Cytotoxic Activity of Epidermal Growth Factor-Genistein against Breast Cancer Cells

Fatih M. Uckun, Rama Krishna Narla, Xiao Jun, Tamer Zeren, Taracad Venkatachalam, Kevin G. Waddick, Alexander Rostostev, and Dorothea E. Myers

ABSTRACT

The receptor (R) for epidermal growth factor (EGF) is expressed at high levels on human breast cancer cells and associates with ErbB2, ErbB3, and Src proto-oncogene family protein tyrosine kinases (PTKs) to form membrane-associated PTK complexes with pivotal signaling functions. Recombinant human EGF was conjugated to the soybean-derived PTK inhibitor genistein (Gen) to construct an EGF-R-directed cytotoxic agent with PTK inhibitory activity. The EGF-Gen conjugate was capable of binding to and entering EGF-R-positive MDA-MB-231 and BT-20 breast cancer cells (but not EGF-R-negative NALM-6 or HL-60 leukemia cells) via its EGF moiety, and it effectively competed with unconjugated EGF for target EGF-R molecules in ligand binding assays. EGF-Gen inhibited the EGF-R tyrosine kinase in breast cancer cells at nanomolar concentrations, whereas the IC50 for unconjugated Gen was >10 μM. Notably, EGF-Gen triggered a rapid apoptotic cell death in MDA-MB-231 as well as BT-20 breast cancer cells at nanomolar concentrations. The EGF-Gen-induced apoptosis was EGF-R-specific because cells treated with the control granulocyte-colony stimulating factor-Gen conjugate did not become apoptotic. Apoptosis was dependent both on the PTK inhibitory function of Gen and the targeting function of EGF, because cells treated with unconjugated Gen plus unconjugated EGF did not undergo apoptosis. The IC50s of EGF-Gen versus unconjugated Gen against MDA-MB-231 and BT-20 cells in clonogenic assays were 120 ± 18 μM (P < 0.001) and 30 ± 10 μM versus 112 ± 17 μM (P < 0.001), respectively. Thus, the EGF-Gen conjugate is a >100-fold more potent inhibitor of EGF-R tyrosine kinase activity in intact breast cancer cells than unconjugated Gen and a >100-fold more potent cytotoxic agent against EGF-R+ human breast cancer cells than unconjugated Gen. Taken together, these results indicate that the EGF-R-associated PTK complexes have vital antiapoptotic functions in human breast cancer cells and may therefore be used as therapeutic targets.

INTRODUCTION

Breast cancer is the most common tumor in women, representing 32% of all new cancer cases and causing 18% of the cancer-related deaths among women in the United States (1). Although the majority of patients with metastatic breast cancer will experience an initial response, survival is only modestly improved with contemporary chemotherapy programs (2–4). Consequently, the development of new potent anti-breast cancer drugs has emerged as an exceptional focal point for translational research in treatment of breast cancer (5).

Human EGF3 is a 53-amino acid, single-chain polypeptide (M, 6216) that exerts biological effects by binding to a specific M, 170,000 cell membrane EGF-R (EGF-R/ErbB-1; Refs. 6–9). The human EGF-R consists of an extracellular domain with a high cysteine content and N-linked glycosylation, a single transmembrane domain, and a cytoplasmic domain with PTK activity. Binding of EGF to EGF-R/ErbB-1 results in receptor dimerization with itself or other members of the Erb-B (subtype I) transmembrane PTK family (e.g., Erb-B2 and Erb-B3), resulting in activation with autophosphorylation of the PTK domain (10, 11). Recent studies demonstrated that the EGF-R is physically and functionally associated with the Src proto-oncogene family PTK, including p60c- (10–12). This association is believed to be an integral part of the signaling events in breast cancer cells mediated by the EGF-R and contributes to proliferation and survival of breast cancer cells (12–14). Many types of cancer cells display enhanced EGF-R expression on their cell surface membranes (8). Enhanced expression of the EGF-R on cancer cells has been associated with excessive proliferation and metastasis (9). Examples include breast cancer, prostate cancer, lung cancer, head and neck cancer, bladder cancer, melanoma, and brain tumors (8). In breast cancer, expression of the EGF-R

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3 The abbreviations used are: EGF, epidermal growth factor; rhEGF, recombinant human EGF; EGF-R, EGF receptor; PTK, protein tyrosine kinase; Gen, genistein; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; G-CSF, granulocyte colony-stimulating factor; PI, propidium iodide.
is a significant and independent indicator for recurrence and poor relapse-free survival (15–17).

Targeted delivery of Gen, a naturally occurring PTK inhibitory isoflavone (5,7,4'-tri hydrox y isoflavone) from fermentation broth of *Pseudomonas spp.*, to CD19 receptor-associated vital PTK shows considerable promise for more effective treatment of human leukemias and lymphomas (18, 19). We postulated that the EGF-R-associated PTK may be of similar importance for the survival of breast cancer cells and, therefore, may serve as a suitable target for biotherapy of breast cancer using EGF-Gen conjugates. In this study, rhEGF was conjugated to Gen to construct an EGF-R-directed cytotoxic agent with PTK resistance for the survival of breast cancer cells and, therefore, may improve the outcome of human leukemias and lymphomas (18, 19). We postulated that the EGF-R-associated PTK may be of similar importance.

