhibited 1F7-induced apoptosis (not shown). These results indicate that the CD47-mediated killing of breast cancer cells is mediated in large part by the decreased intracellular level of cAMP.

Several reports indicate that cAMP can have positive or negative effects on cell survival depending on the cell type and the death-inducing insult (45–48). Furthermore, some of these cAMP effects depend on the classical pathway of PKA activation, whereas others appear to be independent of PKA activity. Thus, we sought to address this issue using the PKA inhibitor H89 and the more specific PKA inhibitor peptide, PKAI. H-89 or PKAI alone had no effect on the cell viability. However, as seen in Fig. 9, the ability of PTX, 8-Br-cAMP, and forskolin to block CD47-mediated apoptosis, was completely dependent on the activity of PKA.

EGF Opposes CD47-Mediated Cell Death through a Phosphatidylinositol 3'-Kinase (PI-3K)-Dependent Pathway. EGF and its receptors are expressed in many cancers, including breast, ovary, and lung (5, 49). Overexpression of EGF receptors is correlated with an aggressive form of cancer with a poor prognosis (49, 50, 51). EGF has been shown to be antiapoptotic for cancer cells (52, 53) via stimulation of specific signal transduction pathways, including AKT and ERK/mitogen-activated protein kinase. To determine whether the CD47-dependent killing of breast cancer cells could be opposed by EGF, we first assessed EGF receptor expression. MDA-MB-231 and AU-565 cells expressed comparable levels of EGF receptors as shown by FACS analysis (Fig. 10A). Treatment with 4N1K induced about 35% apoptosis in both MDA-MB-231 and AU565 cells, and EGF afforded virtually complete, dose-dependent protection from CD47-induced killing (Fig. 10B). The protective effect of EGF was abolished on treatment with specific inhibitors of the EGF receptor tyro-

Fig. 6. CD47-mediated cell death does not require cytochrome *c* release. One $\times 10^6$ cells were treated with medium alone (Lane C, Control) or with 4N1K, 1F7, etoposide (ETO), or staurosporine (STP) for 10 h. The membrane and cytosolic fractions were prepared as described in "Materials and Methods," run on 10% SDS-PAGE and blotted with rabbit antihuman cytochrome *c* antibody (A and B). For confocal analysis (C), treated cells were stained with monoclonal antihuman cytochrome *c* and secondary antibody. The experiments were repeated three times each with similar results. kDa, M_r in thousands.



