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I. INTRODUCTION

Human epidermal growth factor (EGF) is a 53 amino acid, single-chain polypeptide (Mr 6216 daltons), which exerts biologic effects by binding to a specific 170 kDa cell membrane epidermal growth factor receptor (EGF-R/ErbB-1). Many types of cancer cells display enhanced EGF-R expression on their cell surface membranes. Enhanced expression of the EGF-R on cancer cells has been associated with excessive proliferation and metastasis. Examples include breast cancer, prostate cancer, lung cancer, head and neck cancer, bladder cancer, melanoma, and brain tumors. In breast cancer, expression of the EGF-R is a significant and independent indicator for recurrence and poor relapse-free survival. 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 protein tyrosine kinase (PTK) activity. Binding of EGF to the 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, Erb-B3), resulting in activation with autophosphorylation of the PTK domain. EGF-R is physically and functionally associated with Src protooncogene family PTK, including $p60^{src}$. 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.

Our recent studies provided evidence that the membrane-associated EGF-receptor (R)-protein tyrosine kinase (PTK) complexes serve as endogenous negative regulators of apoptosis in breast cancer cells. We therefore postulated that the EGF-R, similar to the CD19 receptor on leukemia and lymphoma cells, may be a suitable target for biotherapy using tyrosine kinase inhibitors. Genistein (Gen), an isoflavone (5,7,4'-trihydroxyisoflavone) from fermentation broth of *Pseudomonas spp.*, is a naturally occurring tyrosine kinase inhibitor present in soybeans. We found that targeting Gen to the EGF-R-PTK complexes in breast cancer cells using the EGF-Gen conjugate triggers apoptotic cell death. The purpose of this research project is to further evaluate the clinical potential of this membrane-directed apoptosis

induction strategy by examining the in vivo toxicity profile, pharmacokinetics, and efficacy of EGF-Gen in preclinical animal model systems.

II. BODY

II.1. IN VITRO STUDIES

A. EXPERIMENTAL METHODS

Preparation of the EGF-Gen. rhEGF was produced in E. coli harboring a genetically engineered plasmid that contains a synthetic gene for human EGF fused at the N-terminus to a hexapeptide leader sequence for optimal protein expression and folding. 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 crosslinking agent, Sulfosuccinimidyl 6-[4'azido-2'-nitrophenylamino]hexanoate (Sulfo-SANPAH) (Pierce Chemical Co., Rockford, IL) (18) has been employed 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 dimethyl sulfoxide (DMSO)] and then irradiated 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 differentially hydroxyl-protected Gen resulted in the attachment of Gen via its available C7-hydroxyl 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 12 kDa EGF-EGF homoconjugates with or without conjugated Gen as well as higher molecular weight reaction products were removed by size-exclusion high-performance liquid chromatography (HPLC). Reverse phase HPLC using a Hewlett-Packard (HP) 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 (250x4 mm, Hewlett-Packard, Cat.# 79992402-584). Prior to the HPLC runs, a Beckman DU 7400 spectrophotometer was used to generate a UV spectrum for each of the samples to ascertain the λmax 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% to 100% eluent in a time interval of 0 to 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 (CH3CN), 20% H_2O and 0.1% TFA.

Electrospray ionization mass spectrometry (20, 21) was performed using a PE SCIEX API triple quadruple mass spectrometer (Norwalk, CT) to determine the 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% phosphate buffered saline [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 medium 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 incubated for 5-10 min at 37°C until cells detached. Fresh medium was then added, cells 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 x10^8 cpm/μmol), ^{125}I-Gen (3.8 x10^8 cpm/μmol) and ^{125}I-EGF (2.2 x 10^{12} cpm/μmol; Amersham) were performed using standard procedures, as previously described (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 cells lines, NALM-6 (pre-B leukemia) and HL-60 (promyelocytic leukemia).

Immunocytochemistry. Immunocytochemistry was used to (i) examine the surface expression of EGF-R on breast cancer cells, (ii) evaluate the uptake of EGF-Gen by breast cancer cells and (iii) examine the morphologic 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 37oC for 5 min, 10 min, 15 min, 30 min, 60 min, and 24 hours. For EGF-R expression studies, cells were plated on poly-L-lysine coated glass-bottom 35 mm Petri dishes and maintained for 48 hr. At the end of the incubation, cells were washed with PBS and fixed in 2% paraformaldehyde. The cells were permeabilized and non-specific 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 Triton 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 hr at room temperature. After rinsing with PBS, cells were incubated for 1 hr 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 Labhophot upright microscope. Digital images were saved on a Jaz disk and processed with Adobe Photoshop software (Adobe Systems, Mountain View, CA).

***In Vitro* Treatment of Cells with EGF-Genistein.** In order to determine the cytotoxic activity of EGF-Gen against breast cancer cells, cells in alpha-MEM supplemented with 10%(v/v) fetal calf serum were treated with various concentrations of EGF-Gen for 24 hours at 37°C, washed twice in alpha-MEM, and then used in either apoptosis assays or clonogenic assays, as described hereinafter. Controls included (a) cells treated with G-CSF-Gen (an irrelevant cytokine-Gen conjugate which 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 were 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 hours after treatment with EGF-Gen, cells were stimulated with 20 ng/mL EGF for 5 min, lysed in 1% Nonidet-P-40 buffer, and cell lysates were immunoprecipitated with an anti-EGF-R antibody reactive with the sequence Ala^{351}-Asp^{364} of the human EGF-R (Upstate Biotechnology Inc. [UBI] Catalog # 05-104). EGF-R immune complexes were examined for tyrosine phosphorylation by Western blot analysis, as previously described (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 % inhibition value was determined by comparing the density ratios of the tyrosine phosphorylated EGF-R protein bands to those of the baseline sample and using the formula: % Inhibition = 100- 100x [Density of tyrosine phosphorylated EGF-R band]$_{\text{test sample}}$: [Density of tyrosine phosphorylated EGF-R band]$_{\text{baseline control sample}}$. The IC50 values 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 fragmentation in apoptotic cells, thereby providing MC540 binding as an early marker for apoptosis (24). Plasma membrane permeability to propidium iodide (PI, Sigma) develops at a later stage of apoptosis (24). MC540 binding and PI permeability were simultaneously measured in breast cancer cells 24 hours after exposure to EGF-Gen (either without any cytokine preincubation or following 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 $1x10^6$ cells were suspended in 5 μg/mL MC540 and 10 μg/mL PI 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 and a 575 nm band pass filter was placed in front of one photomultiplier tube to measure MC540 emission amd a 635 nm band pass filter was used for PI emission. To detect the DNA fragmentation in apoptotic cells, cells were harvested 24 hours 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 g. This protocol allows the recovery of fragmented DNA in the supernatant. To detect apoptosis-associated DNA fragmentation, supernatants were electropheresed on a 1.2% agarose gel, and the DNA fragments were visualized by ultraviolet 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 alpha-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 colonie were enumerated on a grid using an inverted phase microscope of high optical resolution. Results were expressed as % inhibition of clonogenic cells at a particular concentration of the test agent using the formula: Inhibition = (1 - Mean # of colonies [Test] / Mean # of colonies [Control]) x 100. Furthermore, the

dosesurvival curves were constructed using the percent control survival (= Mean # of colonies[Test] / Mean # c colonies [Control] x 100) results for each drug concentration as the data points and the IC50 values were calculated The IC50 values were determined using an Prism Version II Inplot program (Graphpad Software, In San Diego, CA). The mean IC50 values for EGF-Gen and Gen were compared using Student's t-tests.

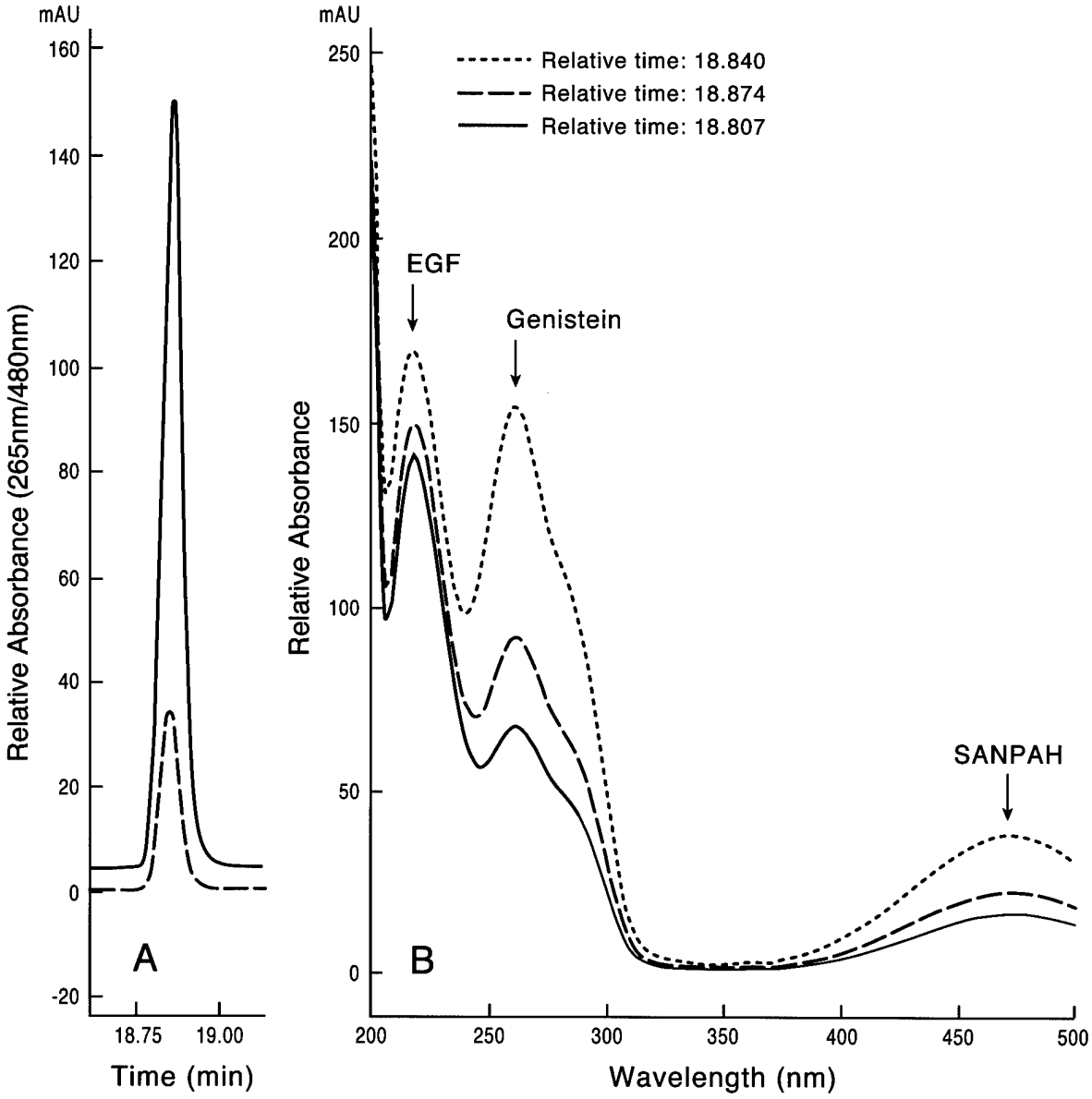
B. RESULTS

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 ^{125}I-Genistein. The electrospray ionization mass spectrum of EGF-Gen also showed a single 7287 kDa EGF-Gen species containing one EGF molecule, three SANPAH molecules, and one Gen molecule. **Figure 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 (i) 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 EGF, (ii) a peak at 267 nm representing Gen, and (iii) a peak at 480 nm corresponding to the nitrobenzene substituted structure in the SANPAH moiety (**Figure 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-^{125}I-Gen, as well as quantitative anti-Gen Western blot analysis of non-radioactive 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-^{125}I-Gen, Final concentration: 260 nM = 1700 ng/ml) to EGF-R on these breast cancer cells in the presence and absence of 100-fold molar excess non-radioactive EGF using standard ligand binding assays (18, 19, 22). EGF-^{125}I-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 x10^6 EGF-Gen molecules/cell for MDA-MB-231 cells and 5.7x10^6 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-^{125}I-Gen did not

FIGURE 1

(A). HPLC Chromatogram of EGF-Gen. A sample of the EGF-Gen conjugate was analyzed on a Spherisorb ODS, 250x4 mm reverse-phase column using a 0.1% TFA-H2O2/0.1% TFA-80% acetonitrile-20% H2O2 gradient as described in the Methods. The retention time of EGF-Gen was 18.84 min. The solid line represents the absorbance at 265 nm and the dotted line reepresents the absorbance at 480 nm. **(B). UV Spectrum of the EGF-Gen.** The EGF-Gen peak obtained from the HPLC run shown in (B) was further analyzed by the diodide array multiple wavelength detector. (see next page)


mAU
160
140
120
100
80
60
40
20
0
-20
Relative Absorbance (265nm/480nm)
18.75
19.00
Time (min)
A
mAU
250
200
150
100
50
0
Relative Absorbance
Relative time: 18.840
Relative time: 18.874
Relative time: 18.807
EGF
Genistein
SANPAH
B
200
250
300
350
400
450
500
Wavelength (nm)

bind to EGF-R negative HL60 or NALM-6 leukemia cell lines. EGF-Gen was as effective as unconjugated EGF in blocking the binding of ^{125}I-EGF to breast cancer cells, whereas GM-CSF did not block the binding of ^{125}I-EGF (**Table 1**). Thus, EGF-Gen was able to bind to EGF-R positive breast cancer cells via its EGF moiety. However, since (1) 35-44% of the EGF-Gen binding to breast cancer cells was not inhibitable by excess unconjugated EGF, (2) EGF-Gen binding not inhibitable by excess EGF was also observed with EGF-R negative leukemia cell lines NALM-6 and HL-60, and (3) unconjugated Gen showed binding to all cell lines, which was not inhibitable by EGF, the Gen moiety as well as non-specific 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 (**Figure 2**) and MDA-MB-231 (**Figure 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 morphologic changes in treated cells. EGF-Gen was very similar to unconjugated EGF with respect to its ability to bind to and induce internalization of EGF-R molecules. Within 5 min after exposure to EGF-Gen, the EGF-R/EGF-Gen complexes begin being internalized, as determined by co-localization of EGF-R (detected by anti-EGF-R antibody, green fluorescence) and EGF-Gen (detected by anti-Gen antibody, red fluorescence) in the cytoplasm of treated cells (**Figure 2 & Figure 3**). By 15-30 min, the EGF-R/EGF-Gen complexes were detected in the perinuclear region of the cells. The examination of the morphologic features of EGF-Gen-treated (but not EGF-treated) cells after 24 hours of exposure showed distinct changes consistent with apoptosis including marked shrinkage, nuclear fragmentation, and formation of apoptotic bodies (**Figure 2**).