### MATERIALS AND METHODS

#### Preparation of the EGF-Gen.

rhEGF was produced in *Escherichia coli* harboring a genetically engineered plasmid that contains a synthetic gene for human EGF fused at the NH$_2$ terminus to a hexapeptide leader sequence for optimal protein expression and folding. The rhEGF fusion protein precipitated in the form of inclusion bodies, and the mature protein was recovered by trypsin-cleavage, followed by purification using ion exchange chromatography and HPLC. rhEGF was 99% pure by reverse-phase HPLC and SDS-PAGE with an isoelectric point of 4.6 ± 0.2. The endotoxin level was 0.172 EU/mg. The recently published photochemical conjugation method using the hetero-bifunctional photoreactive cross-linking agent, sulfo-succinimidyl 6-(4'azido-2'-nitropheny lamino)hexanoate (Sulfo-SANPAH; Pierce Chemical Co., Rockford, IL; Ref. 18) has been used in the synthesis of the EGF-Gen conjugate. Sulfo-SANPAH-modified rhEGF was mixed with a 10:1 molar ratio of Gen (LC Laboratories, Woburn, MA; 50 mm solution in DMSO) and then iodinated with gentle mixing for 10 min with UV light at wavelengths 254–366 nm with a multiband UV light-emitter (model UVGL-15 Mineralight; UVP, San Gabriel, CA). Photolytic generation of a reactive singlet nitrene on the other terminus of EGF-SANPAH in the presence of a 10-fold molar excess of Gen resulted in the attachment of Gen via its available C7-hydroxy group to lysine 28 or lysine 48 residues of EGF. Excess Gen in the reaction mixture was removed by passage through a PD-10 column, and $M_1$, 12,000 EGF-EGF homoconjugates, with or without conjugated Gen, as well as higher molecular weight reaction products were removed by size-exclusion HPLC. Reverse-phase HPLC using a Hewlett-Packard 1100 series HPLC instrument was used for separation of EGF-Gen from EGF-SANPAH. After the final purification, analytical HPLC was performed using a Spherisorb ODS-2 reverse-phase column (250 × 4 mm; Hewlett-Packard). Prior to the HPLC runs, a Beckman DU 7400 spectrophotometer was used to generate a UV spectrum for both samples to ascertain the $\lambda_{max}$ for EGF-Gen, EGF-SANPAH, and unmodified EGF. Each HPLC chromatogram was subsequently run at wavelengths of 214, 265, and 480 nm using the multiple wavelength detector option supplied with the instrument to ensure optimal detection of the individual peaks in the chromatogram. Analysis was achieved using a gradient flow consisting of 0–100% eluent in a time interval of 0–30 min. Five-μL samples applied to the above column were run using the following gradient program: 0–5 min, 0–20% eluent; 5–20 min, 20–100% eluent; 25–30 min, 100% eluent; and 30–35 min, 100–0% eluent. The eluent was a mixture of 80% acetonitrile (CH$_3$CN), 20% H$_2$O, and 0.1% TFA.

Electrospray ionization mass spectrometry (20, 21) was performed using a PE SCIEX API triple quadrupole mass spectrometer (Norwalk, CT) to determine the stoichiometry of Gen and EGF in EGF-Gen. $^{125}$I-Gen was also used to confirm the stoichiometry of Gen and EGF in EGF-Gen and to verify the removal of free genistein and genistein-labeled EGF-EGF homoconjugates by the described purification procedure. Gen (in 65% ethanol, 35% PBS, pH 7.5; LC Laboratories, Woburn, MA) was radioiodinated at room temperature in Reacti-Vials containing Iodo-beads (Pierce Chemical Co., Rockford, IL) and $^{125}$I (Na, carrier-free, 17.4 Ci/mg; NEN, Boston, MA) as per manufacturer’s instructions (18, 19). The purity of EGF-$^{125}$I-Gen was assessed by SDS-PAGE (20% separating gels, nonreducing conditions) and autoradiography using intensifying screens and Kodak XAR-5 film. EGF-$^{125}$I-Gen was also used for *in vitro* ligand binding assays (18, 21) and EGF-Gen internalization studies (18).

#### Breast Cancer Cells.

MDA-MB-231 (ATCC HTB-26) is an EGF-R-positive breast cancer cell line initiated from anaplastic carcinoma cells of a 51-year-old patient. BT-20 (ATCC HTB-19) is another EGF-R-positive breast cancer cell line isolated from the primary breast tumor of a 74-year-old patient with grade II mammary adenocarcinoma. MDA-MB-231 and BT-20 breast cancer cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum. For subculturing, medium was removed from the flasks containing a confluent layer of cells, and fresh 0.25% trypsin was added for 1–2 min. Trypsin was removed, and cultures were incubated for 5–10 min at 37°C until cells detached. Fresh medium was then added, and cells were aspirated and dispensed into new flasks.

