Induction of programmed cell death in ErbB2/HER2-expressing cancer cells by targeted delivery of apoptosis-inducing factor

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Abstract
Apoptosis-inducing factor (AIF) is a mitochondrial flavoprotein with NADH oxidase activity that has a vital function in healthy cells but is also an important mediator of caspase-independent programmed cell death in stressed and damaged cells. Here, we generated a truncated AIF derivative (AIFΔ100) that lacks the mitochondrial import signal of the protein. Bacterially expressed AIFΔ100 was functionally active and induced cell death on microinjection into Vero cells accompanied by clear signs of apoptosis. For specific targeting to tumor cells, AIFΔ100 was genetically fused to the scFv(FRP5) antibody fragment that recognizes the ErbB2 (HER2) receptor tyrosine kinase frequently overexpressed in many human cancers. Recombinant scFv(FRP5)-AIFΔ100 (5-AIFΔ100) protein and a similar scFv(FRP5)-ETA252-366-AIFΔ100 (5-E-AIFΔ100) molecule harboring in addition the nontoxic translocation domain of Pseudomonas exotoxin A as an endosome escape function displayed binding to ErbB2-expressing cells followed by protein internalization and accumulation in intracellular vesicles. In the presence of the endosomolytic reagent chloroquine 5-E-AIFΔ100 but not the similar 5-AIFΔ100 protein displayed potent cell killing activity, which was strictly dependent on the expression of ErbB2 on the target cell surface. Our results show that recombinant AIF specifically targeted to human cancer cells and delivered into the cytosol has potent cell killing activity, suggesting this molecule as an effector function suitable for the development of humanized immunotoxin-like molecules.

Introduction
Recombinant immunotoxins are chimeric molecules that combine specific recognition of cancer cells with selective delivery of a potent protein toxin of plant or bacterial origin (1, 2). Successful application in cancer patients has revived interest in these targeted therapeutics (3–5). Nevertheless, repeated or prolonged treatment with antibody-toxins is complicated by frequent development of neutralizing antibodies directed against the toxin domain (6), prompting attempts to reduce immunogenicity of the toxin by chemical modification or elimination of immunodominant epitopes (7, 8). Using a cytotoxic protein of human origin for immunotoxin-like molecules constitutes an alternative approach to circumvent the problem of high immunogenicity. Target cell killing by bacterial toxins such as Pseudomonas exotoxin A (ETA) is mediated by the inhibition of protein synthesis followed by the induction of apoptosis via indirect mechanisms (9, 10). Consequently, human molecules that transmit strong proapoptotic signals are primary candidates for the development of antibody fusion proteins for cancer therapy (11, 12).

Here, we investigated targeted delivery of recombinant human apoptosis-inducing factor (AIF) as a strategy to selectively induce tumor cell death. AIF plays an important role in caspase-independent programmed cell death, and its activation in cancer cells may be crucial for the effectiveness of a broad range of cytotoxic and antiproliferative substances (13). AIF is a phylogenetically old flavoprotein that shares significant homology with bacterial ferredoxins and NADH oxidoreductases (14). Transcription and translation of the mammalian AIF gene results in a 67-kDa precursor molecule, which harbors two putative mitochondrial localization signals at the N-terminal and mitochondrial intermembrane space, where it is required for correct assembly and maintenance of the respiratory chain complex I (15). On stimulation of cell death, AIF is cleaved further by calpains and/or cathepsins to a 57-kDa protein (16, 17) and released into the cytosol, from where it translocates to the nucleus (18, 19). Nuclear AIF interacts with cyclophilin A to form an active DNase (20), whereby the positively charged surface of AIF facilitates binding to DNA in a sequence-independent manner, which is essential for the apoptogenic function of AIF (21).

We used recombinant human AIF to selectively induce cell death in tumor cells overexpressing the ErbB2 (HER2, Neu) receptor tyrosine kinase. Elevated ErbB2 levels have been found in many tumors of epithelial origin and have been shown to contribute to cellular transformation (22),
making this tumor-associated surface antigen an attractive target for specific therapies including monoclonal antibodies and antibody-derived reagents such as immunotoxins (2, 23). Bacterially expressed AIF lacking the mitochondrial import signal of the protein was functionally active and induced apoptotic cell death on microinjection. For targeting to ErbB2-expressing tumor cells, this truncated AIF derivative (AIF −Δ100) was fused to the ErbB2-specific single-chain Fv antibody fragment scFv(FRP5) (24). The resulting chimeric scFv(FRP5)-AIF −Δ100 fusion protein displayed specific binding to ErbB2-expressing tumor cells and, on inclusion of an endogenous protein translocation domain and addition of an endosomolytic activity, selectively killed ErbB2-expressing cancer cells.