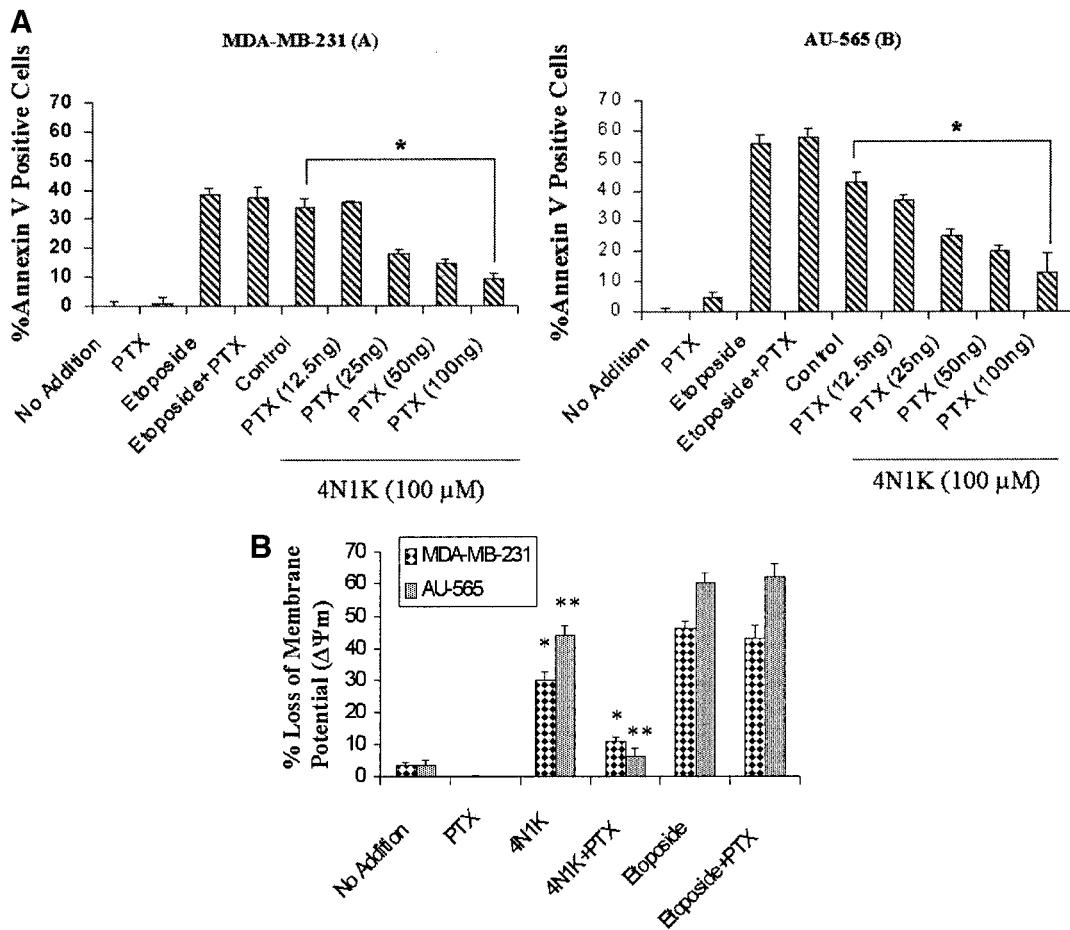


Fig. 7. CD47-mediated annexin V binding and loss of mitochondrial membrane potential require Gi. One $\times 10^5$ MDA-MB-231 (A) or AU-565 (B) cells were treated with medium alone or with different concentrations of pertussis toxin (PTX) for 24 h. The cells were treated with 4N1K (100 μM) for another 24 h to induce apoptosis. Treatment with etoposide (10 $\mu\text{g}/\text{ml}$) was used as a control. Apoptosis was determined with FITC-conjugated annexin V as assessed by flow cytometry. The data are presented as means \pm SD of percentage of annexin V-positive cells with triplicate determinations. The experiment was repeated three times with identical results. *, $P < 0.0002$ between 4N1K versus 4N1K+PTX at highest concentrations in both "(A)" and "(B)" within A. In B, MDA-MB-231 or AU-565 cells were treated with medium alone or with PTX (100 ng/ml) for 24 h. The cells were treated with 4N1K or etoposide for another 24 h for induction of apoptosis. The cells were harvested and stained with CMX-ROS, and were analyzed by flow cytometry. The data are presented as percentage loss of mitochondrial transmembrane potential ($\Delta\psi\text{m}$). The experiment was repeated three times with similar results. *, $P < 0.0002$; and **, $P < 0.0001$; between cells treated with 4N1K versus 4N1K+PTX.

sine kinase, AG1478 and PD153035. Both compounds restored apoptosis to the same level as 4N1K treatment alone (Fig. 11). Furthermore, preincubation of cells with an antibody that blocks binding of EGF to its receptor also prevented EGF rescue of 4N1K-treated cells (Fig. 11).

To examine the signaling pathway(s) by which EGF protected the cells from CD47-mediated death, specific inhibitors of PI-3K or MEK/ERK were used. Neither wortmannin nor the MEK inhibitor PD98059 alone had an affect on annexin V binding (Fig. 12). However, wortmannin completely blocked the ability of EGF to protect against CD47-mediated cell death, suggesting that PI-3K activation is required for the EGF survival response in the face of 4N1K-induced apoptosis. In contrast to wortmannin, pretreatment with PD98059 did not alter the ability of EGF to block 4N1K-induced apoptosis indicating that ERKs are not required (Fig. 12). Thus EGF activation of the PI-3K signaling pathway is required for the prevention of CD47-induced apoptosis, suggesting that the demonstrated role of AKT in EGF signaling may be involved.