#### Binding of EGF-$^{125}$I-Gen to Breast Cancer Cells.

Ligand binding assays using EGF-$^{125}$I-Gen (2.0 × 10$^6$ cpm/μmol), $^{125}$I-Gen (3.8 × 10$^6$ cpm/μmol), and $^{125}$I-EGF (2.2 × 10$^4$ cpm/μmol; Amersham) were performed using standard procedures, as described previously (18, 22). The cell lines in ligand binding assays included the EGF-R-positive breast cancer cell lines, MDA-MB-231 and BT-20, as well as the EGF-R-negative human leukemia cell lines, NALM-6 (pre-B leukemia) and HL-60 (promyelocytic leukemia).

#### Immunocytochemistry.

Immunocytochemistry was used to: (a) examine the surface expression of EGF-R on breast cancer cells; (b) evaluate the uptake of EGF-Gen by breast cancer cells; and (c) examine the morphological features of EGF-Gen-treated cancer cells. In uptake studies, the culture medium was replaced with fresh medium containing 10 μg/ml EGF or EGF-Gen, and cells were incubated at 37°C for 5, 10, 15, 30, and 60 min, and 24 h. For EGF-R expression studies, cells were plated on poly-l-lysine-coated, glass-bottomed, 35-mm Petri dishes and maintained for 48 h. At the end of the incubation, cells were washed with PBS and fixed in 2% paraformaldehyde. The cells were permeabilized, and nonspecific binding sites were blocked with 2.5% BSA in PBS containing 0.1% Triton X-100 for 30 min. To detect the EGF-R/EGF-Gen complexes, cells were incubated with a mixture of a monoclonal antibody (1:10 dilution in PBS containing BSA and...
Trion X-100) directed to the extracellular domain of the human EGF-R (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA) and a polyclonal rabbit anti-Gen antibody (1:500 dilution) for 1 h at room temperature. After rinsing with PBS, cells were incubated for 1 h with a mixture of a goat anti-mouse IgG antibody conjugated to FITC (Amersham Corp., Arlington Heights, IL) at a dilution of 1:40 in PBS and donkey anti-rabbit IgG conjugated to Texas Red (Amersham Corp.). Cells were washed in PBS and counterstained with toto-3 (Molecular Probes, Inc., Eugene, OR) for 10 min at a dilution of 1:1000. Cells were washed again with PBS, and the coverslips were mounted with Vectashield (Vector Labs, Burlingame, CA) and viewed with a confocal microscope (Bio-Rad MRC 1024) mounted in a Nikon Labophot upright microscope. Digital images were saved on a Jaz disc and processed with Adobe Photoshop software (Adobe Systems, Mountain View, CA).

**In Vitro Treatment of Cells with EGF-Genistein.** To determine the cytotoxic activity of EGF-Gen against breast cancer cells, cells in α-MEM supplemented with 10% (v/v) FCS were treated with various concentrations of EGF-Gen for 24 h at 37°C, washed twice in α-MEM, and then used in either apoptosis assays or clonogenic assays, as described hereafter. Controls included: (a) cells treated with G-CSF-Gen (an irrelevant cytokine-Gen conjugate that does not react with EGF-R); (b) cells treated with unconjugated EGF plus unconjugated Gen; (c) cells treated with unconjugated Gen or unconjugated EGF; and (d) cells treated with PBS, pH 7.4. In some experiments, excess G-CSF or EGF was added to the EGF-Gen-containing treatment medium to show that the cytotoxicity of EGF-Gen can be selectively blocked by excess EGF but not G-CSF.

**Immune-Complex Kinase Assays and Anti-Phosphotyrosine Immunoblotting.** Twenty-four h after treatment with EGF-Gen, cells were stimulated with 20 ng/ml EGF for 5 min and lysed in 1% NP40 buffer, and cell lysates were immunoprecipitated with an anti-EGF-R antibody reactive with the sequence Ala-Asp-Asp of the human EGF-R (Upstate Biotechnology, Inc.). EGF-R immune complexes were examined for tyrosine phosphorylation by Western blot analysis, as described previously (23). All anti-phosphotyrosine Western blots were subjected to densitometric scanning using the automated AMBIS system (Automated Microbiology System, Inc., San Diego, CA), and for each time point, a percentage inhibition value was determined by comparing the density ratios of the tyrosine-phosphorylated EGF-R protein bands with those of the baseline sample and using the formula:

\[
\% \text{ inhibition} = 100 - \frac{(\text{Density of tyrosine phosphorylated EGF-R band})_{\text{test sample}}}{(\text{Density of tyrosine-phosphorylated EGF-R band})_{\text{baseline control sample}}} \times 100
\]

The IC₅₀s were determined using an Inplot program (Graphpad Software, Inc., San Diego, CA). The Src immune complexes were then subjected to immune complex kinase assays, as described (18, 19, 23).