Biologic Activity of EGF-Gen. EGF-Gen treatment resulted in decreased tyrosine phosphorylation of the EGF-R in a dose-dependent fashion (**Figure 4A**). Whereas EGF-Gen exhibited marked PTK-inhibitory activity in MDR-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 (**Figure 4A).** The inhibitory effect of EGF-Gen was blocked by preincubation of cells

Table 1. Specific binding of EGF-^{125}I-Gen to Breast Cancer Cells

EGF-^{125}I-Gen Binding to Breast Cancer Cells

Cell line	-cold EGF (cpm)	+cold EGF (cpm)	Specific binding	Inhibitable binding	p mol/10^6 cells	Molecules per cell
MDA-MB-231	4531	1983	2548	56%	7.5	$4.5x10_6$
BT-20	7511	2663	4848	65%	9.5	$5.7x10_6$
NALM-6	2708	3091	0	None	None	None
HL-60	788	1346	0	None	None	None

^{125}I-EGF Binding to Breast Cancer Cells

Cell line	-cold EGF (cpm)	+cold EGF (cpm)	Specific binding (cpm)	Inhibitable binding	+cold EGF-Gen (cpm)	Inhibition by EGF-Gen	+cold GM-CSF (cpm)	Inhibition by GM-CSF
MDA-MB-231	15,102	1,100	14,002	93%	1,398	91%	15,440	0%
BT-20	17,351	ND	ND	ND	1,624	91%	16,486	5%

^{125}I-Gen Binding to Breast Cancer Cells

Cell line	-cold EGF (cpm)	+cold EGF (cpm)	Specific binding	Inhibitable binding	p mol/10^6 cells	Molecules per cell
MDA-MB-231	852	860	0	None	None	None
BT-20	2439	2540	0	None	None	None
NALM-6	1098	N.D.	N.D.	N.D.	N.D.	N.D.
HL-60	814	N.D.	N.D.	N.D.	N.D.	N.D.

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.

FIGURE 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, A'; 5 min: B, B'; 30 min: C, C'; 24 hr: D, 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 represents the nuclei stained with toto-3. A, A': BT-20 cells showed high level EGF-R expression; no red fluorescent staining was observed in untreated cells incubated with the anti-Gen antibody. B, B': Following 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. C, C': By 30 min, most of the EGF-R/EGF-Gen complexes were internalized and deposited in the perinuclear region (arrows). D, D': Following 24 hr exposure, the cells lost their adherent features and showed morphologic changes consistent with apoptosis (open arrow).

(see next page)

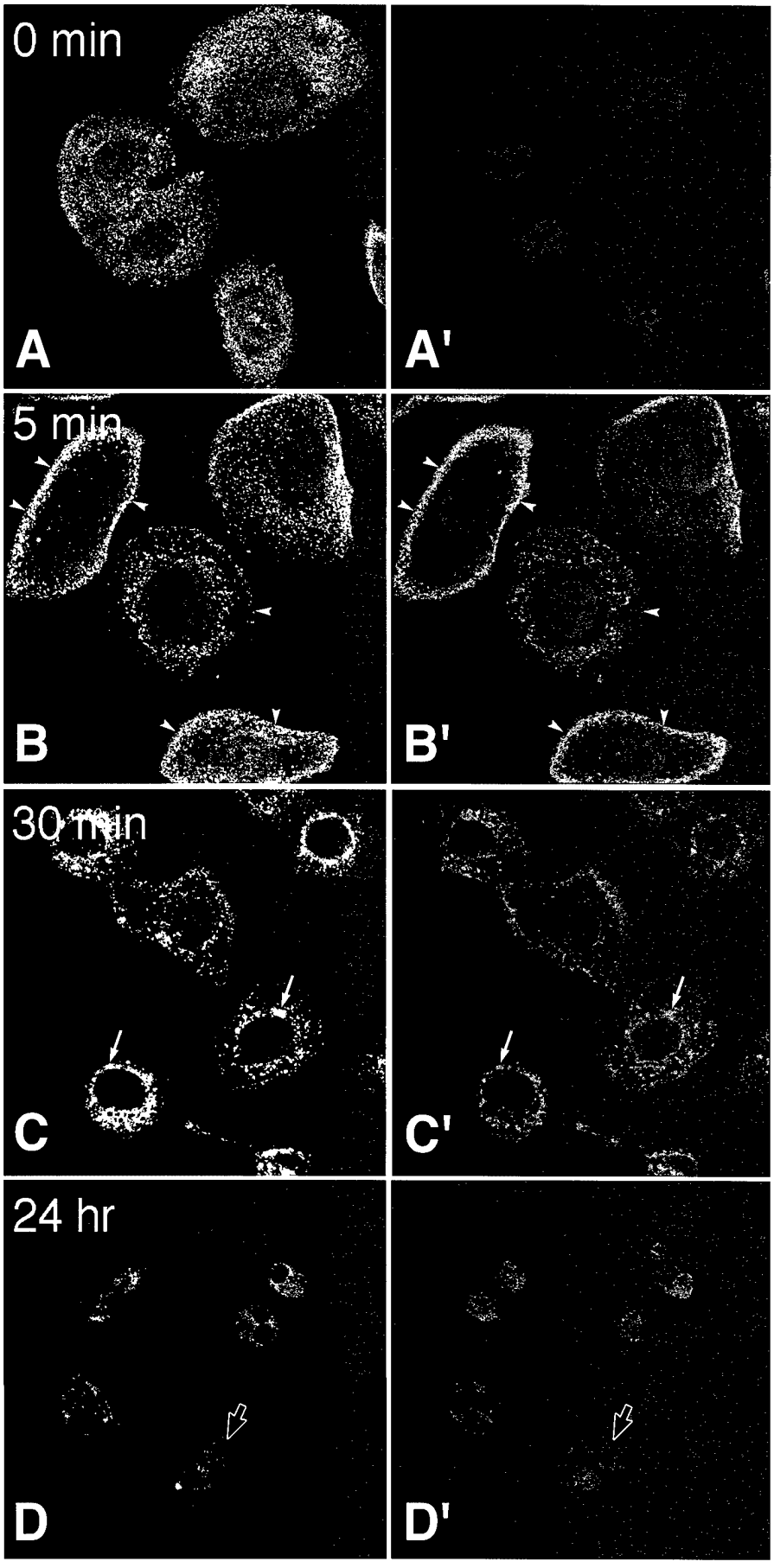
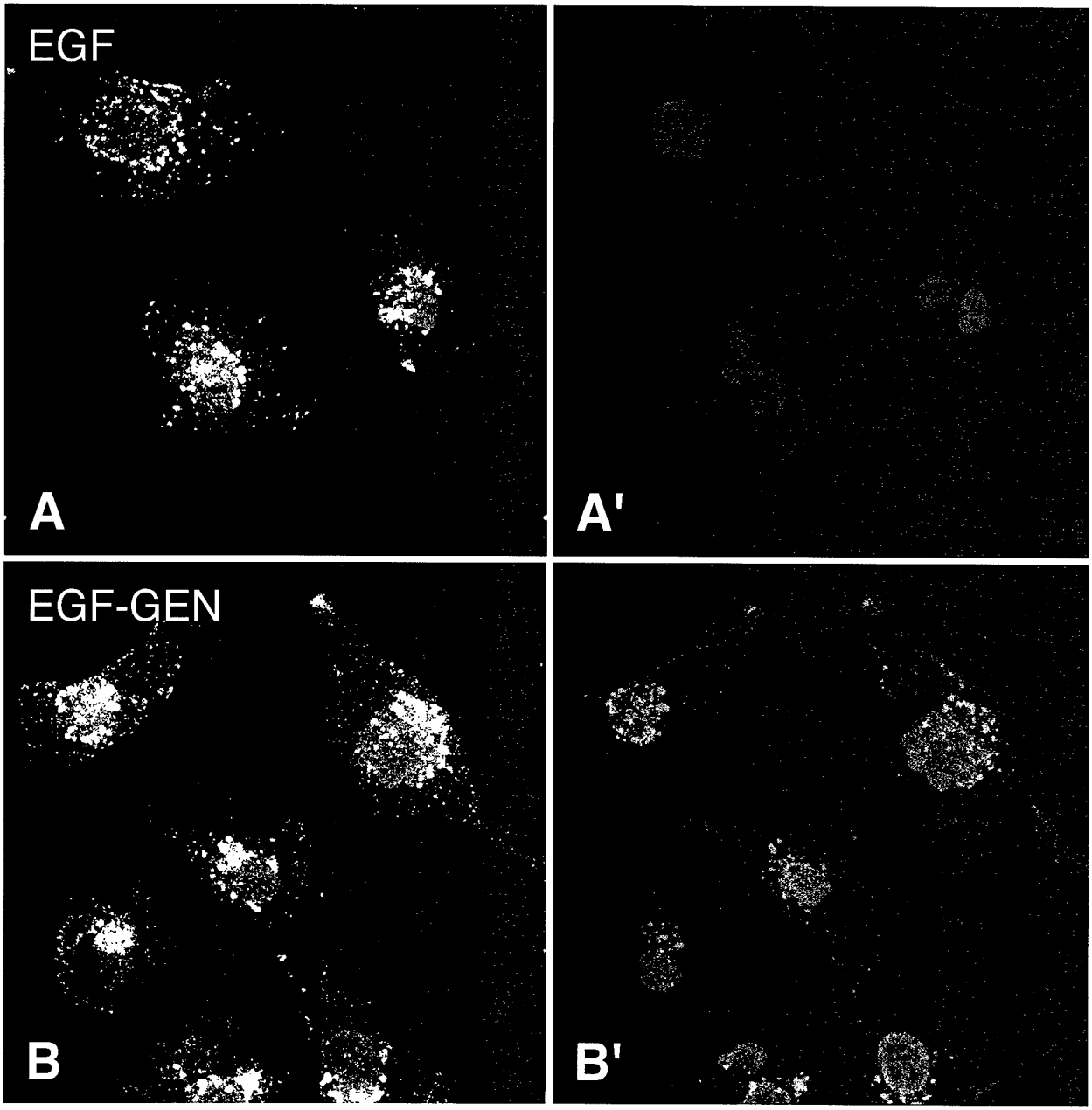

0 min
A
A'
5 min
B
B'
30 min
C
C'
24 hr
D
D'

FIGURE 3

Binding and Internalization of EGF-Gen in MDA-MB-231 Cells. Cells were incubated with either unconjugated EGF (10 μg/ml) (A, A') or EGF-Gen (10 μg/ml) (B, B') for 15 min and processed for the detection of EGF-R (green fluorescence; left panel) and EGF-Gen (red fluorescence; right panel) by immunocytochemistry. EGF-Gen was very similar to 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. (see next page)


EGF
A
A'
EGF-GEN
B
B'

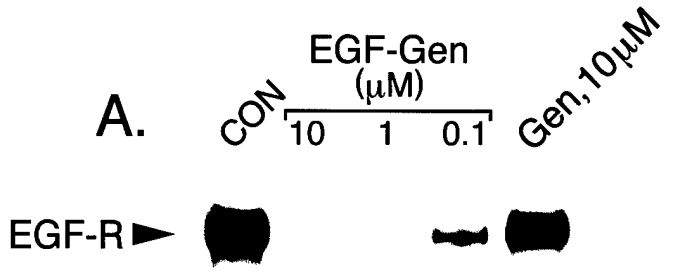
with excess EGF but not by excess G-CSF, a control cytokine which does not react with EGF-R (**Figure 4B**). We next used immune complex kinase assays to assess the effects of EGF-Gen on the enzymatic activities of EGF-R associated Src PTK in MBA-MB-231 cells. As shown in **Figure 4C**, EGF-Gen treatment 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 PTK which are associated with the EGF-R.