Given the known role of CD47 in modulating integrins that can influence cell adhesion, we investigated the effects of 4N1K on cell morphology. 4N1K treatment resulted in no observable effects on cell shape, and, importantly, the peptide did not cause cells to detach or round up (Fig. 13A). There was no effect of 4N1K or 1F7 on the

surface expression of $\beta 1$ integrins as judged by FACS analysis (not shown). However, staining of 4N1K-treated cells for $\alpha v\beta 3$ expression indicated a slight up-regulation, whereas treatment with etoposide caused a sharp decline in $\alpha v\beta 3$ integrin expression (Fig. 13A). Perhaps because of this decrease in the expression of $\alpha v\beta 3$, etoposide significantly affected the cell morphology (Fig. 13B).

DISCUSSION

Both natural and antibody ligands of CD47 induced the death of four different breast tumor cell lines. Although the degree or time course of apoptosis varied among the different cell lines tested, all of the cell lines were susceptible. The cell death seen here was characterized by some features characteristic of classical apoptosis such as cell shrinkage, externalization of phosphatidyl serine, and decreased mitochondrial membrane potential. However, this mode of cell death was novel in that it proceeded without active caspases, DNA laddering, cytochrome *c* release or Bcl-2 loss. All of these characteristics are in common with CD47-mediated death of lymphocytes described by us (29) and others (28, 30). Expression of the neuronal form of CD47 in cultured cerebral cortical neurons has been reported to induce apoptosis (54). However, this effect was blocked by ZVAD-FMK, indicating the involvement of caspases, and nucleosomal DNA cleav-