**Apoptosis Assays.** Loose packing of membrane phospholipid head groups and cell shrinkage precede DNA fragmen-
tation in apoptotic cells, thereby providing MC540 binding as an early marker for apoptosis (24). Plasma membrane permeability to PI (Sigma) develops at a later stage of apoptosis (24). MC540 binding and PI permeability were simultaneously measured in breast cancer cells 24 h after exposure to EGF-Gen (either without any cytokine preincubation or before preincubation with excess unconjugated EGF or G-CSF), unconjugated Gen, unconjugated EGF + unconjugated Gen, or G-CSF-Gen, as described (24). Stock solutions of MC540 and PI, each at 1 mg/ml, were passed through a 0.22 μm filter and stored at 4°C in the dark. Shortly before analysis, suspensions containing 1 x 10^6 cells were suspended in 1 ml MCS40 and 10 μl P1 and kept in the dark at 4°C. Whole cells were analyzed with a FACStar Plus flow cytometer (Becton Dickinson, San Jose, CA). All analyses were done using 488-nm excitation from an argon laser. MC540 and PI emissions were split with a 600-nm short pass dichroic mirror; a 575-nm band pass filter was placed in front of one photomultiplier tube to measure MC540 emission, and a 635-nm band pass filter was used for P1 emission. To detect apoptosis-associated DNA fragmentation, cells were harvested 24 h after treatment with EGF-Gen, and DNA was prepared from Triton-X-100 detergent lysates for analysis of fragmentation, as described (24). In brief, cells were lysed in hypotonic 10 mM Tris-Cl (pH 7.4), 1 mM EDTA, 0.2% Triton-X-100, and subsequently centrifuged at 11,000 X g. This protocol allows the recovery of fragmented DNA in the supernatant. To detect apoptosis-associated DNA fragmentation, supernatants were electrophoresed on a 1.2% agarose gel, and the DNA fragments were visualized by UV light after staining with ethidium bromide.

**Clonogenic Assays.** After treatment with EGF-Gen, G-CSF-Gen, unconjugated EGF, unconjugated Gen, or PBS, cells were resuspended in clonogenic medium consisting of α-MEM supplemented with 0.9% methylcellulose, 30% fetal bovine serum, and 50 μM 2-mercaptoethanol. Cells were plated in duplicate Petri dishes at 100,000 cells/ml/dish and cultured in a humidified 5% CO2 incubator for 7 days. Cancer cell colonies were enumerated on a grid using an inverted phase microscope of high optical resolution. Results were expressed as percentage inhibition of clonogenic cells at a particular concentration of the test agent using the formula:

\[
\text{% inhibition} = \frac{1 - \text{mean no. of colonies (test)}}{\text{Mean no. of colonies (control)}} \times 100
\]

Furthermore, the dose-survival curves were constructed using the percentage of control survival [mean no. of colonies (test)/mean no. of colonies (control) X 100] results for each drug concentration as the data points, and the IC_{50} values were calculated. The IC_{50} were determined using an Prism Version II Inplot program (Graphpad Software, Inc.). The mean IC_{50} for EGF-Gen and Gen were compared using Student’s t tests.

**RESULTS**

**Composition of EGF-Gen Conjugate.** EGF-Gen was consistently found to contain, in four independent conjugations, one molecule of Gen per each EGF molecule, as determined by the specific activity of EGF-Gen prepared with 125I-Gen. The electrospray ionization mass spectrum of EGF-Gen also showed a single M, 7287 EGF-Gen species containing one EGF molecule, three SANPAH molecules, and one Gen molecule. Fig. 1A depicts the analytical HPLC chromatogram of purified EGF-Gen, which eluted as a single peak at 18.84 min. The UV spectral scan of this HPLC peak revealed: (α) a peak at a wavelength of 220 nm (due to peptide bonds) and a shoulder at 280 nm (due to aromatic amino acid residues) representing

<table>
<thead>
<tr>
<th>Cell line</th>
<th>−cold EGF (cpm)</th>
<th>+cold EGF (cpm)</th>
<th>Specific binding</th>
<th>Inhibitable binding pmol/10^6 cells</th>
<th>Molecules per cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>4531</td>
<td>1983</td>
<td>2548</td>
<td>56%</td>
<td>7.5</td>
</tr>
<tr>
<td>BT-20</td>
<td>7511</td>
<td>2663</td>
<td>4848</td>
<td>65%</td>
<td>9.5</td>
</tr>
<tr>
<td>NALM-6</td>
<td>2708</td>
<td>3091</td>
<td>0</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>HL-60</td>
<td>788</td>
<td>1346</td>
<td>0</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

**Table 1** Specific binding of EGF-125I-Gen to Breast Cancer Cells

The binding of EGF-Gen, unconjugated Gen, and unconjugated EGF to EGF-R breast cancer cells and EGF-R leukemia cells was examined in ligand binding assays, as described in "Materials and Methods." Each cpm determination was performed in duplicate.