Materials and Methods

Cell Lines and Culture Conditions

Human A431 squamous carcinoma cells, MDA-MB453 and MDA-MB468 breast carcinoma cells, and Vero African green monkey kidney cells were maintained in DMEM (Cambrex) supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin. Murine Renca-lacZ renal carcinoma cells and Renca-lacZ/ErbB2 cells stably expressing human ErbB2 (25) were cultured in RPMI 1640 (Cambrex) containing the same supplements and 0.2 mg/mL Zeocin (all Renca-lacZ derivatives) and 0.48 mg/mL G418 (Renca-lacZ/ErbB2). All cells were cultivated at 37°C in a humidified atmosphere of air and 5% CO2.

Construction of AIF Derivatives

A cDNA fragment encoding truncated human AIF that lacks residues 1 to 100 of the protein (AIF −Δ100) was derived from full-length AIF cDNA from macrophages by PCR with oligonucleotides 5′-KpmI-AIF Δ100, 5′-AAAAGGTACCGGTATGGGCTACCCAGAACAGAAACAGAAAAAGG-3′ and 3′-EcoRI-AIF 5′-AAGAAATTCTCAGTCTTCTCAT-GAAGTGGATTGTTGCTACTTC-3′ as primers. Constructs for expression of antibody-AIF fusion proteins were then derived by stepwise assembly of cDNA encoding the ErbB2-specific scFv(FRP5) antibody fragment and AIF −Δ100 cDNA, or scFv(FRP5) and AIF −Δ100 fragments linked by a sequence encoding the translocation domain (residues 252–366) of ETA in derivatives of the bacterial expression vector pSW5 (26, 27). The resulting plasmids pSW5-5-AIF −Δ100 and pSW5-5-E-AIF −Δ100 encode under the control of a TATA promoter the 5′-AIF −Δ100 and 5′-E-AIF −Δ100 fusion proteins that consist of an NH2-terminal ompA signal peptide, a FLAG tag, and a hexahistidine tag provided by the vector backbone followed by scFv(FRP5) and AIF −Δ100, or scFv(FRP5), ETA252–366, and AIF −Δ100 domains, respectively. For expression of recombinant AIF −Δ100 without cell targeting domain, the AIF −Δ100 cDNA fragment was reamplified with oligonucleotides 5′-Ndel-AatrI-AIF −Δ100, 5′-TGTACA-TATGGGTTAGGGCTACCCAGAACAGAAAC-3′ and 3′-EcoRI-AIF and, after digestion with Ndel and EcoRI, inserted 5′ of sequences encoding Myc and hexahistidine tags in the bacterial expression vector pSW5 (28), resulting in plasmid pSW5-AIF −Δ100. All constructs were verified by restriction analysis and DNA sequencing.

Bacterial Expression and Purification of Recombinant Proteins

Escherichia coli BL21(ADE3) carrying the respective expression plasmids was grown to an A600 of 0.8 at 37°C in LB medium containing 0.1 mol/L glucose and 100 μg/mL ampicillin. Then, protein expression was induced by addition of IPTG to a final concentration of 0.5 mmol/L. Cells were harvested 2 h later by centrifugation at 6,000 × g for 15 min at 4°C. Bacterial pellets were resuspended in buffer A [0.6 mol/L NaCl, 8 mol/L urea, 2.7 mmol/L KCl, 4.3 mmol/L Na2HPO4, 1.4 mmol/L KH2PO4 (pH 8.0)] and lysed in a French pressure cell (G. Heinemann). Lysates were cleared by centrifugation at 35,000 × g for 30 min at 4°C and loaded onto a Ni2+-NTA superflow column (Qiagen) connected to a fast protein liquid chromatography system (Amersham Biosciences). Unbound proteins were removed by washing with buffer A, and specifically bound proteins were eluted with buffer B (buffer A containing 0.25 mol/L imidazole). Fractions containing recombinant AIF proteins were identified by SDS-PAGE and immunoblotting with AIF-specific polyclonal goat antibody AIF (D-20; Santa Cruz Biotechnology) followed by horseradish peroxidase-conjugated anti-goat secondary antibody (Sigma-Aldrich) and chemiluminescent detection with the enhanced chemiluminescence kit (Amersham Biosciences). Relevant fractions were pooled, and denaturant was removed by dialysis against PBS containing 400 mmol/L L-arginine followed by dialysis against PBS. Diaoyzed proteins were passed through a 0.45-μm filter (Millipore), divided into aliquots, and stored at −80°C until use.