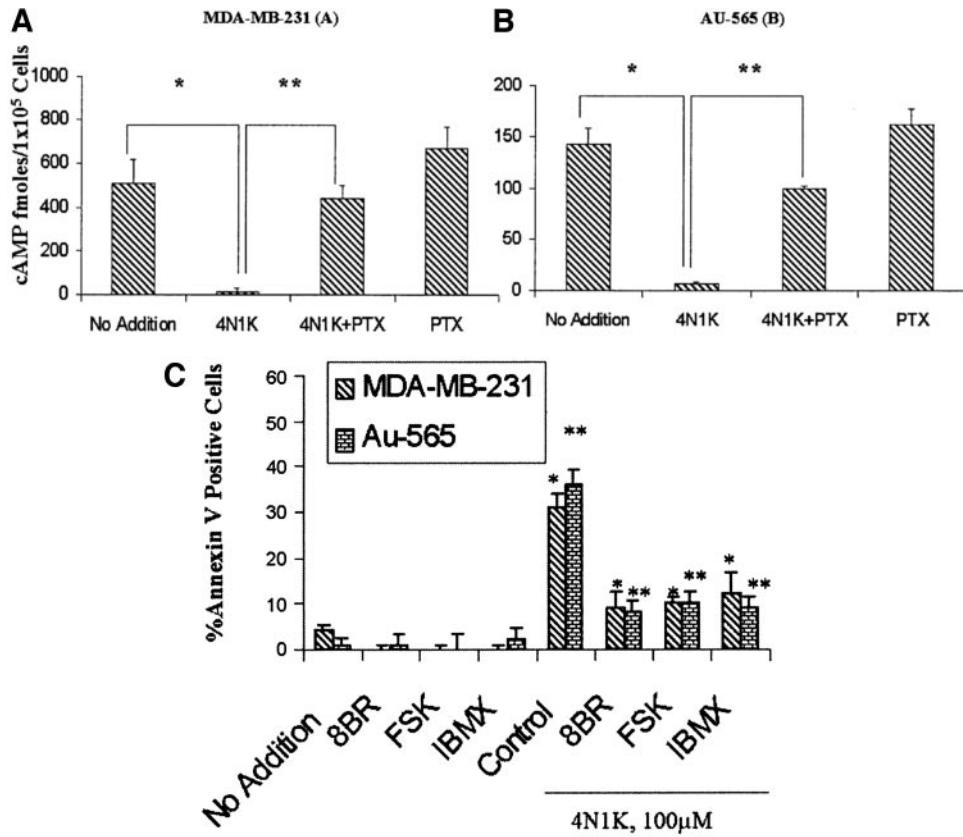


Fig. 8. 4N1K-induced intracellular cAMP loss is prevented by pertussis toxin (PTX). One $\times 10^5$ MDA-MB-231 (A) or AU-565 (B) cells were pretreated with or without PTX (100 ng/ml) for 24 h and then treated with 4N1K (10 μ M) for an additional 24 h. The cells were harvested, and cAMP was determined as indicated in "Materials and Methods." Data presented are picomoles of cAMP/ 1×10^5 cells \pm SD of triplicate determinations. The experiment was repeated three times with similar results (A, B). A, *, $P < 0.001$; **, $P < 0.0005$ between bars. B, *, $P < 0.001$; **, $P < 0.0001$. C, in a similar set of experiments, 1×10^5 MDA-MB-231 or AU-565 cells were pretreated with 8-Br-cAMP (8BR; 100 μ M), forskolin (FSK; 50 μ M), or IBMX (2 μ g/ml) for 1 h before being challenged with 4N1K and were incubated in complete medium for 24 h. The cells were harvested, and apoptosis was determined as above. The data are presented as means \pm SD of percentage of annexin V-positive cells with triplicate determinations. The experiment was repeated three times with similar results. *, $P < 0.002$; **, $P < 0.002$.

age was also reported. Furthermore, coexpression of the CD47 ligand SIRP α (also called BIT or SHPS-1) enhanced neuronal death, perhaps indicating that ligation of CD47 by different ligands could result in activation of different death pathways.

A novel aspect of our studies is the finding that CD47-mediated cell death requires, in large part, a PTX-sensitive G protein, most likely one or more isoforms of Gi (29). In a number of systems in which CD47 modulates the functions of integrins, PTX has been shown to prevent the CD47-dependent augmentation of integrin function (27). Furthermore, it has been suggested that the mechanism of CD47 action in T cells might be distinct from the G protein-dependent functions of CD47 in other cell types (27, 28, 30). Thus, the present study indicates that the mechanism of CD47-dependent killing of breast cancer cells is completely congruent with that discovered in

activated T cells and lymphomas. 4N1K-induced phosphatidylserine exposure on breast carcinoma cells was blocked 70% by PTX treatment (Fig. 7A). In addition to phosphatidylserine exposure as an end point, two-thirds of the drop in mitochondrial membrane potential, $\Delta\Psi_m$, was prevented by PTX treatment of breast carcinoma cells (Fig. 7B), suggesting that CD47 activation of Gi is upstream of all branches of this novel death pathway. It is important to note that PTX alone did not kill cells nor did it affect killing by etoposide. Gi activation by CD47 was accompanied by a sharp decrease in intracellular cAMP levels, which has also been seen in smooth muscle cells (43) and platelets (44) treated with 4N1K peptide or TSP1. We found that 1F7, acting via Gi, dramatically reduced cAMP levels in Jurkat cells and in primary human T cells as well (29). Furthermore, restoring cAMP levels in T cells with 8-Br-cAMP or forskolin, or with