**EGF-125I-Gen binding to breast cancer cells**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>−cold EGF (cpm)</th>
<th>+cold EGF (cpm)</th>
<th>Specific binding</th>
<th>Inhibitable binding pmol/10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>852</td>
<td>860</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>BT-20</td>
<td>2439</td>
<td>2540</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>NALM-6</td>
<td>1098</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>HL-60</td>
<td>814</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

**Table 1** Specific binding of EGF-125I-Gen to Breast Cancer Cells

**125I-Gen binding to breast cancer cells**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>−cold EGF (cpm)</th>
<th>+cold EGF (cpm)</th>
<th>Specific binding</th>
<th>Inhibitable binding pmol/10^6 cells</th>
</tr>
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<tbody>
<tr>
<td>MDA-MB-231</td>
<td>15,102</td>
<td>1,100</td>
<td>14,002</td>
<td>93%</td>
</tr>
<tr>
<td>BT-20</td>
<td>17,351</td>
<td>1,100</td>
<td>14,002</td>
<td>93%</td>
</tr>
<tr>
<td>NALM-6</td>
<td>1098</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>HL-60</td>
<td>814</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND, not done.
Fig. 2 Binding and internalization of EGF-Gen in BT-20 cells. Cells were incubated with EGF-Gen (10 μg/ml) for the indicated times (0 min, A and A'; 5 min, B and B'; 30 min, C and C'; 24 h, D and D'). Cells were then processed for immunocytochemistry using a monoclonal anti-EGF-R antibody and FITC conjugated goat anti-mouse IgG for EGF-R (green fluorescence, left panel). Gen was detected using a polyclonal anti-Gen antibody and Texas Red conjugated anti-rabbit IgG (red fluorescence, right panel), as described in "Materials and Methods." Blue fluorescence, the nuclei stained with toto-3. In A and A', BT-20 cells showed a high level of EGF-R expression; no red fluorescent staining was observed in untreated cells incubated with the anti-Gen antibody. In B and B', after 5 min exposure, EGF-Gen was bound to the cell surface EGF-R (arrowheads), and the internalization of the EGF-R was detected by cytoplasmic green fluorescent staining, whereas the internalization of EGF-Gen molecules was evident from the red fluorescent staining. In C and C', by 30 min, most of the EGF-R/EGF-Gen complexes were internalized and deposited in the perinuclear region (arrows). In D and D', after 24 h exposure, the cells lost their adherent features and showed morphological changes consistent with apoptosis (open arrow).
EGF; (b) a peak at 267 nm representing Gen; and (c) a peak at 480 nm corresponding to the nitrobenzene-substituted structure in the SANPAH moiety (Fig. 1B). The EGF-Gen conjugate was highly stable in mouse, monkey, and human plasma with no detectable decrease in concentration, as examined by quantitative autoradiography of EGF-125I-Gen, as well as quantitative anti-Gen Western blot analysis of nonradioactive EGF-Gen, even after 3 days of continuous incubation at 37°C (data not shown).

Binding of EGF-Genistein to EGF-R-positive Breast Cancer Cells. We examined the in vitro binding of radioiodinated EGF-Gen (EGF-125I-Gen: final concentration, 260 nM = 1700 ng/ml) to EGF-R on these breast cancer cells in the presence and absence of a 100-fold molar excess of nonradioactive EGF using standard ligand binding assays (18, 19, 22). EGF-125I-Gen was able to bind to MDA-MB-231 and BT-20 human breast cancer cells, and this binding was blocked by excess nonradioactive EGF (% EGF-inhibitable binding = 56% for MDA-MB-231 and 65% for BT-20; 4.5 × 10⁶ EGF-Gen molecules/cell for MDA-MB-231 cells and 5.7 × 10⁶ EGF-Gen molecules/cell for BT-20 cells; Table 1), but not by excess nonradioactive GM-CSF, which was used as a control ligand (data not shown). EGF-125I-Gen did not bind to EGF-R-negative HL-60 or NALM-6 leukemia cell lines. EGF-Gen was as effective as unconjugated EGF in blocking the binding of 125I-EGF to breast cancer cells, whereas granulocyte-macrophage-CSF did not block the binding of 125I-EGF (Table 1). Thus, EGF-Gen was able to bind to EGF-R-positive breast cancer cells via its EGF moiety. However, because: (a) 35–44% of the EGF-Gen binding to breast cancer cells was not inhibitable by excess unconjugated EGF; (b) EGF-Gen binding not inhibitable by excess EGF was also observed with EGF-R-negative leukemia cell lines NALM-6 and HL-60; and (c) unconjugated Gen showed binding to all cell lines, which was not inhibitable by EGF, the Gen moiety as well as nonspecific surface adherence may also contribute to the observed binding of EGF-Gen to breast cancer cells.