Binding Assays

DNA-binding activity of recombinant AIF derivatives was determined in gel retardation assays (29). Intact pcDNA3 plasmid DNA or DNA fragments (2.5 μg of 1-kb DNA ladder; Invitrogen) were incubated for 30 min at room temperature with increasing amounts of purified AIF proteins before analysis by agarose gel electrophoresis (1%) in the presence of ethidium bromide to visualize the DNA. To inhibit interaction of AIF with DNA, recombinant proteins (320 ng) were preincubated with 1 unit heparin (Liquermin; Roche Pharma) for 30 min at room temperature before addition to 100 ng of plasmid DNA and agarose gel electrophoresis.

Binding of 5-AIF −Δ100 and 5-E-AIF −Δ100 proteins to the surface of tumor cells was investigated by fluorescence-activated cell sorting analysis. Trypsinized Renca-lacZ/ErbB2, Renca-lacZ, MDA-MB453, MDA-MB468, or A431 cells (5 × 105) were incubated with 2.5 μg of purified AIF proteins for 1 h at 4°C. Unbound proteins were removed, cells were washed, and bound proteins were detected with 1 μg of anti-FLAG antibody M2 (Sigma-Aldrich) followed by phycoerythrin (PE)-conjugated goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch). Fluorescence of cells was analyzed using a FACS Calibur fluorescence-activated cell sorter (Becton Dickinson). For competition experiments,
5-E-AIFΔ100 was pretreated with a 5-fold molar excess of a recombinant ErbB2 protein fragment encompassing the scFv(FRP5)-binding epitope (28) for 30 min at room temperature before addition to A431 cells. To investigate the contribution of the positively charged AIFΔ100 domain to cell binding, 2.5 μg of 5-E-AIFΔ100 were preincubated with 10 units heparin for 30 min at room temperature before cell-binding analysis.

**Immunofluorescence Analysis**

Cells were grown overnight in chamber slides, treated with 2.5 μg of purified 5-E-AIFΔ100 for 1 h at 37°C, washed, and incubated for another 1.5 h in fresh medium. Following fixation with 4% paraformaldehyde in PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. Then, cells were sequentially incubated with ETA-specific polyclonal rabbit antibody (24) and Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes) in PBS containing 3% bovine serum albumin. Nucleic acids were stained with propidium iodide. Finally, the samples were analyzed with a Leica TCS SL laser scanning microscope (Leica Mikrosysteme).

**Microinjection Experiments**

Vero cells (5 × 10^4) were seeded onto a 35-mm Petri dish in normal growth medium. One day later, 5-E-AIFΔ100 and AIFΔ100 proteins at a concentration of 72 μg/mL were each mixed with recombinant glutathione S-transferase (GST)-enhanced green fluorescent protein (eGFP) fusion protein, and mixtures were diluted 3:1 in PBS and microinjected into the cytosol of 100 to 140 cells per sample as described previously (12, 30). Control cells were injected with GST-eGFP in the absence of AIF proteins. After microinjection, cells were incubated at 37°C for 48 h. Cell morphology was analyzed and eGFP-positive cells were counted at 0, 24, and 48 h by fluorescence microscopy.

**Cell Viability and Apoptosis Assays**

Cells were seeded in 96-well plates at a density of 1 × 10^4 per well and incubated for 72 h at 37°C with varying concentrations of purified AIF proteins in triplicate in the presence or absence of 50 or 100 μM of chloroquine. The relative number of viable cells in comparison with cells grown without AIF proteins was determined in 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) metabolization assays as described (31), and data were statistically evaluated using the t test. To confirm dependence of cytotoxic activity on binding to ErbB2, 5-E-AIFΔ100 was pretreated with a 5-fold molar excess of a recombinant ErbB2 protein fragment for 30 min at room temperature before addition to A431 cells.