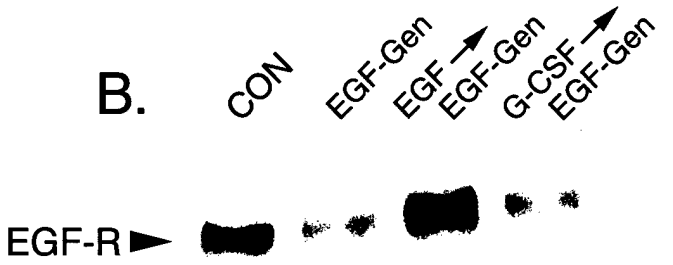
Targeting Gen to vital PTK in leukemia cells results in apoptotic cell death (18, 19). Furthermore, the examination of the morphologic features of EGF-Gen treated BT-20 and MDA-MB-231 cells by immunocytohemistry suggested that these cells might be undergoing apoptosis. Therefore, we decided to formally 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 propidium iodide (PI) permeability of MDA-MB-231 breast cancer cells were simultaneously measured before and after treatment with 1μg/ml EGF-Gen (=0.1 μM), 10μg/ml EGF (1 μM) plus 10μg/ml unconjugated Gen (=37 μM), or 1μg/ml G-CSF-Gen. Whereas less than 10% 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 hours after EGF-Gen treatment (95.1% = 57.9% $MC540^+$ early stage apoptosis plus 37.2% $MC540^+/PI^+$ advanced stage apoptosis at 24 hours) (**Figure 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 **Figure 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

FIGURE 4

Inhibitory Activity of EGF-Gen on EGF-R-associated PTKs of Human Breast Cancer Cells. **[A]** After a 24-hour incubation with EGF-Gen (0.1 μM, 1.0 μM, or 10.0 μM) or unconjugated Gen (10 μM), MDA-MB-231 cells were lysed in 1% Nonidet-P-40 buffer, and cell lysates were immunoprecipitated with an anti-EGF-R antibody reactive with the sequence Ala^{351}-Asp^{364} of the human EGF-R. The EGF-R immune complexes were then subjected to APT immunoblotting. **[B]** After a 24-hour incubation with 10.0 μg/mL (=1.3 μM) EGF-Gen, MDA-MB-231 cells were lysed in 1% Nonidet-P-40 buffer, cell lysates were immunoprecipitated with an anti-EGF-R antibody, and the EGF-R immune complexes were subjected to APT immunoblotting as in [A]. Controls included untreated cells as well as cells pretreated with 10-fold molar excess of unconjugated EGF (=13 μM) or unconjugated G-CSF (13 μM) prior to EGF-Gen incubation. **(C)** Src immune complex kinase assays in the presence of [γ-^{32}P]ATP (50 μCi/μmol) were performed on the lysates of MDA-MB-231 cells treated with 1 μg/mL EGF-Gen (0.1 μM), 1 μg/mL G-CSF-Gen, or 1 μg/mL EGF (0.1 μM) + 1 μg/mL Gen (3.7 μM). Controls included untreated cells. (see next page)


A.
CON
EGF-Gen
(μM)
10
1
0.1
Gen,10μM
EGF-R


B.
CON
EGF-Gen
EGF
EGF-Gen
G-CSF
EGF-Gen
EGF-R

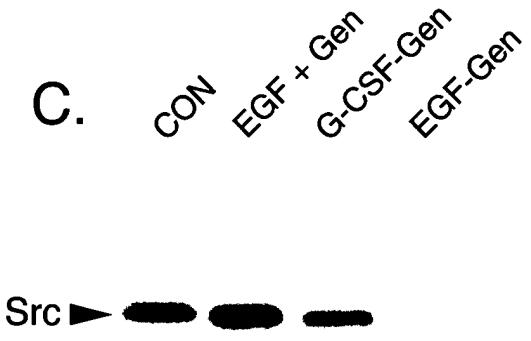
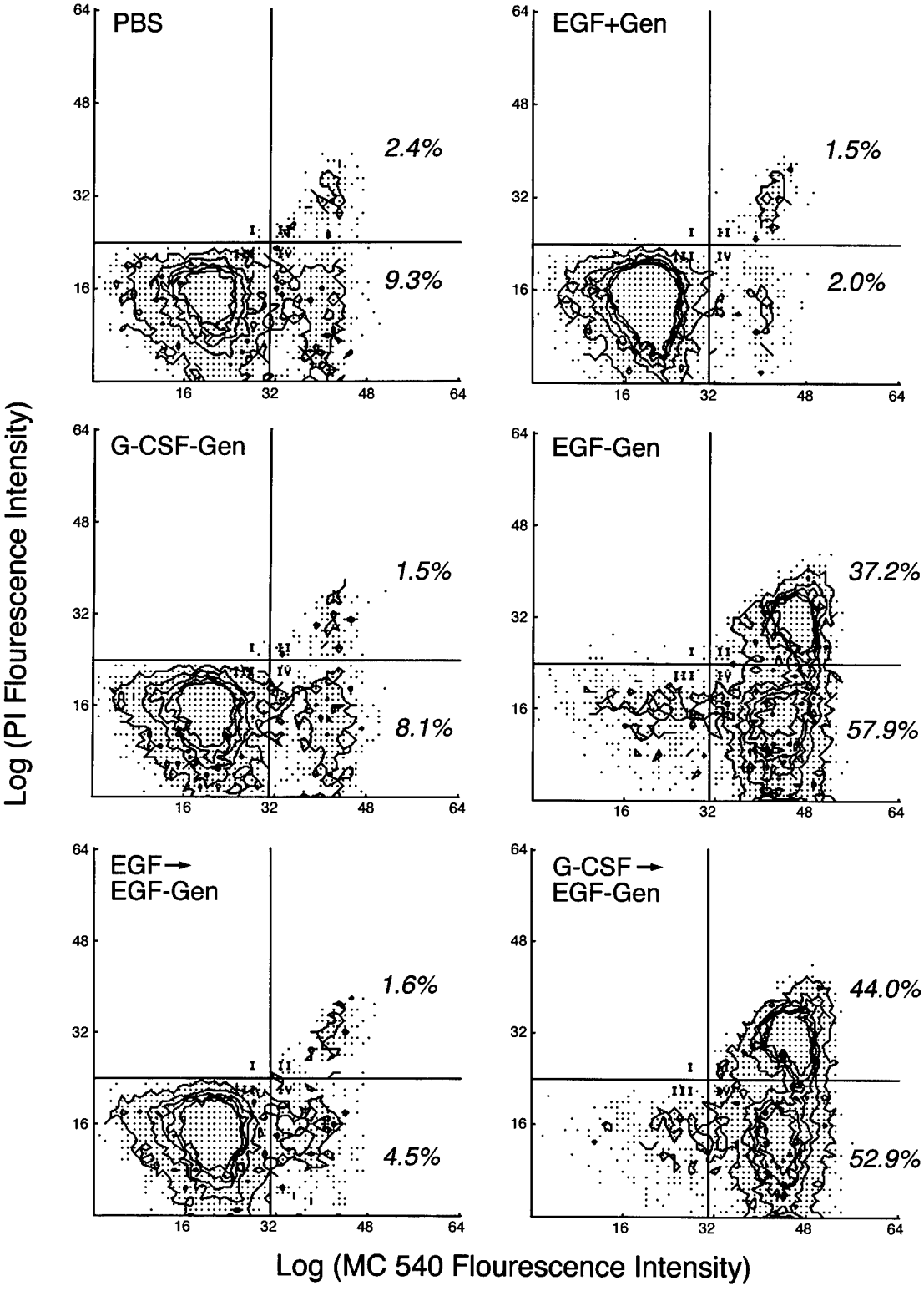

C.
CON
EGF + Gen
G-CSF-Gen
EGF-Gen
Src

FIGURE 5

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 hours 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, 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. (see next page)


PBS
2.4%
9.3%
EGF+Gen
1.5%
2.0%
G-CSF-Gen
1.5%
8.1%
EGF-Gen
37.2%
57.9%
EGF→
EGF-Gen
1.6%
4.5%
G-CSF→
EGF-Gen
44.0%
52.9%
64
48
32
16
Log (PI Flourescence Intensity)
Log (MC 540 Flourescence Intensity)

EGF because cells treated with unconjugated Gen plus unconjugated EGF did not show apoptotic DNA fragmentation (**Figure 6**).

We compared the ability of equimolar concentrations of 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 (**Figure 7).**

We next tested the anti-cancer activity of EGF-Genistein against MDA-B-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 hour 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 which 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 **Figure 8,** EGF-Gen inhibited in each of 3 independent experiments the clonogenic growth of MDA-MB-231 as well as BT-20 cells at nanomolar concentrations with mean IC50 values 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 >1,000 fold higher mean IC50 values (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 P-values for the Student's t-test comparisons of the IC50 values for EGF-Gen vs Gen were <0.001 for both cell lines. The IC50 values derived from the composite

FIGURE. 6

Internucleosomal DNA Fragmentation in EGF-Gen-Treated Breast Cancer Cells. Cells were harvested 24 hours after treatment with PBS (CON), 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 base pairs. (see next page)

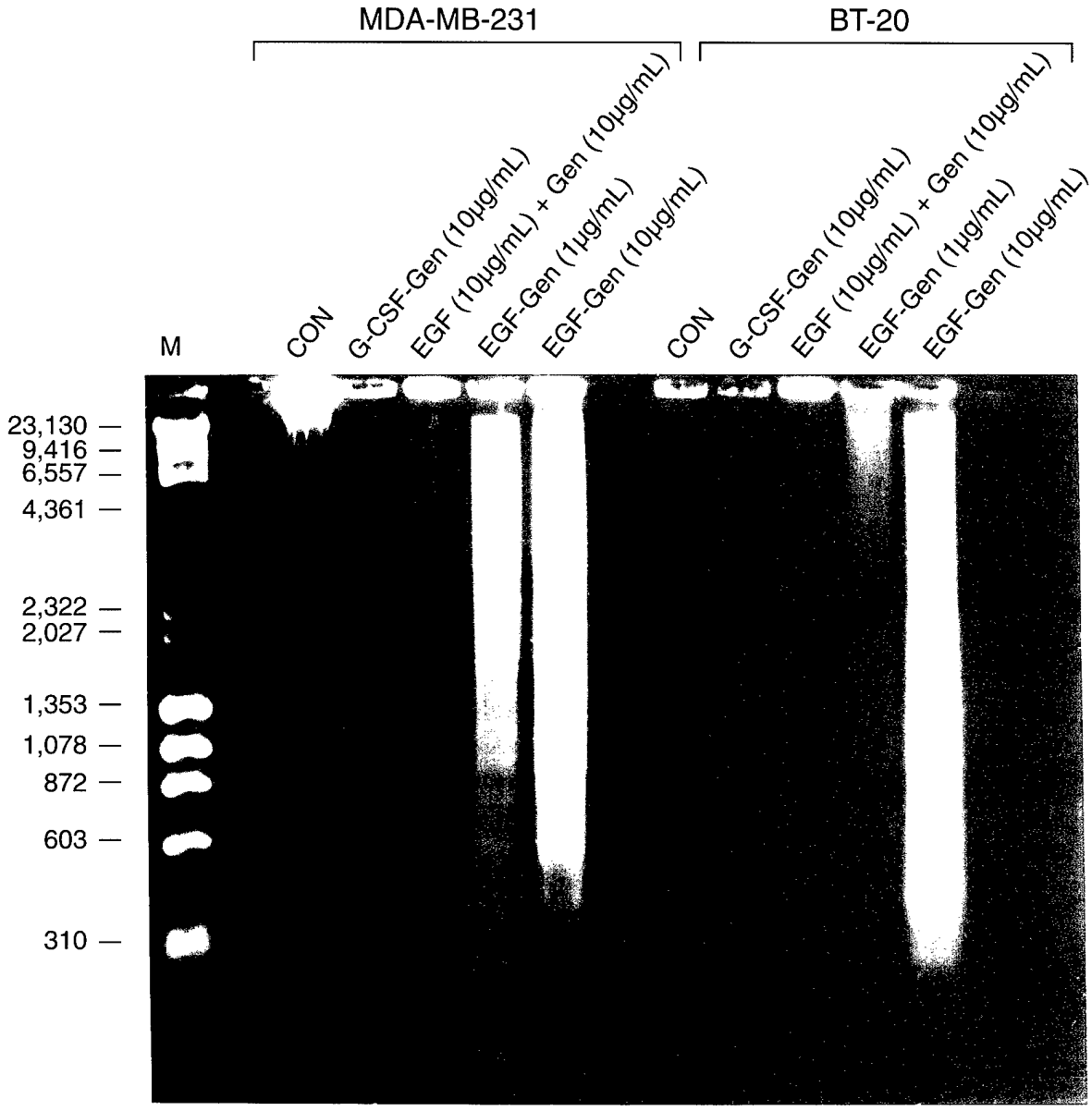

MDA-MB-231
BT-20
M
CON
G-CSF-Gen (10μg/mL)
EGF (10μg/mL) + Gen (10μg/mL)
EGF-Gen (1μg/mL)
EGF-Gen (10μg/mL)
CON
G-CSF-Gen (10μg/mL)
EGF (10μg/mL) + Gen (10μg/mL)
EGF-Gen (1μg/mL)
EGF-Gen (10μg/mL)
23,130
9,416
6,557
4,361
2,322
2,027
1,353
1,078
872
603
310

FIGURE 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 hours after treatment with PBS, EGF-Gen (0.1 μM, 1.0 μM, 10 μM), or unconjugated Gen (1.0 μM, 10 μM, 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) (= % MC540 single fluorescent + % MC540/PI double fluorescent) is also provided. (see next page)

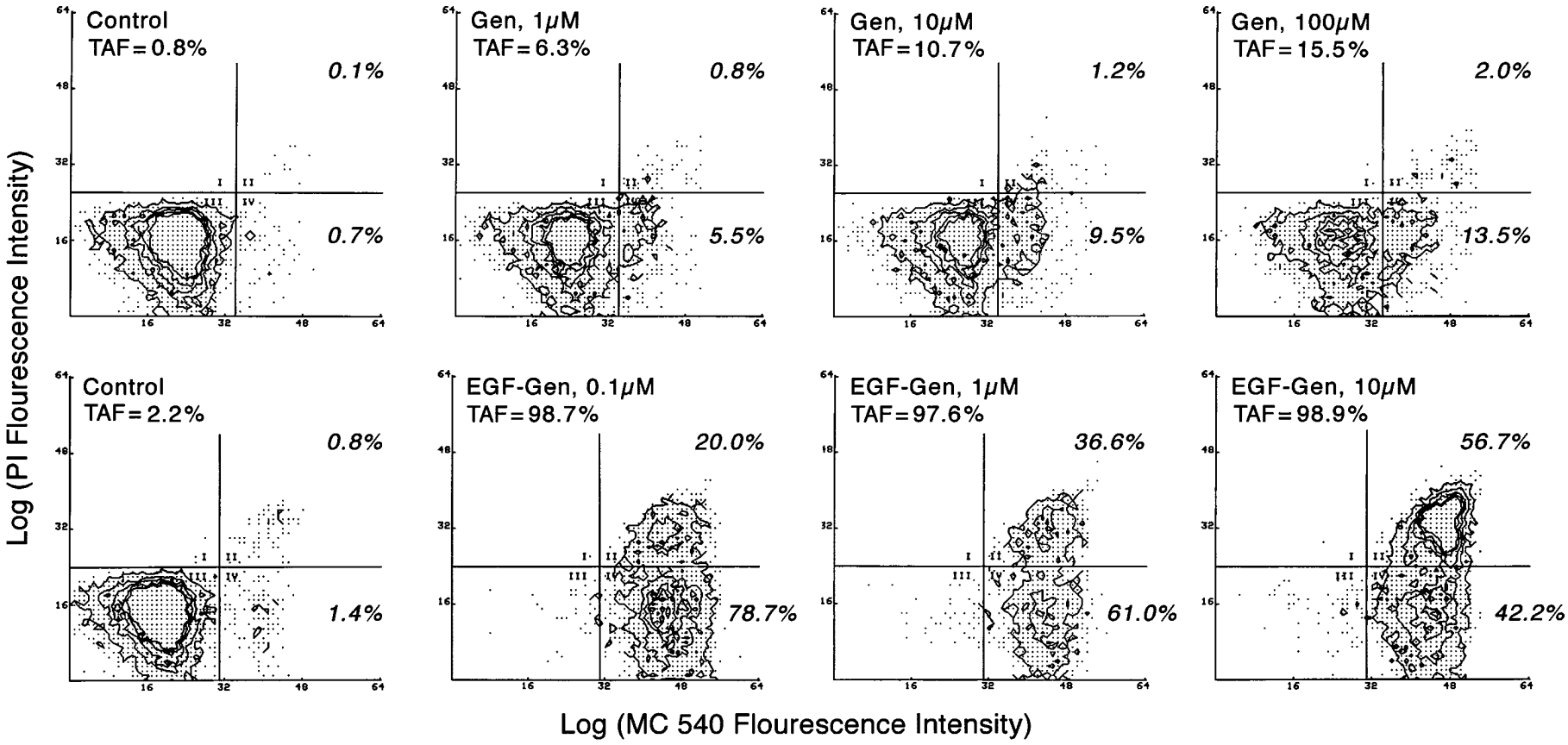

Control
TAF=0.8%
0.1%
0.7%
Gen, 1μM
TAF=6.3%
0.8%
5.5%
Gen, 10μM
TAF=10.7%
1.2%
9.5%
Gen, 100μM
TAF=15.5%
2.0%
13.5%
Control
TAF=2.2%
0.8%
1.4%
EGF-Gen, 0.1μM
TAF=98.7%
20.0%
78.7%
EGF-Gen, 1μM
TAF=97.6%
36.6%
61.0%
EGF-Gen, 10μM
TAF=98.9%
56.7%
42.2%
Log (PI Flourescence Intensity)
Log (MC 540 Flourescence Intensity)