We next examined the kinetics of uptake and cytotoxicity of unlabeled EGF-Gen in BT-20 (Fig. 2) and MDA-MB-231 (Fig. 3) human breast cancer cells using immunocytochemistry and confocal laser microscopy for tracing the internalized EGF-R and EGF-Gen molecules as well as evaluating the morphological changes in treated cells. EGF-Gen was very similar to unconjugated EGF with respect to its ability to induce internalization of the surface EGF-R molecules. Notably, the intracellular staining patterns for EGF-R and EGF-Gen were very similar.
EGF-R/EGF-Gen complexes were detected in the perinuclear region of the cells. The examination of the morphological features of EGF-Gen-treated (but not EGF-treated) cells after 24 h showed distinct changes consistent with apoptosis including marked shrinkage, nuclear fragmentation, and formation of apoptotic bodies (Fig. 2).

Biological Activity of EGF-Gen. EGF-Gen treatment resulted in decreased tyrosine phosphorylation of the EGF-R in a dose-dependent fashion (Fig. 4A). Whereas EGF-Gen exhibited marked PTK-inhibitory activity in MDA-MB-231 cells at concentrations as low as 0.1 μM in the treatment medium, unconjugated Gen did not significantly affect the EGF-R tyrosine phosphorylation, even at a 10 μM concentration (Fig. 4A). The inhibitory effect of EGF-Gen was blocked by preincubation of cells with excess EGF but not by excess G-CSF, a control cytokine that does not react with EGF-R (Fig. 4B). We next used immune complex kinase assays to assess the effects of EGF-Gen on the enzymatic activities of EGF-R-associated Src PTK in MDA-MB-231 cells. As shown in Fig. 4C, EGF-Gen treatment inhibited the Src kinase. Unlike EGF-Gen, a mixture of unconjugated Gen and EGF or G-CSF-Gen did not inhibit the Src kinase activity in MDA-MB-231 cells. Thus, EGF-Gen is a potent inhibitor of both the EGF-R tyrosine kinase as well as other PTKs that are associated with the EGF-R.

Targeting Gen to vital PTKs in leukemia cells results in apoptotic cell death (18, 19). Furthermore, the examination of the morphological features of EGF-Gen-treated BT-20 and MDA-MB-231 cells by immunocytochemistry suggested that these cells might be undergoing apoptosis. Therefore, we decided to formally study whether EGF-Gen could trigger apoptosis in breast cancer cells. To this end, we first used a quantitative, flow cytometric apoptosis detection assay. MC540 binding and PI permeability of MDA-MB-231 breast cancer cells were simultaneously measured before and after treatment with 1 μg/ml EGF-Gen (0.1 μM), 10 μg/ml EGF (1 μM), or 10 μg/ml G-CSF-Gen. Whereas >90% of MDA-MB-231 or BT-20 cells showed apoptotic changes after EGF plus unconjugated Gen treatment or G-CSF-Gen treatment, a significant portion of cells underwent apoptosis within 24 h after EGF-Gen treatment (95.1% = 57.9% MC540⁺ early stage apoptosis plus 37.2% MC540⁻/PI⁺ advanced stage apoptosis at 24 h; Fig. 5). Excess EGF (10 μg/ml), but not excess G-CSF (10 μg/ml), could prevent EGF-Gen-induced apoptosis. Thus, EGF-Gen causes apoptosis in an EGF-R-specific fashion, and this activity requires both its EGF-R binding growth factor moiety as well as its PTK inhibitory Gen moiety.

As shown in Fig. 6, DNA from Triton-X-100 lysates of EGF-Gen-treated MDA-MB-231 or BT-20 breast cancer cells showed a ladder-like and dose-dependent fragmentation pattern, consistent with apoptosis. The EGF-Gen-induced DNA fragmentation was EGF-R-specific because DNA from cells treated with the control cytokine-Gen conjugate G-CSF-Gen showed no fragmentation. DNA fragmentation was dependent both on the PTK inhibitory function of Gen and the targeting function of EGF because cells treated with unconjugated Gen plus unconjugated EGF did not show apoptotic DNA fragmentation (Fig. 6).

We compared the ability of equimolar concentrations of
Fig. 5  EGF-Gen induces apoptosis in human breast cancer cells. Fluorescence-activated cell sorting correlated two-parameter displays of MDA-MB-231 cells stained with MC540 and PI 24 h after treatment with PBS, 10 μg/ml EGF + 10 μg/ml Gen (37 μM), 1 μg/ml G-CSF-Gen, 1 μg/ml EGF-Gen (0.1 μM), 10 μg/ml EGF + 1 μg/ml EGF-Gen, 10 μg/ml G-CSF + 1 μg/ml EGF-Gen, or 10 μg/ml G-CSF + 1 μg/ml EGF-Gen. The percentages indicate the fraction of cells at an early stage of apoptosis, as measured by single MC540 fluorescence, and the fraction of cells at an advanced stage of apoptosis, as measured by dual MC540/PI fluorescence.
EGF-Gen and unconjugated Gen to induce apoptosis in dose-response studies using the MDA-MB-231 breast cancer cell line. Whereas EGF-Gen caused apoptosis in 98.7% of treated breast cancer cells at concentrations as low as 0.1 μM, Gen was significantly less active and caused apoptosis in only 15.5% of the breast cancer cells, even at a 100 μM concentration (Fig. 7).