Induction of apoptosis was measured after treatment of 2 × 10^5 A431 cells per well in 24-well plates with 1 μg/mL 5-E-AIFΔ100 protein for 24 or 48 h in the presence of 100 μmol/L of chloroquine. Control cells were treated with PBS and chloroquine. For each sample, adherent cells were detached with trypsin and combined with cells floating in the culture medium. Harvested cells were pelleted at 300 × g, washed twice with PBS, and labeled with Annexin V-FITC and propidium iodide for 20 min using the Annexin V-FLUOS staining kit (Roche Molecular Biochemicals) according to the manufacturer’s recommendations. Fluorescence of cells was analyzed using a FACSCalibur fluorescence-activated cell sorter (Becton Dickinson). To investigate dependence of apoptosis induction on the activity of the positively charged AIF domain,
Results

Generation of ErbB2-Specific Antibody-AIF Fusion Proteins

cDNA encoding truncated human AIF (amino acid residues 101–613; AIFΔ100) that lacks mitochondrial localization sequences was derived from mRNA of macrophages by reverse transcription-PCR and either fused directly to a sequence encoding the ErbB2-specific scFv antibody fragment scFv(FRP5) in the bacterial expression plasmid pSW50 or connected to the scFv sequence via the translocation domain of ETA (amino acid residues 252–366) as a potential endosome escape sequence (Fig. 1A). Likewise, the AIFΔ100 cDNA fragment was inserted into plasmid pSW5 for bacterial expression of recombinant AIFΔ100 without cell targeting domain. In the resulting plasmids, pSW5-5-AIFΔ100, pSW50-5-AIFΔ100 and pSW5-AIFΔ100 expression of scFv(FRP5)-AIFΔ100 (5-AIFΔ100) and scFv(FRP5)-ETA252-366-AIFΔ100 (5-E-AIFΔ100) fusion proteins carrying NH2-terminal FLAG and hexahistidine tags and of truncated AIFΔ100 carrying COOH-terminal Myc and hexahistidine tags is controlled by the IPTG-inducible tac promoter. The recombinant proteins were expressed in E. coli strain BL21 (DE3) and purified from bacterial lysates prepared in 8 mol/L urea by Ni2+-affinity chromatography under denaturing conditions. SDS-PAGE analysis revealed a purity of ∼80% after refolding (Fig. 1B). Identity of the proteins was confirmed by immunoblot analysis with AIF-specific antibody (Fig. 1B).

DNA-Binding Activity of AIF Fusion Proteins

Binding of AIF to DNA depends on the positively charged surface of the molecule and is indispensable for its apoptosis-inducing activity (21). To test whether the bacterially expressed antibody-AIF fusion proteins retained the ability to bind to DNA, gel retardation experiments were done. Increasing amounts of purified 5-E-AIFΔ100 and 5-AIFΔ100 proteins were incubated with 2.5 μg of DNA fragments (1-kb DNA ladder). Then, the electrophoretic mobility of the resulting protein-DNA complexes in a 1% agarose gel was determined. In the absence of antibody-AIF fusion proteins, the DNA fragments migrated with the expected distribution pattern (Fig. 2A, lane 1). Addition of purified 5-E-AIFΔ100 (Fig. 2A, lanes 2–4) or 5-AIFΔ100 (Fig. 2A, lanes 5–7) retarded the DNA fragments substantially and in a concentration-dependent manner. Similar retardation was found after complexing pcDNA3 plasmid DNA with purified AIFΔ100 (Fig. 2A, lanes 8 and 9), showing that the bacterially expressed AIF derivatives retained DNA-binding ability irrespective of the inclusion of the heterologous cell binding and ETA translocation domain in the fusion proteins.

Binding of AIF Fusion Proteins to Tumor Cells

Binding of 5-E-AIFΔ100 to ErbB2 on the surface of tumor cells was investigated by fluorescence-activated cell sorting analysis with murine Renca-lacZ/ErbB2 renal carcinoma cells stably transfected with human ErbB2 cDNA construct (25) and human MDA-MB453 breast carcinoma cells endogenously expressing high levels of ErbB2 (32). After incubation with purified 5-E-AIFΔ100 fusion protein followed by FLAG tag–specific antibody and PE-conjugated secondary antibody, strong binding of 5-E-AIFΔ100 to Renca-lacZ/ErbB2 and MDA-MB453 cells was detected (Fig. 2B). Although less pronounced, also significant binding of the fusion protein to parental Renca-lacZ cells and human MDA-MB468 breast carcinoma cells was found, which do not express detectable

![Figure 2](image-url)