Table 2. Cytotoxic Activity of EGF-Gen Against Clonogenic Breast Cancer Cells

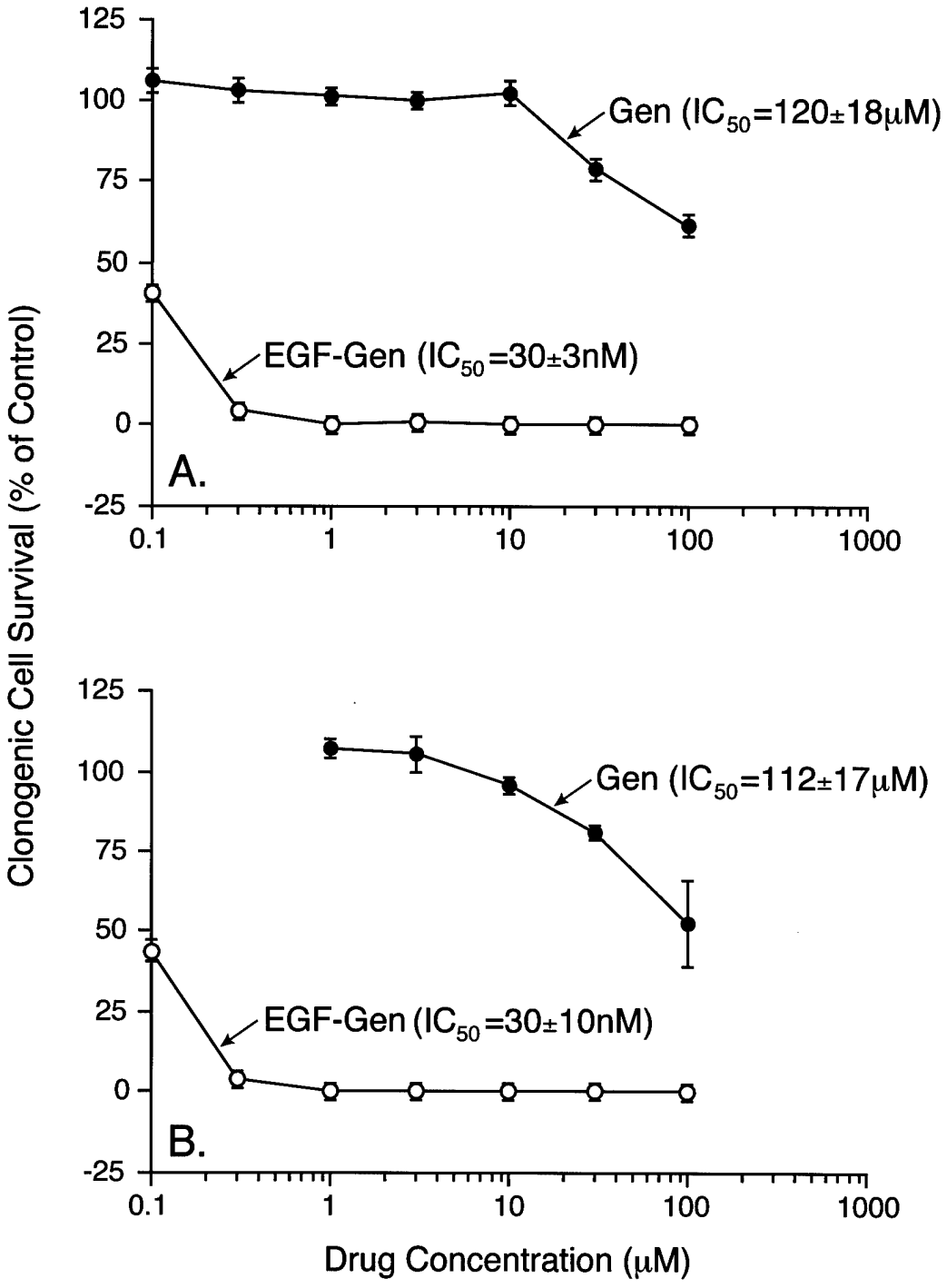
Cell Line	Treatment	Mean No. Colonies Per 1 x 10^5 Cells	Percent Inhibition of Clonogenic Cells
MDA-MB-231	PBS	394 (368,420)	–
Breast Cancer	EGF, 10 µg/mL	524 (512,536)	0.0
	Gen, 10 µg/mL	275 (268,282)	30.2
	EGF-Gen, 10 µg/mL	0 (0,0)	>99.4
	GCSF-Gen, 10 µg/mL	395 (390,400)	0.4
BT-20	PBS	155 (143,167)	–
Breast Cancer	EGF, 10 µg/mL	161 (152,170)	0.0
	Gen, 10 µg/mL	117 (113,121)	24.5
	EGF-Gen, 10 µg/mL	0 (0,0)	>99.4
	GCSF-Gen, 10 µg/mL	154 (150,158)	0.6
PC-3	PBS	298 (287,309)	–
Prostate	EGF, 10 µg/mL	355 (307,403)	0.0
Cancer	Gen, 10 µg/mL	256 (253,259)	14.1
	EGF-Gen, 10 µg/mL	0 (0,0)	>99.7
	GCSF-Gen, 10 µg/mL	309 (298,320)	0.0
NALM-6	PBS	214 (209,219)	–
Pre-B ALL	Gen, 10 µg/mL	175 (162,188)	18.2
$EGFR_c(-)$	EGF-Gen, 10 µg/mL	210 (187,233)	0.0
	B43-Gen, 10 µg/mL	0 (0,0)	>99.5

Cancer cells were treated with the indicated agents for 24 hours at 37°C, washed twice, and then plated in duplicate Petri dishes at 10^5 cells/mL. The clonogenic medium was alpha-MEM supplemented with 0.9% methylcellulose, 30% fetal bovine serum, and 50 µM 2-mercaptoethanol. Colonies were enumerated on day 7 using an inverted phase microscope of high optical resolution.

FIGURE 8

EGF-Gen is more cytotoxic against clonogenic MDA-MB-231 and BT-20 human breast cancer cells than unconjugated Gen. MDA-MB-231 (shown in [A]) and BT-20 (shown in [B] cells were treated with 0.1, 0.3, 1.0, 3.0, 10, 30, or 100 μM EGF-Gen or equimolar concentrations of unconjugated Gen for 24 hours. Subsequently, cells were assayed for clonogenic growth in vitro, as described in Materials and Methods. . The colony numbers for MDA-MB-231 cells ranged from $385/10^5$ cells to 510 colonies/10^5 cells (mean ± SE = 450 ± 36 colonies/10^5 cells). The colony numbers for BT-20 cells ranged from $193/10^5$ cells to 276 colonies/10^5 cells (mean ± SE = 224 ± 26 colonies/10^5 cells). Composite clonogenic cell survival curves were generated using the dose response data from 3 independent experiments, each performed in duplicate. Each data point on the composite survival curve represents the mean % control clonogenic cell survival at a given drug concentration. The error bars for each data point are the standard error to the mean. The individual IC50 values for EGF-Gen from the 3 experiments ranged from 21nM to 42nM for MDA-MB-231 cells (mean ± SE = 30 ± 3 nM), and 17nM to 64 nM for BT-20 cells (mean ± SE = 30 ± 10 nM). The corresponding IC50 values for Gen ranged from 99 μM to 154

μM for MDA-MB-231 cells (mean ± SE = 120 ± 18 μM), and from 80 μM to 139 μM for BT-20 cells (mean ± SE = 112 ± 17 μM). The EGF-Gen IC50 values derived from the composite MDA-MB-31 and BT-20 clonogenic cell survival curves were 39 nM and 20 nM, respectively. By comparison, the IC50 values of the composite clonogenic survival curves for unconjugated Gen were 160 μM and 147, respectively. (see next page)


125
100
75
50
25
0
-25
Gen (IC50=120±18μM)
EGF-Gen (IC50=30±3nM)
A.
0.1
1
10
100
1000
125
100
75
50
25
0
-25
Gen (IC50=112±17μM)
EGF-Gen (IC50=30±10nM)
B.
0.1
1
10
100
1000
Clonogenic Cell Survival (% of Control)
Drug Concentration (μM)

MDA-MB-31 clonogenic cell survival curves were 39 nM for EGF-Gen and 160 μM for unconjugated Gen. The IC50 values derived from the composite BT-20 clonogenic cell survival curves were 20 nM for EGF-Gen and 147 μM for unconjugated Gen (**Figure 8**).

C. SUMMARY

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 protooncogene family protein tyrosine kinases (PTK) 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 with an IC50 value of 2.9 nM, whereas the IC50 value for unconjugated Gen was >100 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 receptor-specific because cells treated with the control granulocyte-colony stimulating factor (G-CSF)-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 IC50 values of EGF-Gen versus unconjugated Gen against MDA-MB-231 and BT-20 cells in clonogenic assays were 30± 3 nM versus 120 ± 18 μM ($P<0.001$) and 30 ± 10 nM versus 112 ± 17 μM ($P<0.001$), respectively. Thus, the EGF-Gen conjugate is a >1,000-fold more potent inhibitor of EGF-R tyrosine kinase activity in intact breast cancer cells than unconjugated Gen and a >1,000-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 anti-apoptotic functions in human breast cancer cells and may therefore be used as therapeutic targets.

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II.2. IN VIVO STUDIES

A. EXPERIMENTAL METHODS

Preparation of the EGF-Genistein Conjugate. EGF-Gen was produced by conjugating recombinant human EGF to genistein (Gen) according to a recently published photochemical conjugation method using the hetero-bifunctional photoreactive crosslinking agent, Sulfosuccinimidyl 6-[4'azido-2'-nitrophenylamino]hexanoate (Sulfo-SANPAH) (Pierce Chemical Co., Rockford, IL), as previously described in detail (18). The chemical composition and *in vitro* biologic activity of EGF-Gen were previously reported (18).

Cross-reactivity of Human EGF and Anti-Human EGF-R Antibodies with Mouse EGF-R. Livers and thymus of BALB/c mice were frozen in liquid nitrogen and 5 μm thick tissue sections were prepared using a cryostat. The sections were fixed in 2% paraformaldehyde (pH 7.4) and processed for standard indirect immunofluorescence using a monoclonal antibody directed to the extracellular domain of the human EGF-R (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA) as the primary antibody and a goat anti-mouse IgG conjugated to FITC (Amersham Corp., Arlington Heights, IL) as the secondary antibody. In parallel, sections were also stained by direct immunofluorescence staining techniques with FITC-conjugated EGF (Molecular Probes, Inc., Eugene, OR) in Hank's Balanced Salt buffer containing BSA, 0.1% sodium azide, and 20 mM HEPES (pH 7.0) according to the manufacturer's recommendations. Coverslips were mounted with Vectashield containing propidium iodide (Vector Labs, Burlingame, CA) to stain the nuclei.

Mouse Toxicity Studies. The toxicity profile of EGF-Gen in BALB/c mice was examined, as previously reported for other biotherapeutic agents (19, 21). All BALB/c mice used in the toxicity studies were obtained from the specific pathogen free (SPF) breeding facilities of the National Institutes of Health (NIH; Bethesda, MD) at 6 - 8 weeks of age. The mice were housed in an American Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved specific pathogen-free mouse facility. All husbandry and experimental contact made with the mice maintained SPF conditions.

The mice were kept in Micro-Isolator cages (Lab Products, Inc., Maywood, NY) containing autoclaved food, water and bedding. Female BALB/c mice were used and monitored daily for lethargy, cleanliness and morbidity. At the time of death, necropsies were performed and the toxic effects of immunoconjugate administration were assessed. For histopathologic studies, tissues were fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin by routine methods. Glass slides with affixed 6 micron tissue sections were prepared and stained with Hemotoxylin and Eosin (H&E).

In single dose toxicity studies, female BALB/c mice were administered an i.p. bolus injection of EGF-Gen in 0.2 ml PBS, or 0.2 ml PBS alone (control mice). In cumulative toxicity studies, mice received a total of 2800 μg (=140 mg/kg) EGF-Gen i.p. over 28 consecutive days. No sedation or anesthesia was used throughout the treatment period. Mice were monitored daily for mortality for determination of the day 30 LD50 values. Mice surviving until the end of the 30 days monitoring were sacrificed and the tissues were immediately collected from randomly selected mice, and preserved in 10% neutral buffered formalin. Standard tissues collected for histologic evaluation included: bone, bone marrow, brain, cecum, heart, kidney, large intestine, liver, lung, lymph node, ovary, pancreas, skeletal muscle, skin, small intestine, spleen, stomach, thymus, thyroid gland, urinary bladder, and uterus (as available).