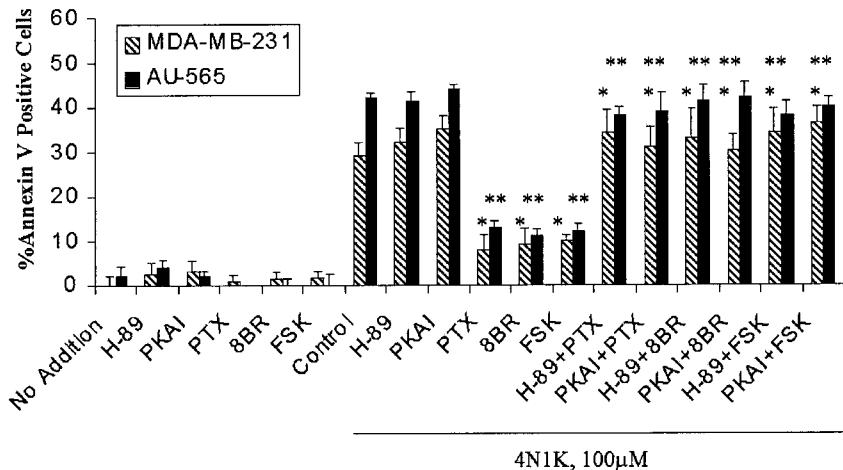


Fig. 9. Inhibition of protein kinase A suppresses the antiapoptotic effect of pertussis toxin (PTX), 8-bromo cAMP (8BR), and forskolin (FSK). One $\times 10^5$ MDA-MB-231 or AU-565 cells were pretreated with PTX (100 ng/ml), 8-bromo-cAMP (100 μ M), or forskolin (50 μ M) in the presence or absence of H-89 or protein kinase A inhibitor peptide (PKAI; 1 μ g/ml each) before being challenged with 4N1K in complete medium for 24 h. The cells were harvested, and apoptosis was determined as above. The data are presented as means \pm SD of percentage annexin V-positive cells with triplicate determinations. The experiment was repeated three times with similar results. *, $P < 0.05$; **, $P < 0.01$.

Fig. 10. Epidermal growth factor (*EGF*) protects against CD47-induced apoptosis. In A, MDA-MB-231 and AU-565 cells were stained with antihuman EGF receptor antibody SC-120 and FITC-conjugated goat antihuman IgG. The dark colored histogram, the isotype control; the light histogram, the corresponding staining with the EGF receptor antibody. In B, 1×10^5 MDA-MB-231 or AU-565 cells were treated either with medium alone or with various concentrations of recombinant EGF before challenged with 4N1K for 24 h. The cells were harvested, and apoptosis was determined as above. The data are presented as means \pm SD of percentage annexin V-positive cells with triplicate determinations. The experiment was repeated three times. *, $P < 0.001$; **, $P < 0.0003$.

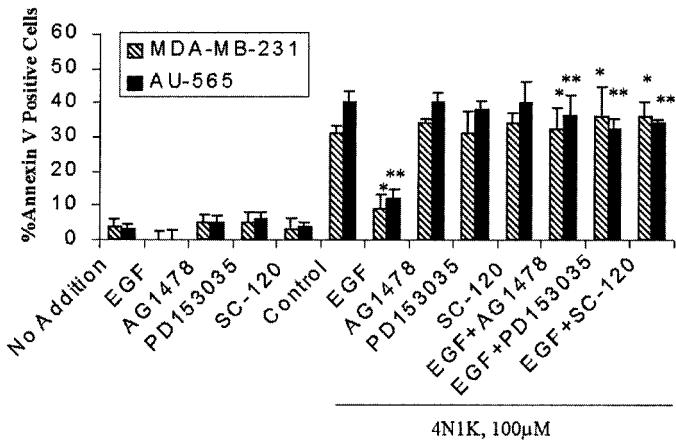
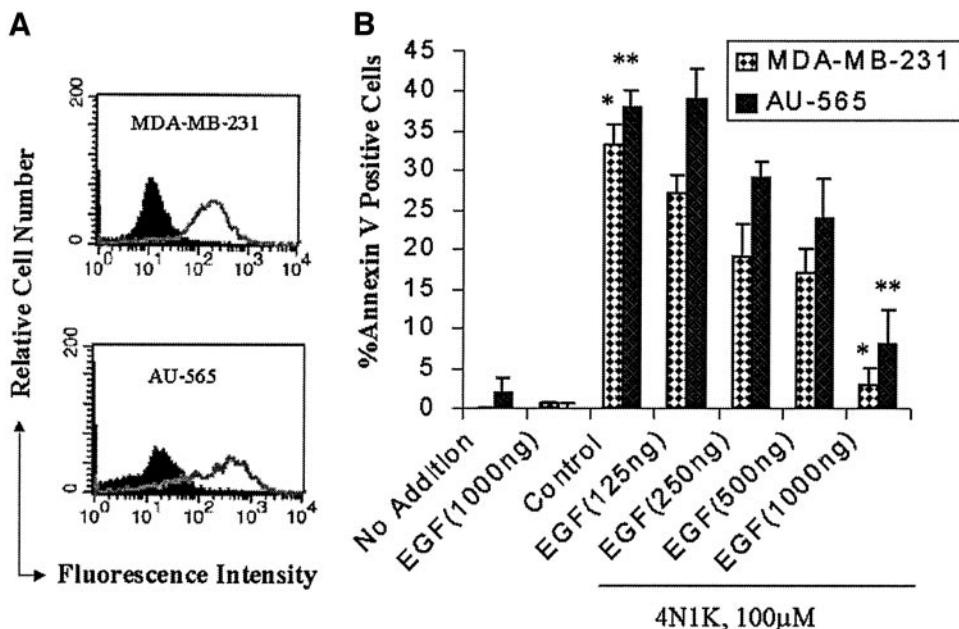