**Figure 2.** A, binding of antibody-AIF fusion proteins to DNA. Increasing amounts of purified 5-E-AIFΔ100 (lanes 2–4) and 5-AIFΔ100 (lanes 5–7) fusion proteins were incubated with 2.5 μg of DNA fragments (1-kb DNA ladder; lane 1), and formation of protein-DNA complexes was analyzed by agarose gel electrophoresis. For comparison, migration of 0.5 μg of pcDNA3 plasmid DNA (lane 10) and plasmid DNA after incubation with untargeted AIFΔ100 (lanes 8 and 9) was analyzed. B, cell binding of 5-E-AIFΔ100 protein. Murine Renca-lacZ/ErbB2 renal carcinoma cells stably transfected with human ErbB2 cDNA and human MDA-MB453 breast carcinoma cells endogenously expressing high ErbB2 levels, and ErbB2-negative murine Renca-lacZ and human MDA-MB468 breast carcinoma cells were incubated with purified 5-E-AIFΔ100 protein followed by monoclonal anti-FLAG antibody M2 and PE-conjugated secondary antibody for detection by flow cytometry (filled areas). Open areas, control cells were treated with primary and secondary antibodies in the absence of 5-E-AIFΔ100. For comparison of binding to ErbB2-positive and ErbB2-negative cells (Renca-lacZ/ErbB2 versus Renca-lacZ; MDA-MB453 versus MDA-MB468), mean fluorescence intensities (MFI) are indicated.
ErbB2 levels. Similar results were obtained for 5-AIFΔ100 fusion protein that lacks the ETA domain (data not shown), suggesting a contribution of the AIF domain to cell binding independent of the presence of ErbB2. To investigate this possibility further, binding of untargeted AIFΔ100 and 5-E-AIFΔ100 fusion protein to Renca-lacZ/ErbB2 was investigated with or without preincubation of the proteins with the highly sulfated glycosaminoglycan heparin to prevent possible interaction of the positively charged surface of AIF with negatively charged molecules on the cell surface. Thereby, in the absence of heparin, binding of untargeted AIFΔ100 to the surface of Renca-lacZ/ErbB2 was comparable with that of 5-E-AIFΔ100. However, whereas cell binding of AIFΔ100 was markedly reduced by heparin, binding of 5-E-AIFΔ100 was only marginally affected (shown as Supplementary Data). This suggests that binding of the fusion protein to ErbB2-expressing cells is primarily mediated by interaction of the antibody domain with its antigen.

**Effect of Chimeric 5-E-AIFΔ100 Protein on Cell Viability**

Induction of cell death by 5-E-AIFΔ100 was investigated in cell viability assays with Renca-lacZ/ErbB2 and ErbB2-negative Renca-lacZ cells. Cells were cultured for 72 hours with increasing concentrations of purified 5-E-AIFΔ100 protein before the proportion of viable cells in comparison with PBS-treated controls was determined in MTT assays. Thereby, independent from the expression of ErbB2, no cell killing by 5-E-AIFΔ100 was observed (Fig. 3A). Similar results were obtained for 5-AIFΔ100 (data not shown). To test whether this lack of cell killing activity was due to insufficient intracellular uptake of antibody-AIF fusion protein, Renca-lacZ/ErbB2 cells were treated with 5-E-AIFΔ100 and uptake of the protein after 2.5 hours at 37°C was analyzed by confocal laser scanning microscopy using anti-ETA and secondary Alexa Fluor 488-conjugated antibodies for detection. In the majority of cells, punctuate staining within the cytoplasm was found (Fig. 3B), suggesting efficient uptake of 5-E-AIFΔ100 and routing to vesicular structures such as endosomes. Diffuse intracellular staining indicative of cytosolic localization was not observed.

To investigate whether direct delivery of 5-E-AIFΔ100 to the cytosol could induce cell death, Vero cells were microinjected with purified 5-E-AIFΔ100 or untargeted AIFΔ100 for comparison. Recombinant GST-eGFP protein was coinjected to follow injected cells. As shown in Fig. 4A, injection with 5-E-AIFΔ100 had no effect on the cells, with cellular morphology after 30 hours indistinguishable from that of control cells injected with PBS (Fig. 4A, left and right). Likewise, there was no decline in the number of apparently viable cells with normal morphology up to 48 hours after injection of 5-E-AIFΔ100 (Fig. 4B). In marked contrast, a large proportion of cells injected with untargeted AIFΔ100 showed typical apoptotic morphology, including membrane blebbing and formation of apoptotic bodies after 30 hours (Fig. 4A, middle). Another 18 hours later, 61% of cells injected with AIFΔ100 displayed apoptotic morphology or could no longer be identified as intact cells due to disintegration into small cell fragments (Fig. 4B). These data show that recombinant AIFΔ100 can induce cell death if delivered into the cytosol of target cells. However, fusion to the NH2-terminal antibody domain apparently interfered with the proapoptotic activity of the AIF domain in 5-E-AIFΔ100, preventing the induction of apoptosis on direct intracellular delivery by microinjection.