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. MDA-MB-231 cell line was maintained in RPMI 1640 medium 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 incubated for 5-10 min at 37°C until cells detached. Fresh medium was then added, cells aspirated and dispensed into new flasks.

In some experiments, the cytotoxic activity of plasma samples from EGF-Gen treated cynomolgus monkeys was examined using a methylcellulose colony assay system (18, 22). In brief, MDA-MB-231 cells (10^7/mL in RPMI + 10% FBS) were treated overnight at 37°C with 1: 20 (v/v) PBS-diluted plasma samples from EGF-Gen-treated monkeys. After treatment, cells were washed twice, plated at 10^6

cells/mL in RPMI + 10% FBS + 0.9% methylcellulose in Petri dishes, and cultured for 7 days at 37°C in a humidified 5% CO2 incubator. Subsequently, MDA-MB-231 colonies containing > 20 cells were enumerated using a inverted phase-contrast microscope and the % inhibition of colony formation was calculated using the formula: % Inhibition = 1 - (Mean No. Colonies in Test Culture/Mean No. Colonies in Control Culture)x100. In some experiments, excess unconjugated EGF was added to the plasma samples to block the action of EGF-Gen by competing for the EGF-R on cancer cells. Excess G-CSF was used as a control.

Maintenance of SCID Mouse Colony. The SCID mice were housed in an AAALAC-approved specific pathogen-free facility. Animal housing was located in a secure indoor facility with controlled temperature, humidity, and noise levels. The SCID mice were housed in microisolater cages which were autoclaved with rodent chow. Water was also autoclaved and supplemented with trimethoprim/sulfomethoxazol 3 days/week.

SCID Mouse Xenograft Model of Human Breast Cancer. The left hind legs of the CB. 17 SCID mice were inoculated s.c. with $1x10^6$ MDA-MB-231 breast cancer cells in 0.2 mL PBS.
SCID mice inoculated with human breast cancer cells were treated with EGF-Gen (0.2 μg/dose = 10 μg/kg/dose or 2.0 μg/dose = 100 μg/kg/dose in 0.2 ml PBS) with daily i.p doses for 10 treatment days starting the day after inoculation of cancer cells. Daily treatments with PBS, 10 μg (=500 μg/kg) G-CSF-Gen, 10 μg Gen (=500 μg/kg) combined with 10 μg (500 μg/kg) EGF or 10 μg (500 μg/kg) Gen alone were used as controls. 50 μg (2.5 mg/kg) Adriamycin (Ben Venue Laboratories, Inc., Bedford, OH 44146) or 9.3 μg (= 465 μg/kg) methotrexate (Lederle Parenterals, Inc., Carolina, Puerto Rico 00630) were given as single dose i.p. bolus injections on the day after inoculation of cancer cells. 1 mg (50 mg/kg) cyclophosphamide (Bristol-Myers Squibb Co., Princeton, New Jersey 08543) was injected i.p. on two consecutive days starting the day after inoculation of cancer cells. Mice were monitored daily for health status and tumor growth, and were sacrificed if they became moribund, developed tumors which impeded their ability to attain food or water, or at the end of the 7-month observation period. For histopathologic studies, tissues were fixed in 10% neutral buffered formalin, dehydrated, and embedded

in paraffin by routine methods. Glass slides with affixed 6 micron tissue sections were prepared and stained with hematoxylin/eosin. Primary endpoints of interest were tumor growth and tumor -free survival outcome. Estimation of life table outcome and comparisons of outcome between groups were done, as previously reported (19-21). The efficacy of EGF-Gen against established tumors was examined by treating SCID mice with subcutaneous MDA-MB-231 xenografts of 0.5 cm or 1.0 diameter with 100 μg/kg/day EGF-Gen i.p on 10 consecutive days and determining the tumor diameter daily for 20 days from the start of therapy. Control mice were treated with 0.2 ml PBS for 10 consecutive days.

Cynomolgus Monkey Experiments. Female cynomolgus monkeys were obtained from BioMedical Resources Foundation of Houston, TX. The monkeys were housed in the AAALAC-accredited primate facility. The monkeys were singly housed in stainless steel cages and provided with toys and treats for enrichment. Prior to entering the study, the monkeys were housed in a quarantine-room in the same facility for 6 weeks. During this time, they were TB-tested three times, serologically screened for Herpes virus simiae, and screened for enteric bacterial, protozoal, and helminth pathogens. In pharmacodynamic studies, monkeys were fasted overnight prior to anesthesia and treatment. After induction of anesthesia (Ketamine hydrochloride 10-15 mg/kg), a catheter was placed percutaneously either into the right or left cephalic vein using a sterile disposable kit. This catheter was taped in place for administration of EGF-Gen or maintenance fluids (Normal saline at 4 ml/kg/hr via an infusion pump) and for drawing of blood samples. A Harvard infusion pump was used to administer EGF-Gen as a constant intravenous infusion over a 1 hour period.

Pharmacokinetic Studies. Tissue distribution studies in SCID mice were performed using EGF-^{125}I-Gen and ^{125}I-Gen, as described in detail in previous publications from our laboratory (19). A flow-limited physiological pharmacokinetic model was used to characterize the tissue disposition of EGF-Gen in non-tumor bearing as well as tumor bearing SCID mice (19, 23). Tissue volumes and plasma flow rates were those previously described for mice (24). A set of linear differential equations describing the mass balances of each model compartment was used to estimate tissue partition coefficients (i.e., the ratio of the drug concentration in the tissue of interest to the drug concentration in the plasma at equilibrium)

for each organ. These differential equations were simultaneously solved using the ADAPT II software (25). Biliary excretion and gut reabsortion were incorporated into the physiological model in the form of saturable processes, based on previous studies establishing saturable biliary excretion of unconjugated recombinant human EGF (26). In plasma half-life studies in SCID mice and cynomolgus monkeys, the EGF-Gen conjugate concentrations were measured in the plasma samples using the EGF Quantikine ELISA kit from R&D Systems, a quantitative sandwich enzyme immunoassay, which allows the detection of the EGF-Gen conjugate via its EGF moiety. In these studies, EGF-Gen was administered to SCID mice by intraperitoneal injection at doses of 100 μg/kg and 1 mg/kg. Four mice were used at each dose level, and blood samples were obtained at six non-overlapping time points from each pair of mice. Mice were serially bled by retroorbital puncture at 0 min, 10 min, 30 min, 1 hour, 2 hours, 4 hours, and 12 hours following the administration of the first bolus dose of EGF-Gen. In cynomolgus monkeys, EGF-Gen was administered on each of the 10 treatment days intravenously over 1 hour and plasma samples were obtained at 0 min, 30 min, 45 min, 1 hour, 2 hours, 4 hours, 8 hours, and 12 hours post-infusion time points after the first dose of EGF-Gen. A two compartment first-order pharmacokinetic model was fit to the plasma concentration-versus-time data for EGF-Gen. Maximum likelihood estimation as implemented in ADAPT II software, was used to estimate the central compartment volume of distribution, elimination rate constant, and distribution rate constants for EGF-Gen, as previously described (19-21, 25).

B. RESULTS

Biodistribution and Toxicity of EGF-Gen in Mice. Human EGF binds to murine EGF-R in mouse tissues, as determined by immunocytochemistry (**Figure 9**). Because of the crossreactivity of human EGF and EGF-Gen with murine EGF-R, we decided to use mice in the initial evaluation of the biodistribution and toxicity of EGF-Gen. Tissue distribution studies were performed using EGF-^{125}I-Gen and a flow-limited physiological pharmacokinetic model was used to characterize the *in vivo* tissue disposition of EGF-Gen in non-tumor bearing as well as tumor bearing SCID mice. For each organ, the partition coefficients (i.e., R values = the tissue-to-plasma equilibrium distribution ratios for linear

binding) were determined, as previously described (19). When compared to unconjugated Gen, a much greater amount of EGF-Gen partitioned to bone marrow (R bone marrow: 0.00008 ml/g for Gen vs 0.53 ml/g for EGF-Gen), spleen (R spleen: 0.04 ml/g for Gen vs 5.28 ml/g for EGF-Gen), liver (R liver: 0.50 ml/g for Gen vs 7.40 ml/g for EGF-Gen), kidney (R kidney: 0.25 ml/g for Gen vs 0.93 ml/g for EGF-Gen), and lungs (R lungs: 0.53 ml/g for Gen vs 1.44 ml/g for EGF-Gen) (**Table 3**). In both tumor bearing and non-tumor bearing mice, EGF-Gen most extensively partitioned to the liver with tissue drug concentrations exceeding plasma concentrations more than seven times ($P<0.05$) (**Table 3**). By contrast, very little EGF-Gen partitioned to the subcutaneous xenografts in tumor bearing SCID mice. The partition coefficients for the liver and tumor were 8.2 and 0.2, respectively (**Table 3**).

In toxicity studies, 28 female BALB/c mice were injected intraperitoneally with a single bolus dose of 2 μg (=100 μg/kg) - 800 μg (=40 mg/kg) EGF-Gen in 0.2 ml PBS. EGF-Gen was not toxic to mice at any of these dose levels; none of the mice experienced any side effects or died of toxicity during the 30 day observation period. Even at the highest doses of 400 μg or 800 μg (= 40 mg/kg) EGF-Gen, mice did not become weak or lethargic, lose weight, or develop diarrhea or a scruffy skin. When mice were treated with multiple doses of EGF-Gen at a total dose level of 2.8 mg (=140 mg/kg) according to a 100 μg/mouse/day (= 5 mg/kg/day) x 28 days schedule, no significant toxicity was observed and none of the 10 mice died. No histopathologic lesions were found in any of the organs of EGF-Gen treated mice receiving a single dose or multiple doses of EGF-Gen, including the liver which had the highest partition coefficient in tissue distribution studies. Thus, the maximum tolerated dose (MTD ~ LD10) of EGF-Gen was not reached at the 40 mg/kg single dose level or the 140 mg/kg cumulative dose level.

***In Vivo* Anti-tumor Activity of EGF-Gen in a SCID Mouse Xenograft Model of Human Breast Cancer.** CB.17 SCID mice developed rapidly growing tumors after subcutaneous inoculation of $1x10^6$ MDA-MB-231 cells. We examined the *in vivo* anti-tumor activity of EGF-Gen in this SCID mouse xenograft model of human breast cancer. EGF-Gen significantly improved tumor-free survival in a dose-dependent fashion, when it was administered 24 hours after inoculation of tumor cells. At a dose level of 100 μg/kg/d x 10 days (1 mg/kg total dose), which is >100-fold less than the highest tested and

FIGURE 9

Binding of Anti-Human EGF-R Antibody and Human EGF to EGF-R on Murine Hepatocytes. [A] Expression of EGF-R (green fluorescence) on the hepatocytes as shown by crossreactivity of anti-human EGF-R antibody. [B] Lack of binding of FITC-conjugated human EGF (green fluorescence) to murine thymocytes. [C] Binding of FITC-conjugated human EGF (green fluorescence) to murine hepatocytes. Red fluorescence represents the propidium iodide staining of the nuclei. (see next page)

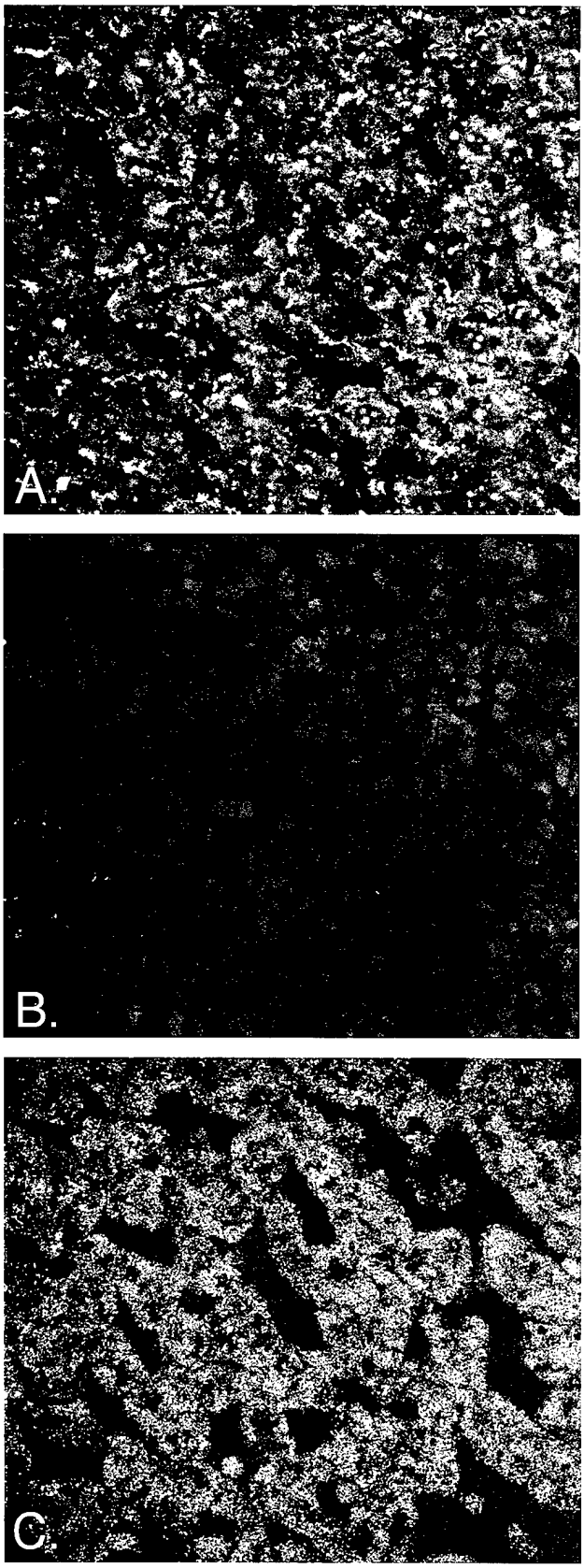

A.
B.
C.

Table 3. Tissue Distribution Parameters for EGF-Genistein and Genistein in SCID Mice.