Fig. 11. Epidermal growth factor (*EGF*) protection against CD47-induced apoptosis is specific. Both MDA-MB-231 and AU-565 cells were incubated with EGF-specific tyrosine kinase inhibitors AG-1478 (2 μ M) and PD153035 (2 μ M) or an EGF receptor-blocking antibody, SC-120 (1 μ g/ml), before being challenged with 4N1K and incubated for another 24 h. The cells were harvested, and apoptosis was determined as above. The data are presented as means \pm SD of percentage of annexin V-positive cells with triplicate determinations. *, $P < 0.05$; **, $P < 0.05$.

the phosphodiesterase inhibitor IBMX, substantially inhibited CD47-mediated death. The sparing effects of all of these agents, as well as that of PTX, were completely blocked by PKA inhibitor H89 and the highly specific PKA inhibitor peptide, suggesting that one or more downstream substrates of PKA strongly opposes the death signal initiated by the CD47 activation of Gi.

There are a number of reports in the literature of cAMP inhibiting apoptosis in various cell types. Cyclic-AMP analogs and forskolin have been shown to oppose apoptosis initiated by diverse agents in neutrophils, promonocytic leukemia cells, and smooth muscle cells (45–48). However, the mechanisms by which cAMP does this are often not known, although most require active PKA. There are, however, rare reports of cAMP preventing apoptosis by a mechanism independent of both PKA activity and transcription (45). One well-described antiapoptotic role of PKA is the phosphorylation of BAD, a BH3-only protein (55) that, when dephosphorylated, can bind to and

neutralize antiapoptotic Bcl-2 family members on mitochondria, thus leading to mitochondrial damage, release of cytochrome *c*, and other pro-apoptotic factors (56). The impact of CD47 ligation on mitochondrial function seen here suggests that the modulation of BAD or a similar BH3-only protein by cAMP-dependent regulation of PKA activity may be involved.

Treatment of epithelial cells with EGF has been shown to protect against FAS and tumor necrosis factor- α -induced apoptosis (57, 58), whereas ErbB2/neu overexpression protects against apoptosis by the chemotherapeutic drug Taxol (59). A protective role of EGF has been reported in TRAIL-induced apoptosis of breast cancer cells as well as in tumor necrosis factor- β -induced apoptosis of cultured fetal hepa-