**Selective Cytotoxicity of 5-E-AIFΔ100 Toward ErbB2-Expressing Tumor Cells**

The results presented above indicate that cell binding by the scFv(FRP5) domain and DNA binding by the AIFΔ100 domain as a measure for AIF activity are preserved in 5-E-AIFΔ100. In contrast, the observed entrapment of 5-E-AIFΔ100 in intracellular vesicles suggests that the translocation domain of ETA is not fully functional in the context of the fusion protein. To test whether chimeric AIF molecules...
can be released from such intracellular vesicles by an exogenous endosomolytic reagent, we incubated target cells with increasing concentrations of 5-AIFΔ100 or 5-E-AIFΔ100 in the presence of chloroquine and measured effects on cell viability in MTT assays. Although addition of chloroquine resulted in pronounced and concentration-dependent cytotoxicity of 5-E-AIFΔ100 toward Renca-lacZ/ErbB2 cells, this was not the case for ErbB2-negative Renca-lacZ control cells, showing dependence of this effect on recognition of ErbB2 on the cell surface (Fig. 5A). Cytotoxicity of 5-E-AIFΔ100 toward human A431 epidermoid cancer cells endogenously expressing ErbB2 was even more pronounced but, as in the case of Renca-lacZ/ErbB2 cells, was strictly dependent on concurrent treatment with chloroquine (Fig. 5B, left). Unexpectedly, chloroquine had no significant effect on 5-AIFΔ100, which did not affect viability of A431 cells irrespective of the presence of the endosomolytic reagent (Fig. 5B, right). This suggests that the ETA translocation domain in the longer 5-E-AIFΔ100 protein, while on its own not facilitating release from endosomes, contributes another function to the antibody-AIF molecule important for uptake and/or cytotoxic activity.

To investigate specificity of cell killing, 5-E-AIFΔ100 protein was pretreated with a 5-fold molar excess of a recombinant ErbB2 protein fragment (ErbB22zz) encompassing the scFv(FRP5)-binding epitope (28) before addition to A431

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**Figure 4.** Induction of apoptotic morphology after cytosolic delivery of recombinant AIF derivatives. Purified 5-E-AIFΔ100 and AIFΔ100 proteins were microinjected into the cytosol of Vero cells together with GST-eGFP for detection. Control cells were injected with GST-eGFP and PBS. A, at the indicated time points, injected cells were identified by fluorescence microscopy and cellular morphology was analyzed. Representative fields are shown. Bottom, a cell undergoing apoptosis after injection with AIFΔ100 is shown at higher magnification (fluorescence and phase-contrast microscopy). B, for quantification, the percentage of morphologically normal eGFP-positive cells was determined directly after microinjection (0 h) and 24 and 48 h later.