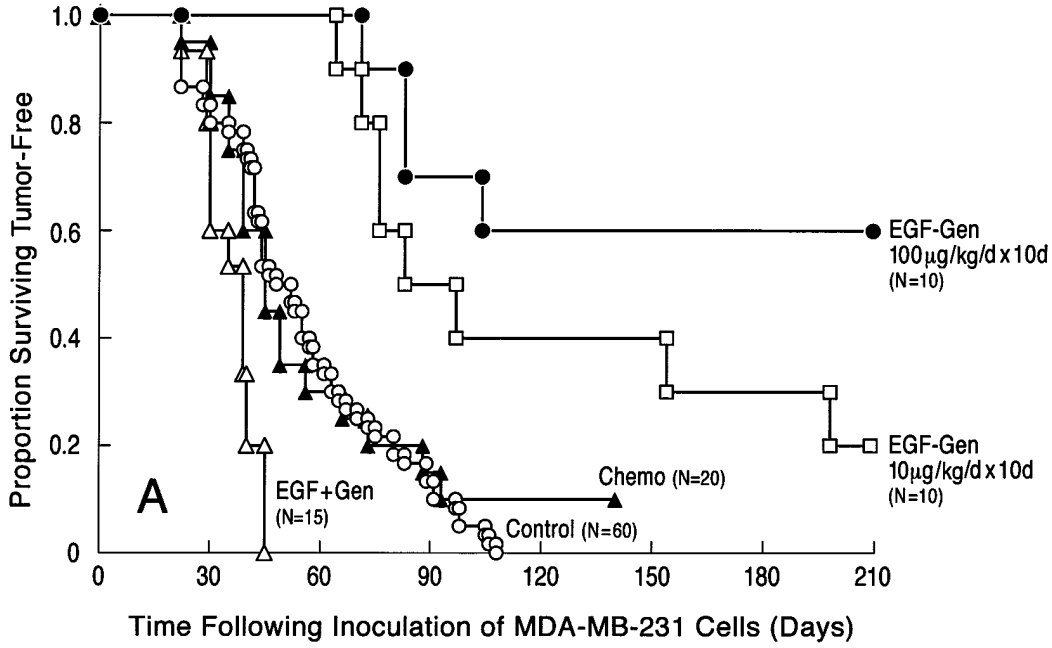
Tissue	EGF-Gen		Gen
	No Tumor	Tumor	No Tumor
	Linear binding constant (R)		
Brain	0.05	0.05	0.03
Heart	0.38	0.35	0.34
Skin	0.20	0.15	0.21
Muscle	0.11	0.09	0.09
Bone marrow	0.53	0.42	0.00008
Stomach	1.00	1.13	N.D.
Spleen	5.28	3.87	0.04
Lungs	1.44	0.84	0.53
Kidney	0.93	0.86	0.25
Liver	7.40	8.15	0.50
Intestine	0.86	1.08	N.D.
Tumor	N.A.	0.16	N.A.

A flow-limited physiological pharmacokinetic model was used to characterize the tissue disposition of both drugs in mice. Volume terms and flow rates were those previously described for mice (*23*). A set of differential equations describing the mass balances of each model compartment was used to estimate linear binding constants for each organ. These differential equations were simultaneously solved with the use of ADAPT II software (*25*). R, tissue-to-plasma equilibrium distribution ratio for linear binding in ml/g. N.A., not applicable.

nontoxic cumulative dose (i.e., 140 mg/kg) in mice, EGF-Gen was more effective than cyclophosphamide (50 mg/kg/d x 2 days), adriamycin (2.5 mg/kg x 1) or methotrexate (0.5 mg/kg x 1), the most widely used standard chemotherapeutic drugs for breast cancer. **Figure 10** shows the tumor-free survival outcome of SCID mice treated with EGF-Gen, chemotherapeutic drugs (CHEMO = cyclophosphamide, adriamycin, or methotrexate), or control agents after inoculation with MDA-MB-231 breast cancer cells. None of the 60 control mice (CON) treated with PBS (N=40), G-CSF-Gen (100 μg/kg/day) (N=10), or unconjugated Gen (100 μg/kg/day) (N=10) remained alive tumor-free beyond 108 days (median tumor-free survival = 52 days) (**Figure 10A**). All of the 10 mice treated with EGF plus Gen developed tumors within 45 days with a median tumor-free survival of only 39 days. Tumors reached a size of 0.5 cm^3 by 84±3 days in PBS, G-CSF-Gen, or Gen treated CON mice, 62± 1 days in EGF plus Gen treated mice, and 77 ± 16 days in the CHEMO group (**Figure 10B**). Within the CHEMO group, all of the adriamycin-treated mice developed rapidly growing tumors with a median tumor-free survival time of only 42 days. Tumors reached a size of 0.5 cm^3 by 92 ± 6 days in cyclophosphamide-treated mice, 72 ± 5 days in adriamycin treated mice, and 71 ± 5 days in methotrexate-treated mice. By comparison, 40 ± 16% of mice treated for 10 consecutive days with 10 μg/kg/day EGF-Gen survived tumor-free beyond 3 months and 20 ± 13% were still alive tumor-free at 7 months (median tumor-free survival time = 97 days; CON vs 10 μg/kg/day EGF-Gen, $P<0.0001$ by log-rank test). Remarkably, 60 ± 16% of mice treated for 10 consecutive days with 100 μg/kg/day EGF-Gen remained alive free of detectable tumors for more than 7 months (CON vs 100 μg/kgday EGF-Gen, $P<0.00001$ by log-rank test) (**Figure 10**). Tumors developing in EGF-Gen-treated mice reached the 0.5 cm^3 tumor size much later than control mice (114 ± 8 days [10 μg/kg/day dose level] and 129 ± 14 days [100 μg/kg/day dose level] vs 84± 3 days, P=0.007 (10 μg/kg/day dose level) and $P<0.001$ (100 μg/kg/day dose level)). The average size (mean ± SE) of tumors at 90 days and 120 days were 0.257±0.059 cm^3 and 0.822±0.146 cm^3, respectively for mice in the CON group. By comparison, the average size (mean ± SE) of tumors at 90 days and 120 days were significantly smaller at 0.013± 0.007 cm^3 (P=0.009) and 0.166 ± 0.083 cm^3 (P=0.006) for mice treated with EGF-Gen at 100 μg/kg/day dose level. Thus, EGF-Gen elicited significant *in vivo* anti-tumor activity at non-toxic doses. The inability of 10 μg (=500 μg/kg)/day x 10 days of unconjugated Gen (= 37,000 pmols) in combination with unconjugated EGF to confer tumor-free

FIGURE 10

Antitumor Activity of EGF-Gen Against Human Breast Cancer in SCID Mice. Tumor free survival curves (shown in A) and life-table analysis of tumor-free survival outcome (shown in B) of SCID mice challenged with $1x10^6$ MDA-MB-231 cells. Twenty-four hours after s.c. inoculation of cancer cells, mice received EGF-Gen (10 μg/kg/day x 10 days, N=10, or 100 μg/kg/day x 10 days, N=10), EGF (500 μg/kg/day x 10 days) + Gen (500 μg/kg/day x 10 days) (N=15), or chemotherapy (N=20) (i.e., cyclophosphamide , 50 mg/kg/day x 2 days; adriamycin, 2.5 mg/kg single bolus dose; or methotrexate, 0.5 mg/kg single bolus dose), as described in Materials and Methods. Controls (N=60) were treated with PBS, G-CSF-Gen (500 μg/kg/day x 10 days), or Gen (500 μg/kg/day x 10 days). [1] The P-values for tumor-free survival comparisons were determined using the log-rank test, whereas the P-values for the average time to 0.5 cm^3 tumor size were determined using student t-tests. (see next page)



B. Life Table Analysis

Treatment Group	# of Mice	Proportion Surviving Tumor-Free (%)				Median Tumor-Free Survival (days)	P-value (log rank)		Time to 0.5cm³ tumor size	P-value
		30 days	60 days	90 days	180 days		vs PBS	vs EGF-Gen 2 μg/day		vs PBS
CON	60	83±5	35±6	13±4	0±0	52	—	<0.00001	84±3	—
EGF+Gen	15	80±10	0±0	0±0	0±0	39	0.003[1]	<0.0001	62±1	0.02[1]
EGF-Gen, 10μg/kg/day	10	100±0	100±0	50±16	30±15	97	<0.00001	0.2	114±8	0.007
EGF-Gen, 100μg/kg/day	10	100±0	100±0	70±15	60±16	>210	<0.00001	—	129±14	<0.001
CHEMO	20	95±5	30±10	15±8	N.D.	48	NS	<0.0001	77±16	NS

survival in this SCID mouse model in contrast to the potency of 2 μg (=100 μg/kg)/day x 10 days EGF-Gen containing 309 pmols of Gen demonstrates that (a) the in vivo anti-tumor activity of EGF-Gen cannot be attributed to its EGF moiety alone and (b) conjugation to EGF enhances the anti-tumor activity of Gen against breast cancer cells by >100-fold.

In contrast to EGF-Gen, cyclophosphamide (50 mg/kg/day x 2 days; N=5), adriamycin (2.5 mg/kg, N=10), or methotrexate (0.5 mg/kg, N=5) did not significantly affect tumor development in this SCID mouse model. Of the 20 mice treated with one of these chemotherapeutic drugs, only 10±7 % remained tumor-free beyond 3 months, which indicates no improvement over the control group and a worse tumor-free survival outcome compared to the 2.0 μg/day EGF-Gen group (median tumor-free survival = 48 days; CON vs CHEMO, P= 0.32; EGF-Gen, 2.0 μg/day vs CHEMO, P<0.05) (**Figure 10B**)

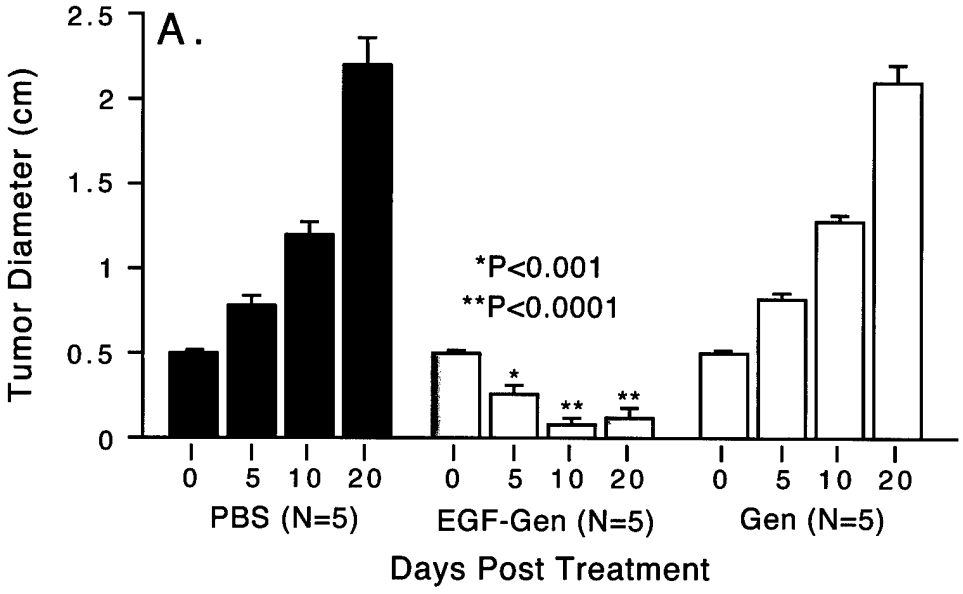
Furthermore, treating SCID mice with established subcutaneous human breast cancer xenografts of 0.5 cm diameter with EGF-Gen at this dose level resulted in eradication of the tumors in 2 of 5 mice and >50% shrinkage in 3 of 5 mice within 10 days. The day 10 diameters of tumors in the EGF-Gen treated group were 0.1 cm, 0.0 cm, 0.0 cm, 0.2 cm, and 0.1 cm with a mean (± SE) diameter of 0.08 ± 0.04 cm (**Figure 11A**). There was no significant tumor progression between days 10 and 20 in this group of mice. The day 20 tumor diameters were 0.3 cm, 0.0 cm, 0.0 cm, 0.1 cm, and 0.2 cm with a mean (±SE) diameter of 0.12 ± 0.06 cm. In contrast to the tumors in EGF-Gen-treated mice, all of the control tumors in 5 PBS treated mice as well as 5 mice treated with unconjugated Gen (1 mg/kg/day x 10 days) showed >200% increase in diameter within 10 days: The day 10 tumor diameters ranged from 1.0 to 1.4 cm (mean ±SE = 1.24 ± 0.08 cm) in PBS-treated mice and from 1.2 to 1.4 cm (mean ±SE= 1.28 ±0.04 cm) in Gen-treated mice (P values <0.0001 for EGF-Gen vs PBS as well as EGF-Gen vs Gen). These tumors continued their rapidly progressive growth and the day 20 dimaters ranged from 1.8 to 2.6 cm (mean ±SE= 2.20±0.16 cm) in PBS-treated mice and from 1.9 to 2.4 (mean ±SE = 2.12 ± 0.10 cm) in Gen-treated mice (**Figure 11A**).

EGF-Gen treatment significantly reduced the growth rate of breast cancer xenografts of 1.0 cm diameter during the 20-day observation period but unlike with tumors of 0.5 cm diameter, it failed to cause shrinkage or disappearance of these larger tumors (**Figure 11B**). The day 10 tumor diameters ranged from 2.4 to 3.3 cm (mean ± SE = 2.90 ± 0.21 cm) in PBS-treated mice and from 2.3 to 3.5 cm (mean ± SE = 2.90 ± 0.25 cm) in Gen-treated mice. By comparison, the tumor diameters in EGF-Gen-treated mice ranged from 1.3 to 2.3 cm (mean ± SE = 1.70 ± 0.23 cm) (P valuues: EGF-Gen vs PBS = 0.008; EGF-Gen vs Gen = 0.009) (**Figure 11B**).