We next tested the anticancer activity of EGF-Gen against MDA-MB-231 and BT-20 breast cancer cell lines using in vitro clonogenic assays. The EGF-R-negative leukemia cell line NALM-6 was used as a negative control, and the EGF-R-positive prostate cancer cell line PC-3 was used as a positive control. As shown in Table 2, 24-h treatment with 10 μg/ml EGF-Gen killed >99% of clonogenic MDA-MB-231 and BT-20 cells as well as >99% of PC-3 cells, under conditions that did not affect the clonogenic growth of EGF-R-negative NALM-6 leukemia cells. The lack of toxicity to NALM-6 cells was not caused by a cellular resistance to Gen, because B43-Gen, an anti-CD19 immunoconjugate (18), killed >99% of NALM-6 cells. Unlike EGF-Gen, neither EGF (10 μg/ml; unmodified or Sulfo-SANPAH-modified) nor Gen (10 μg/ml) were able to inhibit the clonogenic growth of EGF-R-positive cancer cell lines. Similarly, G-CSF-Gen (10 μg/ml) did not affect the clonogenic growth of these breast and prostate cancer cell lines (Table 2). To more accurately compare the cytotoxic activities of EGF-Gen and unconjugated Gen, we performed detailed dose-response studies using in vitro clonogenic assays. As shown in Fig. 8, EGF-Gen inhibited in each of three independent experiments the clonogenic growth of MDA-MB-231

![Internucleosomal DNA fragmentation in EGF-Gen-treated breast cancer cells. Cells were harvested 24 h after treatment with PBS (CON, control), EGF-Gen, G-CSF-Gen, or unconjugated EGF + unconjugated Gen, and DNA was prepared for analysis of fragmentation. DNA was then separated by electrophoresis through a 1% agarose gel, and the DNA bands were visualized by UV light after staining with ethidium bromide. Lane M, molecular size markers in bp.](image-url)
Fig. 7 EGF-Gen induces apoptosis in human breast cancer cells. FACS correlated two-parameter displays of MDA-MB-231 cells stained with MC540 and PI 24 h after treatment with PBS, EGF-Gen (0.1, 1.0, and 10 μM), or unconjugated Gen (1.0, 10, and 100 μM). The percentages indicate the fraction of cells at an early stage of apoptosis, as measured by single MC540 fluorescence, and the fraction of cells at an advanced stage of apoptosis, as measured by dual MC540/PI fluorescence. For each treatment, the total apoptotic fraction (TAF, percentage of MC540 single fluorescent cells + percentage of MC540/PI double fluorescent cells) is also provided.

Table 2 Cytotoxic activity of EGF-Gen against clonogenic breast cancer cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>Mean no. of colonies per 1 × 10⁴ cells</th>
<th>% inhibition of clonogenic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231 (breast cancer)</td>
<td>PBS</td>
<td>394 (368,420)</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>EGF, 10 μg/ml</td>
<td>524 (512,536)</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Gen, 10 μg/ml</td>
<td>275 (268,282)</td>
<td>30.2</td>
</tr>
<tr>
<td></td>
<td>EGF-Gen, 10 μg/ml</td>
<td>0 (0.0)</td>
<td>&gt;99.4</td>
</tr>
<tr>
<td></td>
<td>GCSF-Gen, 10 μg/ml</td>
<td>395 (390,400)</td>
<td>0.4</td>
</tr>
<tr>
<td>BT-20 (breast cancer)</td>
<td>PBS</td>
<td>155 (143,167)</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>EGF, 10 μg/ml</td>
<td>161 (152,170)</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Gen, 10 μg/ml</td>
<td>117 (113,121)</td>
<td>24.5</td>
</tr>
<tr>
<td></td>
<td>EGF-Gen, 10 μg/ml</td>
<td>0 (0.0)</td>
<td>&gt;99.4</td>
</tr>
<tr>
<td></td>
<td>GCSF-Gen, 10 μg/ml</td>
<td>154 (150,158)</td>
<td>0.6</td>
</tr>
<tr>
<td>PC-3 (prostate cancer)</td>
<td>PBS</td>
<td>298 (287,309)</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>EGF, 10 μg/ml</td>
<td>355 (307,403)</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Gen, 10 μg/ml</td>
<td>256 (253,259)</td>
<td>14.1</td>
</tr>
<tr>
<td></td>
<td>EGF-Gen, 10 μg/ml</td>
<td>0 (0.0)</td>
<td>&gt;99.7</td>
</tr>
<tr>
<td></td>
<td>GCSF-Gen, 10 μg/ml</td>
<td>309 (298,320)</td>
<td>0.0</td>
</tr>
</tbody>
</table>
| NALM-6 [Pre-B ALL "EGFR(-)"
     cell line] | PBS             | 214 (209,219)                          | 18.2                            |
|                                   | EGF-Gen, 10 μg/ml | 175 (142,188)                      | 18.2                            |
|                                   | B43-Gen, 10 μg/ml | 0 (0.0)                             | >99.5                           |
|                                   | MDA-MB-231      | 395 (390,400)                          | 0.0                             |
|                                   | BT-20           | 154 (150,158)                          | 0.6                             |