**Figure 5.** Selective cytotoxicity of 5-E-AIFΔ100 toward ErbB2-expressing cells in the presence of chloroquine. A, ErbB2-expressing Renca-lacZ/ErbB2 (black columns) and Renca-lacZ control cells (white columns) were incubated with the indicated concentrations of 5-E-AIFΔ100 in the presence of 50 μmol/L chloroquine. B, ErbB2-expressing human A431 epidermoid cancer cells were treated with increasing concentrations of 5-E-AIFΔ100 (left) or 1 μg/mL 5-AIFΔ100 (right) in the presence (black columns) or absence (white columns) of 100 μmol/L chloroquine. After 72 h, the relative number of viable cells in comparison with PBS-treated controls was determined using an MTT metabolism assay. C, to confirm specificity, 5-E-AIFΔ100 was treated with a 5-fold molar excess of recombinant ErbB2 protein (ErbB22zz). Binding of pretreated 5-E-AIFΔ100 (left, shaded area) and 5-E-AIFΔ100 in the absence of competitor (left, open area) to A431 cells was measured by flow cytometry. Bound 5-E-AIFΔ100 was detected by monoclonal anti-FLAG antibody M2 and PE-conjugated secondary antibody. Left, filled area, control cells were treated with primary and secondary antibodies in the absence of 5-E-AIFΔ100. Right, shaded bar, to analyze cytotoxicity, 5-E-AIFΔ100 pretreated with recombinant ErbB2 protein was added to A431 cells at a final concentration of 1 μg/mL in the presence of 100 μmol/L chloroquine. Black column, control cells were treated with 5-E-AIFΔ100 and chloroquine in the absence of competitor. After 72 h, the relative number of viable cells was determined as described above. Bars, SD. ***, P < 0.001; **, P < 0.01; *, P < 0.05; ns, P > 0.05.
cells. This resulted in a marked reduction of cell binding (Fig. 5C, left) and cell killing (Fig. 5C, right). To confirm that cell death observed on treatment with 5-E-AIFΔ100 was due to AIF-induced apoptosis, A431 cells were incubated in the presence of chloroquine with 1 μg/mL of the AIF fusion protein or 5-E-AIFΔ100 pretreated with heparin to block the activity of the AIF domain. Induction of apoptosis was measured by determining the percentage of Annexin V and propidium iodide double-positive cells (Fig. 6A). After 24 hours, 28% of 5-E-AIFΔ100-treated cells were apoptotic. Heparin significantly reduced the proapoptotic activity of the fusion protein (16% apoptotic cells), resulting in apoptosis similar to that in cells incubated without 5-E-AIFΔ100 (12% apoptotic cells). After 48 hours, this effect was even more pronounced, with 61% apoptotic cells on treatment with 5-E-AIFΔ100 in the absence and 27% apoptotic cells in the presence of heparin. As it was the case for Renca-lacZ/ErbB2 cells (shown as Supplementary Data),3 binding of 5-E-AIFΔ100 to ErbB2-expressing A431 cells was not affected by heparin (Fig. 6B). Hence, the observed reduction in cell killing activity in the presence of heparin must be due to inhibition of the intracellular activity of the AIF domain. Indeed, neutralization of the positively charged AIF surface by heparin strongly inhibited DNA binding of 5-E-AIFΔ100 considered to be crucial for the proapoptotic activity of the protein (Fig. 6C).

Discussion

During their progression to malignancy, cancer cells accumulate mutations that promote proliferation and limit their sensitivity to proapoptotic signals (33). Radiation and chemotherapy depend on the induction of apoptosis, which explains why development of radioresistance and chemoresistance in cancer cells is frequently accompanied by a blockade of relevant proapoptotic signaling pathways (34, 35). Introducing into such cells functional proapoptotic effector molecules may bypass endogenous resistance mechanisms and prove useful for cancer therapy. Here, we evaluated targeted delivery of recombinant AIF as a means to selectively induce cell death in tumor cells expressing the tumor-associated ErbB2 cell surface antigen. Many cytotoxic and antiproliferative substances rely for their activity at least in part on AIF (13). AIF activation has been shown on treatment of glioma and leukemic cells with the cyclin-dependent kinase inhibitor flavopiridol (36, 37), and release of endogenous AIF from mitochondria and nuclear translocation was shown to be crucial for cisplatin-induced apoptosis in prostate and ovarian cancer cells (38, 39). Likewise, the activity of etoposide in combination with a histone deacetylase inhibitor against drug-resistant non–small cell lung carcinoma cells was strictly dependent on AIF (40).

To test its suitability as an effector domain in cytotoxic antibody fusion proteins, we first generated a truncated AIF derivative that lacks the mitochondrial import signal of the protein but retains its endogenous nuclear localization signal. Microinjection of this recombinant AIFΔ100 molecule into the cytoplasm of Vero cells resulted in massive cell death, accompanied by clear signs of apoptosis such as membrane blebbing and formation of apoptotic bodies. Hence, increasing the concentration of activated AIF in the cytoplasm is sufficient to induce apoptosis without the requirement for exogenous chemotherapeutic agents, and as previously shown for an AIF fragment similar to AIFΔ100, this effect may be further amplified by the release of endogenous AIF and cytochrome c from mitochondria (41). For specific targeting to tumor cells, we either fused the AIFΔ100 fragment directly to the COOH terminus of the ErbB2-specific scFv(FR5P) antibody domain (5-AIFΔ100) or connected antibody and AIFΔ100 sequences via the translocation domain of ETA (5-E-AIFΔ100) as a potential endosome escape activity.

Recombinant AIFΔ100, 5-AIFΔ100, and 5-E-AIFΔ100 proteins formed complexes with plasmid DNA and DNA fragments in a concentration-dependent manner, as expected for functional AIF. Endogenous cellular AIF displays a strong positive electrostatic potential on its surface, which allows...
it to bind to DNA in a sequence-independent fashion. This is crucial for the apoptosis-inducing activity of AIF, and AIF mutants defective in DNA binding fail to induce cell death (21). DNA binding and proapoptotic activity of 5-E-AIF\(_{100}\) were abolished on preincubation with heparin. This shows that interaction of the chimeric protein with DNA is indeed dependent on the positive surface charge of the AIF domain and strongly indicates that induction of apoptosis by 5-E-AIF\(_{100}\) relies on the same mechanism described for AIF.