***In Vivo* Pharmacokinetic Features of EGF-Gen in SCID mice.** We sought to determine the therapeutic systemic exposure levels of EGF-Gen by examining its pharmacokinetics when administered at dose levels which were effective in the SCID mouse xenograft model of human breast cancer. Thus, SCID mice were treated with daily intraperitoneal (i.p) bolus injections of 10 μg/kg or 100 μg/kg EGF-Gen for 10 consecutive days. The differences between various pharmacokinetic parameters of healthy mice versus mice with breast cancer xenografts were calculated based on 95% confidence intervals provided by ADAPT II software. EGF-Gen was cleared rapidly from blood with an elimination half-life of 1.3 (± 0.2)-1.6 (± 0.4) hours (**Table 4, Figure 12A**). At the lower dose level, EGF-Gen was cleared more rapidly from blood [13.1 (± 2.4) vs 6.4 (± 0.6) ml/hr/g], had a larger central volume of distribution [30.0 (± 3.6) vs 11.6 (± 1.3) ml/g], had a lower measured maximum plasma concentration (0.33 vs 0.45 ng/ml), and yielded a lower systemic exposure level (i.e., area under the concentration-time curve) [7.6 (± 1.4) vs 16.0 (± 1.5) μgxhr/L] in SCID mice with human breast cancer xenografts than in healthy SCID mice that were not inoculated with breast cancer cells ($P<0.05$) (**Table 4, Figure 12A**). The differences between these parameters were calculated based on 95% confidence intervals provided by ADAPT II software. These results suggested that EGF-Gen likely binds to EGF-R^+ human breast cancer cells infiltrating SCID mouse tissues, resulting in more rapid removal from plasma in mice with metastatic human breast cancer. The systemic exposure level, as measured by the area under the serum concentration-time curve (AUC), achieved by the therapeutically effective 10 μg/kg/day dose level of EGF-Gen was 16.0 (± 1.5) μg*hr/L in non-tumor bearing healthy SCID mice and 7.6 (± 1.4) μg*hr/L in SCID mice bearing 1 cm^3 MDA-MB-231 tumors (**Table 4**). By comparison, treatment with 100

FIGURE 11

***In vivo* activity of EGF-Gen against established tumors.** The efficacy of EGF-Gen against established tumors was examined by treating SCID mice with subcutaneous MDA-MB-231 human breast cancer xenografts of 0.5 cm (shown in [A]) or 1.0 diameter (shown in [B]) with 100 μg/kg/day EGF-Gen i.p for 10 consecutive days and determining the tumor diameter daily for 20 days from the start of therapy. Control mice were treated with 0.2 ml PBS for 10 consecutive days or unconjugated Gen at 500 μg/kg/day x 10 days. P-values were determined using student t-tests. (see next page)


A.
Tumor Diameter (cm)
2.5
2
1.5
1
0.5
0
*P<0.001
**P<0.0001
*
**
**
0 5 10 20
0 5 10 20
0 5 10 20
PBS (N=5)
EGF-Gen (N=5)
Gen (N=5)
Days Post Treatment

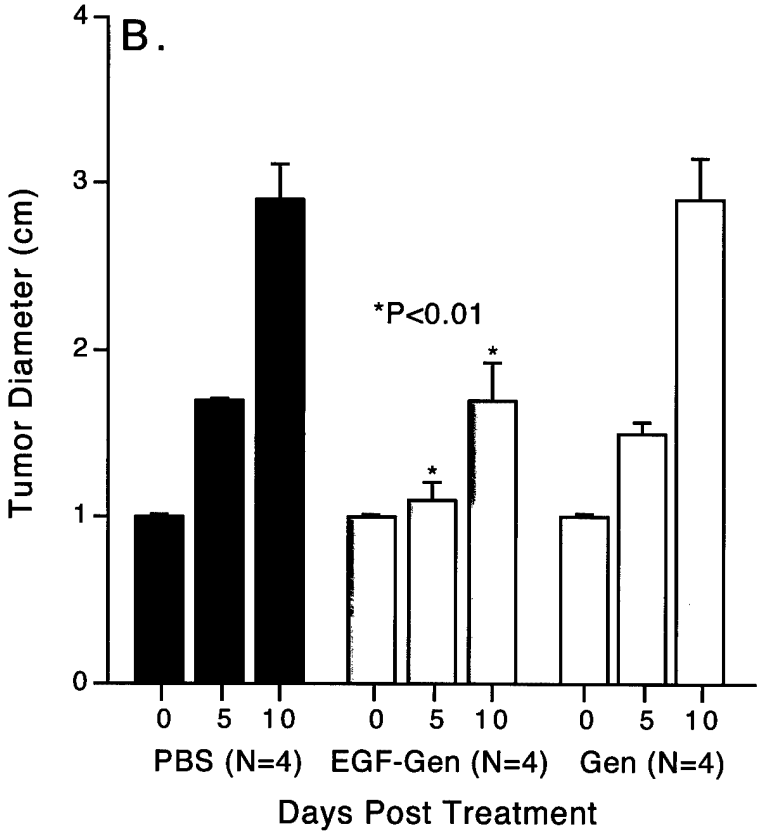
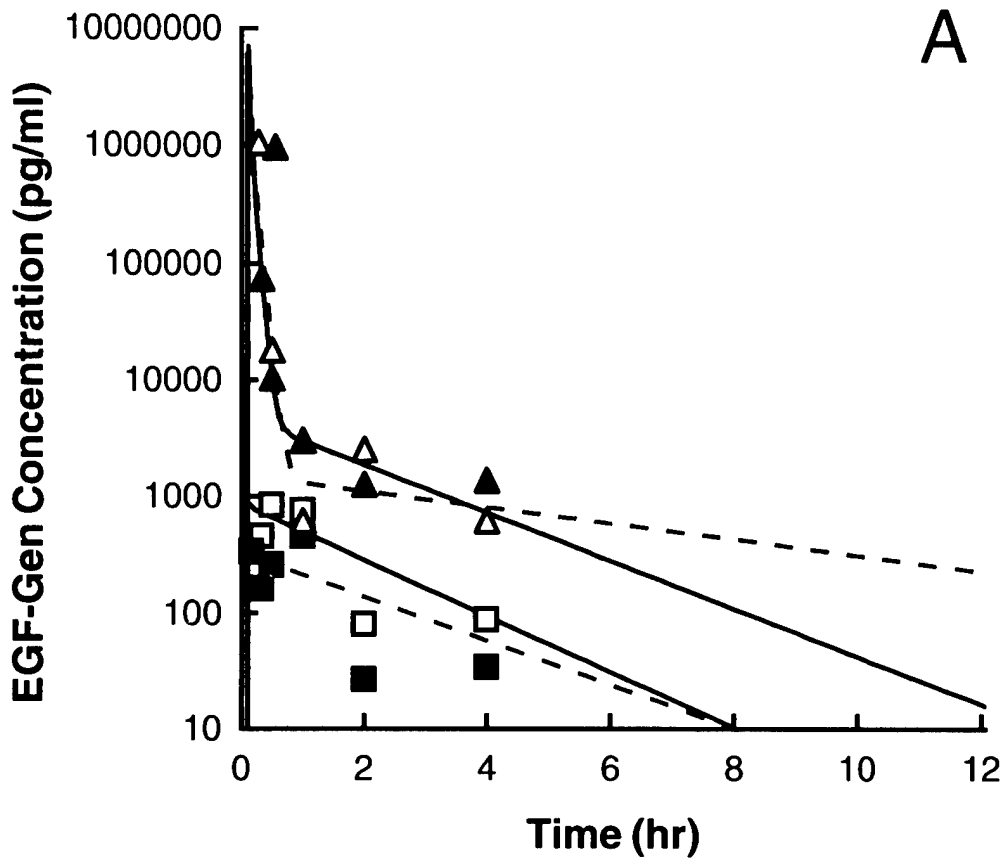

B.
Tumor Diameter (cm)
4
3
2
1
0
*P<0.01
*
*
0 5 10
0 5 10
0 5 10
PBS (N=4)
EGF-Gen (N=4)
Gen (N=4)
Days Post Treatment

FIGURE 12

Pharmacokinetic Features of EGF-Gen in SCID Mice (Panel A) and Cynomolgus Monkeys (Panel B). Plasma concentration-time curves of EGF-Gen after intraperitoneal bolus injection into tumor-free SCID mice at doses of 0.1 μg/g (□) and 1.0 μg/g (Δ), and into MDA-MB-231 tumor xenograft-bearing SCID mice at 0.1 μg/g (■) and 1.0 μg/g (▲). Monkeys received a 1-hour intravenous infusion of EGF-Gen at 0.05 μg/g (○) and 0.1 μg/g (□) dose levels. Lines represent pharmacokinetic model simulations for tumor-free (solid line) and tumor-bearing (dashed line) animals; symbols depict measured plasma concentrations of EGF-Gen. (see next page)


A
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10
EGF-Gen Concentration (pg/ml)
0
2
4
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8
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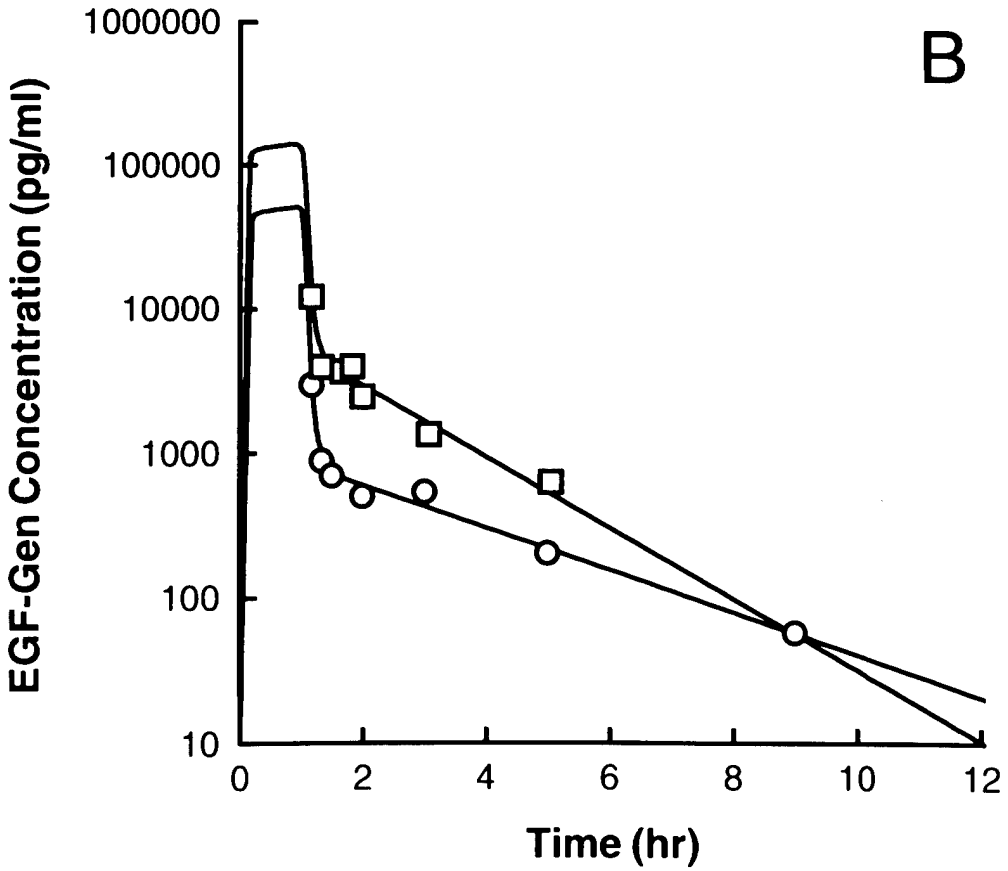
12
Time (hr)


B
1000000
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10
EGF-Gen Concentration (pg/ml)
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2
4
6
8
10
12
Time (hr)

Table 4. Pharmacokinetic Parameters of EGF-Gen in Mice and Monkeys

		Mice without Tumor		Mice with 1 cm^3 Tumor		Monkeys without Tumor	
Parameter	(units)	**100 µg/kg**	**1,000 µg/kg**	**100 µg/kg**	**1,000 µg/kg**	**500 µg/kg**	**1,000 µg/kg**
Vc	(ml/g)	11.6	0.05	30.0	0.05	0.06	0.04
Ke	(1/hr)	0.6	7.8	0.4	6.5	17.3	16.0
Kcp	(1/hr)	0	0.3	0	0.2	1.0	1.4
Kpc	(1/hr)	0	0.3	0	0.7	0.4	0.6
$T_{1/2\alpha}$	(hr)	0	0.09	0	0.1	0.04	0.04
$T_{1/2\beta}$	(hr)	1.3	2.1	1.6	1.0	2.1	1.2
CL	(ml/hr/g)	6.4	0.39	13.1	0.32	1.0	0.7
Vdss	(ml/g)	11.6	0.1	30	0.07	0.2	0.7
Cmax	(ng/ml)	0.45	960	0.33	1100	2.9	12.0
AUC	(µg x hr/L)	16	2564	7.6	3125	500	1400

Vc = central volume of distribution; Ke = elimination rate constant; Kcp, Kpc = distribution rate constants, $T_{1/2\alpha}$ = distribution half-life, $T_{1/2\beta}$ = elimination half-life; CL = systemic clearance from plasma, Vdss = volume of distribution at steady state, Cmax = measured maximum plasma concentration, AUC = area under the concentration-time curve. Dose 1 µg/g was used for efficacy study.