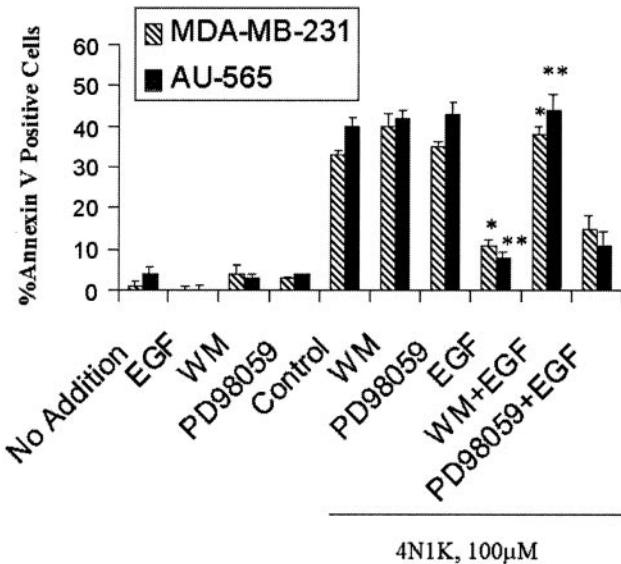


Fig. 12. Epidermal growth factor (*EGF*) protection against CD47-induced apoptosis is phosphatidylinositol 3'-kinase dependent. One $\times 10^5$ MDA-MB-231 or AU-565 cells were incubated with or without 1 μ g/ml EGF and either wortmannin (WM; 100 nM) or PD98059 (PD98059; 50 μ M) before being challenged with 4N1K (100 μ M) for 24 h in complete medium. The cells were harvested, and apoptosis was determined as above. The data are presented as means \pm SD of percentage annexin V-positive cells with triplicate determinations. The experiment was repeated three times with similar results. *, $P < 0.0001$; **, $P < 0.001$.

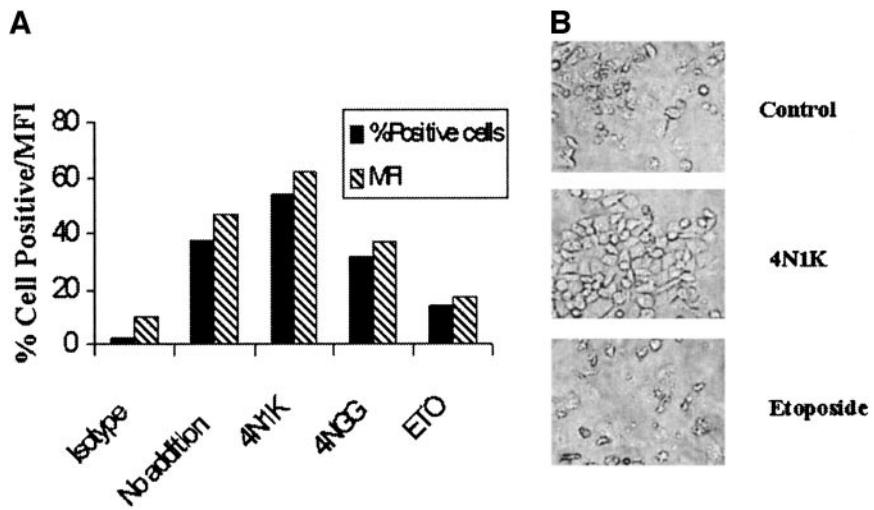


Fig. 13. Effect of 4NIK treatment on expression of $\alpha v\beta 3$ and cell morphology. AU-565 cells were treated in medium alone or with 4NIK, 4NGG, or etoposide (*ETO*) for 24 h. The cells were stained with antihuman $\alpha v\beta 3$ integrin and were analyzed by fluorescence-activated cell sorting. Percentage of positive cells (filled bars) and mean fluorescence intensity (*MFI*; hatched bars) were determined (*A*). After treatment as in *A*, cells were also fixed and stained with Giemsa stain for morphological observation (*B*). The experiment was repeated twice with similar results.

tocytes (52, 60). Breast cancer cells express multiple EGF receptors including ErbB1 and ErbB2/neu. The high expression of ErbB2/neu in specific subsets of breast tumors correlates with poor prognosis and resistance to apoptosis by chemotherapeutic drugs (61). Blocking the activation of EGF receptors (with c225) or ErbB2/neu (with herceptin) exhibits antitumor effects in breast cancer (62). Our data indicate that, along with well-known apoptotic pathways initiated by TRAIL, FAS, or Taxol, CD47-induced apoptosis of breast carcinoma cells is also opposed by EGF. The protective effect of EGF was blocked by EGF receptor-specific tyrosine kinase inhibitors and also by a blocking antibody against the receptor (Fig. 11). Thus even though the mechanism of cell killing is very different for CD47 and CD95/FAS, EGF is able to oppose both forms of cell death.