When cell binding of the 5-E-AIF\(_{100}\) protein was analyzed, we observed strong binding to ErbB2-expressing cancer cells, indicating that the ErbB2-specific antibody domain was functional. Nevertheless, to a lower extent, also binding to ErbB2-negative cells was observed. This was also the case for the shorter 5-AIF\(_{100}\) protein (data not shown), ruling out nonspecific interaction with the cell surface via the ETA translocation domain of 5-E-AIF\(_{100}\). Instead, nonspecific binding to the cell surface could be attributed to the AIF domain because recombinant AIF\(_{100}\) on its own was also able to bind to cells (shown as Supplementary Data). This cell binding was blocked by preincubation of AIF\(_{100}\) with heparin, suggesting electrostatic interaction of the positively charged AIF with negatively charged cell surface molecules as the underlying mechanism. A similar mechanism of cell binding was recently proposed for recombinant derivatives of HMGB2, another positively charged DNA-binding protein (29). Importantly, despite the ability of the AIF domain to mediate cell binding on its own, 5-E-AIF\(_{100}\) showed high selectivity for ErbB2-expressing cells. When ErbB2 was present on the target cell surface, cell binding of 5-E-AIF\(_{100}\) was almost exclusively mediated by the ErbB2-specific antibody domain, indicated by the inability of heparin to significantly inhibit this interaction. Furthermore, cytotoxicity of 5-E-AIF\(_{100}\) in the presence of the endosomolytic reagent chloroquine was strictly dependent on the expression of ErbB2, and binding to ErbB2-expressing cells as well as killing of such cells were markedly reduced in the presence of recombinant ErbB2 as a competitor. This shows that with respect to cell killing, the 5-E-AIF\(_{100}\) protein displays the desired target cell specificity.

The 5-AIF\(_{100}\) and 5-E-AIF\(_{100}\) fusion proteins were bifunctional, displaying both binding to ErbB2-expressing tumor cells via their antibody domain and DNA binding via the AIF fragment as an indicator of AIF activity. Nevertheless, they were unable to facilitate cell killing in the absence of an exogenous endosomolytic activity. To induce DNA fragmentation and cell death, cellular AIF requires proteolytic processing, release from mitochondria into the cytosol, and translocation to the nucleus (18, 19). Recombinant AIF\(_{100}\) corresponding to the active cytoplasmic AIF fragment generated endogenously after stimulation of cell death and was effective in the induction of apoptosis after direct microinjection into the cytoplasm. In contrast, AIF fusion proteins targeted to cell surface receptors such as ErbB2 enter cells from the outside by receptor-mediated endocytosis, resulting in internalization into endosomes. Hence, induction of AIF-dependent apoptosis first requires release of active AIF from these vesicles into the cytoplasm. To enable endo-

Published OnlineFirst June 9, 2009; DOI: 10.1158/1535-7163.MCT-08-1149

Mol Cancer Ther 2009;8(6). June 2009

Molecular Cancer Therapeutics 1533

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gene-modified T cells for expression (48). We identified intracellular processing of the antibody-AIF fusion protein and release from endosomes as critical features. The prototypic 5-E-AIFΔ100 protein still contains the bacterial ETA translocation domain, which could induce antibody responses in humans, albeit presumably at lower levels than full-length toxin. Hence, replacement of the ETA-derived furin cleavage site with a corresponding sequence from a human protein seems as a rational next step and may allow the generation of fully humanized molecules. The low molecular weight compound chloroquine, which is being used for the treatment of malaria and other diseases in humans (49), can also be applied to achieve endosome release in vitro without causing severe side effects (50). In the presence of chloroquine, the prototypic 5-E-AIFΔ100 molecule displayed highly selective cytotoxicity toward ErbB2-expressing cancer cells, holding promise for further optimization and development as a targeted therapeutic of human origin.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Shirley K. Knauer for recombinant GST-eGFP protein and advice on microinjection experiments, Ute Burkhardt for recombinant ErbB2 protein, and Dr. Martin Zörnig for helpful discussions and critical reading of the manuscript.

References


Mol Cancer Ther 2009;8(6). June 2009
Molecular Cancer Therapeutics

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Mol Cancer Ther Published OnlineFirst June 9, 2009.

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