as well as BT-20 cells at nanomolar concentrations with mean IC₅₀s of 30 ± 3 nM (range, 21–42 nM) and 30 ± 10 nM (range, 17–64 nM), respectively (~196 ng/ml), whereas unconjugated Gen elicited substantially less inhibitory activity with >1000-fold higher mean IC₅₀s [120 ± 18 μM; range, 99–154 μM] for MDA-MB-231 cells (~32 μg/ml) and 112 ± 17 μM (range, 80–139 μM) for BT-20 cells (~30 μg/ml). The Ps for the Student’s t test comparisons of the IC₅₀s for EGF-Gen versus Gen were <0.001 for both cell lines. The IC₅₀s derived from the composite MDA-MB-31 clonogenic cell survival curves were
In this report, we presented experimental evidence that the EGF-Gen conjugate inactivates the EGF-R tyrosine kinase as well as ErbB2, ErbB3, and Src proto-oncogene family PTKs in breast cancer cells triggering apoptosis and clonogenic cell death. These results indicate that the EGF-R-associated PTK complexes have vital antiapoptotic functions in human breast cancer cells and may, therefore, be used as therapeutic targets.

EGF-Gen is a cytotoxic drug, and its effects on breast cancer cells are irreversible. We favor the hypothesis that the observed ability of EGF-Gen to cause apoptosis in breast cancer cells is at least in part due to inhibition of EGF-R-associated Src family PTKs rather than inhibition of the EGF-R family PTKs because several studies have demonstrated previously that EGF-R tyrosine kinase activity is not essential for the survival of cancer cells (25–29). For example, Fry et al. (25) reported that the PTK inhibitor PD153035 inhibited EGF-R tyrosine kinase with an IC50 of 29 pm but failed to kill EGF-R positive cells. Tanaka et al. (26) reported that BE-23372M, which inhibits the EGF-R with an IC50 of 30 nM, results only in 50% inhibition of target cell growth. RG-13022, a potent inhibitor of EGF-R tyrosine kinase, also elicited only a transient cytostatic effect on breast cancer cells, even at a 10 μM concentration, and its inhibitory effects were completely abolished after its removal from the culture medium (27). Others similarly reported that inhibition of EGF-R tyrosine kinase with specific inhibitors elicits only transient cytostatic but not cytotoxic effects on cancer cells (28, 29). However, a more recent study using CP-358,774, a potent quinazoline derivative PTK inhibitor, indicates that apoptotic cell death can be induced by inhibition of EGF-R tyrosine kinase (30).

In apoptosis assays, 10 μg/ml (37 μM) Gen was not active against MDA-MB-231 and BT-20 breast cancer cells, whereas 1 μg/ml (0.137 μM) EGF-Gen, which contains 270-fold less Gen, was active. In clonogenic assays, the IC50 for EGF-Gen against MDA-MB-231 and BT-20 breast cancer cells were >1000-fold lower than those of unconjugated Gen (30 nM versus 112–120 μM). Thus, the conjugation of Gen to the targeting EGF molecule substantially enhances its cytotoxic activity against human breast cancer cells. This may in part be due to the delivery of more Gen molecules to cancer cells, thereby increasing the intracellular Gen concentration, by this targeted biotherapy approach. We further postulate that the binding of EGF-Gen to the EGF-R brings Gen in direct contact with EGF-R tyrosine kinase as well as Src family PTK associated with the EGF-R. The inhibitor is held in close proximity to the EGF-R and associated PTK because of its covalent attachment to EGF. Localization of the Gen molecule in close proximity to the ATP-binding domains of the EGF-R-associated PTK may increase the effective binding constant by both reducing entropy and providing additional linker binding assists and lead to sustained inhibition of the PTK. Decreasing the effective off-rate of Gen by conjugating it to EGF may also promote covalent modification of the EGF-R-associated PTK, reminiscent of the oxidative inactivation of CD19-associated Src family PTK by B43-Gen, an anti-CD19 antibody-Gen immunonoconjugate (18).

EGF-R overexpression is found in many types of cancer besides breast cancer (31, 32). Thus, EGF-Gen could potentially be useful in several different types of cancer. The EGF-R on cancer cells represents a potential target for other forms of biotherapy as well (27, 33, 34). Whether EGF-Gen will prove
superior to such anti-EGF-R antibodies or EGF-R-directed recombinant toxins (33, 34) should be examined in appropriate preclinical and clinical settings.

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