The mechanism of EGF protection against apoptosis induced by classical means is well known. Binding of EGF to its receptor leads to activation of PI-3K, which, in turn, activates AKT. AKT then phosphorylates substrates including the Bcl-2 family member BAD. Phosphorylation of BAD blocks its ability to interact with other Bcl-2 family members on mitochondria that would cause cytochrome *c* release and caspase 3 activation (55, 56). In our studies, treatment of breast cancer cells with the PI-3K inhibitor wortmannin prevented the EGF-mediated protection against CD47-induced cell death. On the other hand, the inhibition of ERKs, which are known to be involved in EGF-mediated signaling and to lie downstream of PI-3K, had no effect on the EGF-mediated protection (Fig. 12). Thus, it may be that, in these cells, both PKA and AKT can maintain the phosphorylation of BAD or other BH3-only proteins. When CD47 ligation activates Gi, leading to a decrease in PKA activity, the phosphorylation state of the putative BH3-only protein would decay leading to its attack on mitochondria. Additional studies are required to test this hypothesis.

CD47, originally called integrin-associated protein (27), has been implicated in many aspects of both innate and adaptive immunity, but its potential role in breast cancer has not yet been investigated. TSP1, the first identified ligand of CD47, has been implicated in the growth and progression of human breast carcinoma (7–14). TSP1 exhibits antiangiogenic activity and its overexpression can inhibit tumor growth *in vivo* by blocking neovascularization (8, 19). This function is presumably mediated by CD36, a receptor for the type 1 repeat region of TSP1 and TSP2 that can inhibit the motility and proliferation of endothelial cells (20). TSP1 also induces apoptosis of endothelial cells via CD36 (21) and can activate transforming growth factor β , a cytokine frequently implicated in suppressing tumor growth (63). TSP1 expression has been reported in invasive lobular breast cancer and in stromal cells adjacent to cancer cells

through immunohistochemical and reverse transcription-PCR analysis (7–14). Besides TSP1, TSP2 also exerts strong antiangiogenic effects on squamous cell carcinoma and breast cancer by significantly reducing the density and size of intratumor microvessels (10, 11). Stable, synthetic peptide analogs of TSP1 suppressed breast cancer growth in mouse models in a dose-dependent manner (18). The recent report that a non-metastatic clone of breast cancer cells had up-regulated TSP1 expression some 15-fold but showed no angiostatic phenotype suggested that TSP1 may act through other mechanisms to limit tumor cell functions (22). We have shown here that TSP1 can also kill breast cancer cells directly through its interaction with CD47. Because CD47 signals in conjunction with a number of different integrins (27), the particular set of integrins displayed on the cancer cell and its immediate matrix environment may ultimately determine whether ligation of CD47 can exert the killing effect *in vivo* that we have seen here under simple *in vitro* conditions.

In summary, our data indicate that ligation of CD47 induces Gi-dependent but caspase-independent apoptosis of breast cancer cells *in vitro*. Cell killing depends on Gi-mediated reduction of cAMP levels and is effectively blocked by any agent that can maintain intracellular cAMP and, hence, PKA activity. Because the CD47 agonist sequences occur in all five isoforms of TSP, it is possible that localized secretion or expression of any TSP isoform in the proper milieu could be proapoptotic. It is thus proposed that CD47 may be a potential therapeutic target in breast cancer along with TSP 1 and 2 (7–14). Perhaps activation of the CD47 death pathway along with the inhibition of EGF or ErbB2/neu pathways would provide a more effective therapeutic approach.

ACKNOWLEDGMENTS

We acknowledge Paul Brown for help with confocal microscopy.

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