μg/kg/day x 10 days of EGF-Gen yielded an AUC of 2564 (± 231) μgxhr/L in non-tumor bearing mice and an AUC of 3125 (±281) μgxhr/L in tumor-bearing mice. Thus, the AUC showed a dramatic 160-fold (2564 μgxhr/L vs 16 μgxhr/L, P<0.001) to 411-fold (3125 μgxhr/L vs 7.6 μgxhr/L,P<0.001) increase as the dose of EGF-Gen was increased 10-fold. This dramatic increase in AUC which was accompanied by a dramatic 232-fold [11.6 (± 1.3) ml/g vs 0.05 (± 0.01) ml/g, P<0.001] to 600-fold [30.0 (± 3.6) ml/g vs 0.05 (±0.01) ml/g, P<0.001) decrease of the volume of distribution is most likely due to a saturable receptor-dependent binding and uptake of EGF-Gen, that has been reported to occur with unconjugated human EGF in rats at a dose level of 100 μg/kg (27). As a result of the increase in AUC, the clearance (i.e., Dose/AUC) of EGF-Gen significantly decreased with this dose escalation (**Table 4, Figure 12A**). The dose-dependent decrease in clearance was not associated with significant differences in t1/2β values [1.3 ± 0.2 vs 2.1 ± 0.3 for non-tumor bearing mice, and 1.6 ± 0.4 vs 1.0 ± 0.1 for tumor bearing mice], which is in accord with the published observations of Kim et al. (26, 27). These results taken together with previous reports regarding the pharmacokinetics of unconjugated EGF are consistent with the notion that the initial redistribution of EGF-Gen from plasma to EGF-R^+ cells in various tissues determining the t1/2α values is affected by factors influencing the binding of EGF-Gen to EGF-R^+ cells (e.g. affinity of the EGF-Gen conjugate for EGF-R, number of EGF-R^+ targets in the extravascular compartments), while the later phase of removal from plasma determining the t1/2β values is likely affected by the EGF-R turnover rates and dose-independent disassociation of EGF-Gen from surface EGF-R molecules.

Pharmacodynamic Features and Toxicity of EGF-Gen in Cynomolgus Monkeys. Because EGF-Gen was not toxic to healthy mice even at doses as high as 40 mg/kg given as a single dose or 140 mg/kg given in multiple doses despite the cross-reactivity of human EGF with murine EGF-R, we postulated that such systemic exposure levels could also be achieved in cynomolgus monkeys without excessive toxicity. To test this hypothesis, we measured in cynomolgus monkeys the systemic exposure levels achieved after treatment with 50 μg/kg/day x 10 days and 100 μg/kg/day x 10 days. The plasma concentration-time curves of EGF-Gen in monkeys were also biphasic (**Figure 12B**). The volume of

distribution and clearance tended to decrease as the daily dose increased from 50 μg/kg to 100 μg/kg, similar to what was observed in mice. As shown in **Table 4**, treatment with 100 μg/kg/day EGF-Gen yielded an AUC of 1400 μg*hr/L. This systemic exposure level is much higher than the target AUC of 16 μg*hr/L, which was found to be effective in the SCID mouse model of human breast cancer.

Notably, no clinical or laboratory evidence of significant toxicity was observed in these monkeys, except for a transient alopecia in two of the monkeys (**Table 5**). In particular, we observed no gastrointestinal or hepatic toxicity. No histopathologic lesions were found in the organs of EGF-Gen treated monkeys that were electively euthanized. Thus, EGF-Gen concentrations higher than those which are required to elicit therapeutic efficacy against human breast cancer cells in the SCID mouse xenograft model of human breast cancer were achieved in cynomolgus monkeys without significant systemic toxicity.

We also examined the anti-cancer activity of plasma samples from EGF-Gen-treated monkeys by determining their ability to inhibit the in vitro clonogenic growth of the human breast cancer cell lines MDA-MB-231 and BT-20. As detailed in **Table 6**, 1: 50 PBS-diluted plasma samples obtained at 1 hour after treatment with 1 mg/kg EGF-Gen (but not 1:50 diluted pretreatment plasma samples from the same monkeys) abrogated the in vitro colony formation by these breast cancer cell lines. Notably, excess unconjugated EGF (but not excess G-CSF) could competitively block the cytotoxicity of the EGF-Gen-containing monkey plasma samples (**Table 6**). These results confirmed the biologic activity and stability as well as EGF-R-specificity of the circulating EGF-Gen molecules in cynomolgus monkeys.

Protein tyrosine kinases have long been suspected to play pivotal roles in regulation of cell survival in cancer cells (19, 28-37). Our recent studies provided experimental evidence that the EGF-R-associated PTK complexes are of vital importance for the survival of breast cancer cells and therefore EGF-R may serve as a suitable target for biotherapy of breast cancer using PTK inhibitors (18). EGF-Gen is an experimental anti-cancer drug which targets the naturally occurring PTK inhibitory isoflavone Gen to the membrane-associated anti-apoptotic EGF-R/PTK complexes and triggers apoptotic cell death (18). In the present study, we examined the in vivo anti-cancer activity, pharmacokinetic features, as well as toxicity

Table 5. Toxicity of EGF-Genistein in Cynomolgus Monkeys

System	Grade of Maximum Toxicity (Time)				
	52 B 0.025 mg/kg/day x 10d	**52 A** 0.050 mg/kg/d x 10d	**52 I** 0.100 mg/kg/d x 10d	**63 A** 0.100 mg/kg/d x 10d	**63 F** 0.1 mg/kg/d x 10d
Activity/Feeding	0	2 (d7–9)	2 (d5–8)	1	0
Fever	0	0	0	0	0
Weight loss	0	0	2 (d12)	0	0
Skin (Alopecia)	0	2 (d13–50)	1	0	0
Cardiac					
Tachycardia	1	1	1	2 (d8)	1
Hypotension	0	0	0	0	0
Pulmonary					
Clinical	0	0	2 (d10)	0	0
Respiratory rate	0	2 (d3-7)	0	0	0
Renal					
Creatinine	0	0	0	0	1
Electrolytes	0	0	0	0	0
Proteinuria	0	1	0	1	0
Hematuria	0	0	0	0	0
Liver					
ALT	2 (d14–18)	2 (d2-3)	1	1	1
Bili	0	0	0	0	0
Gastrointestinal					
Nausea/Vomiting	0	0	0	0	0
Diarrhea	1	0	1	0	0
Constipation	0	0	0	0	0
Nervous System					
Central	0	0	0	0	0
Peripheral	0	0	0	0	0
Coagulation					
PT	0	0	1	0	0
PTT	0	0	0	0	0
Infection	0	0	1	0	0
Blood					
Neutropenia	0	0	0	0	0
Anemia	2 (d7–10)	2 (d9-10)	1	2 (d5-6)	1
Thrombocytopenia	0	0	0	0	0
Metabolic	0	0	1	0	1

Cynomolgus monkeys were treated with intravenous infusions of EFG-Gen or Gen, as described in the Methods. For each >Grade I toxicity the onset and duration of toxicity are indicated in parentheses. ALT, alanine aminotransferase: Bili, bilirubin. Monkeys were electively euthanized at the following time points after initiation of therapy: 52 B, 182days; 52 A 186days; 52 I, 127days; 63 A, 15days; 63 F, 30days; 52 F, 135days; 52 G, 135days. No test substance related histopathologic lesions were found in any of the monkeys.

Table 6. In Vitro Anti-tumor Activity of Plasma from EGF-Genistein-treated Cynomolgus Monkeys Against EGF-R+ MDA-MB231 Human Breast Cancer Cell Lines

Plasma Samples	Mean No. Colonies/10^5 Cells	% Inhibition
None	348.5 (344, 353)	—
Monkey 52A (EGF-Gen Dose = 50 µg/kg)		
Pretreatment	354.5 (321, 388)	0
1 hour posttreatment	28.5 (21, 36)	91.8
1 hour posttreatment + 10 µg/ml EGF	301.5 (289, 314)	13.5
1 hour posttreatment + 10 µg/ml G-CSF	21.5 (16, 27)	93.8
Monkey 52I (EGF-Gen Dose = 100 µg/kg)		
Pretreatment	323.5 (320, 327)	7.2
1 hour posttreatment	0 (0, 0)	>99.7
1 hour posttreatment + 10 µg/ml EGF	293.0 (278, 308)	15.9
1 hour posttreatment + 10 µg/ml G-CSF	0 (0, 0)	>99.7
Monkey 63A (EGF-Gen Dose = 100 µg/kg)		
Pretreatment	395.0 (391, 399)	0
1 hour posttreatment	4.5 (2, 7)	98.7
1 hour posttreatment + 10 µg/ml EGF	279.5 (276, 283)	19.8
1 hour posttreatment + 10 µg/ml G-CSF	2.5 (1, 4)	99.3
Monkey 63F (EGF-Gen Dose = 100 µg/kg)		
Pretreatment	323 (306, 340)	7.3
1 hour posttreatment	0 (0, 0)	>99.7
1 hour posttreatment + 10 µg/ml EGF	340 (309, 371)	2.4
1 hour posttreatment + 10 µg/ml G-CSF	0 (0, 0)	>99.7

Cells (10^6/mL RPMI+10% FCS) were incubated overnight at 37°C with 1:50 (v/v) PBS-diluted plasma samples from EGF-Genistein-treated cynomolgus monkeys. After treatment, cells were washed twice, plated at 10^5 cells/ml in RPMI+10% FCS+ 0.9% methylcellulose in Petri dishes, and cultured for 7 days at 37°C in a humidified 5% CO_2 atmosphere. Subsequently, colonies were enumerated and the % inhibition was calculated using the formula:

$$\% \text{ Inhibition} = 1 - \frac{\text{mean no. colonies in test culture}}{\text{mean no. colonies in control culture}} \times 100.$$

Excess EGF was added to some of the plasma samples to block the activity of EGF-Genistein by competing for the EGF-R molecules on MDA-MB231 cells. Excess G-CSF was used as a control for comparison.

profile of EGF-Gen. Here, we presented experimental evidence that EGF-Gen displays significant anti-tumor activity in a SCID mouse xenograft model of human breast cancer. EGF-Gen significantly improved tumor-free survival in a SCID mouse xenograft model of human breast cancer, when it was administered 24 hours after inoculation of tumor cells. Furthermore, treating SCID mice with established subcutaneous human breast cancer xenografts of 0.5 cm diameter with EGF-Gen at this dose level resulted in disappearance of the tumors in 2 of 5 mice and >50% shrinkage in 3 of 5 mice within 10 days, whereas all of the control tumors in 5 PBS treated mice as well as 5 mice treated with unconjugated Gen (1 mg/kg/day x 10 days) showed >200% increase in diameter during the same observation period.

The inability of unconjugated Gen or unconjugated EGF plus unconjugated Gen to exhibit significant anti-tumor activity in this SCID mouse model of metastatic human breast cancer demonstrates that the anti-cancer activity of EGF-Gen cannot be attributed to either the EGF or Gen moieties alone. Daily administration of 2 μg EGF-Gen, which contains 309 pmols of Gen in conjugated form, for a total of 10 days was a highly effective treatment regimen, whereas daily administration of 10 μg Gen, which corresponds to 37,000 pmols, alone or in combination with 10 μg unconjugated EGF (5-fold higher dose of EGF than what is contained in 2 μg EGF-Gen) for 10 days was not effective at all. Thus, the conjugation of Gen to the targeting EGF molecule enhanced its *in vivo* activity against breast cancer cells more than 100-fold. These findings confirm and extend our in vitro data demonstrating that, compared to unconjugated Gen, EGF-Gen is >1,000-fold more potent cytotoxic agent against EGF-R^+ human breast cancer cells.

EGF-Gen improved tumor-free survival in a SCID mouse model of human breast cancer at systemic exposure levels non-toxic to mice or cynomolgus monkeys. Therefore, therapeutic levels of EGF-Gen may also be achievable in women with metastatic breast cancer without excessive toxicity. Notably, EGF-Gen was more effective than cyclophosphamide, adriamycin, or methotrexate in our MDA-MB-231 SCID mouse xenograft model of human breast cancer. Furthermore, plasma samples from EGF-Gen treated cynomolgus monkeys elicited potent and EGF-R-specific *in vitro*. anti-tumor activity against

EGF-R^+ human breast cancer cell lines. These promising preclinical results obtained with EGF-Gen indicate that further clinical development of this promising new anti-breast cancer agent is warranted. EGF-Gen treatment reduced the growth rate of breast cancer xenografts of 1.0 cm diameter, but unlike with tumors of 0.5 cm diameter, it failed to cause shrinkage or disappearance of these larger tumors. This is not surprising especially with the liver having a >40-fold higher partition coefficient than the subcutaneous tumors. Thus, EGF-Gen may be more effective as part of an adjuvant therapy regimen when the disease burden is not very large.

EGF-R overexpression is found in many types of cancer besides breast cancer. Thus, EGF-Gen could potentially be used in several different types of cancer. The EGF-R on cancer cells represents a potential target for other forms of biotherapy as well. Anti-EGF-R antibodies may be useful in the treatment of EGF-R positive malignancies by disrupting EGF-mediated signal transduction events. Whether EGF-Gen is superior to such anti-EGF-R antibodies needs to be examined in appropriate preclinical and clinical settings.

C. SUMMARY

Epidermal growth factor-receptor (EGF-R)-associated protein tyrosine kinase (PTK) complexes have vital anti-apoptotic functions in human breast cancer cells. We have previously shown that targeting the naturally occurring PTK inhibitor genistein to the EGF-R-associated PTK complexes using the EGF-Genistein (Gen) conjugate triggers rapid apoptotic cell death in human breast cancer cells and abrogates their in vitro clonogenic growth. In the present study, we examined the *in vivo* toxicity profile, pharmacokinetics, and anti-cancer activity of EGF-Gen. No toxicities were observed in mice treated with EGF-Gen at dose levels as high as 40 mg/kg administered intraperitoneally (i.p.) as a single dose or 140 mg/kg administered i.p. over 28 consecutive days. EGF-Gen significantly improved tumor-free survival in a SCID mouse xenograft model of human breast cancer, when it was administered 24 hours after inoculation of tumor cells. At 100 μg/kg/d x 10 days (1 mg/kg total dose), which is >100-fold less than the highest tested and nontoxic cumulative dose (i.e., 140 mg/kg) in mice, EGF-Gen was more effective than cyclophosphamide (50 mg/kg/d x 2 days), adriamycin (2.5 mg/kg x 1) or methotrexate (0.5 mg/kg x

1), the most widely used standard chemotherapeutic drugs for breast cancer, and resulted in 60 % long-term tumor-free survival. Furthermore, treating SCID mice with established subcutaneous human breast cancer xenografts of 0.5 cm diameter with EGF-Gen at this dose level resulted in disappearance of the tumors in 2 of 5 mice and >50% shrinkage in 3 of 5 mice within 10 days, whereas all of the control tumors in 5 PBS treated mice as well as 5 mice treated with unconjugated Gen (1 mg/kg/day x 10 days) showed >200% increase in diameter during the same observation period. EGF-Gen treatment reduced the growth rate of breast cancer xenografts of 1.0 cm diameter, but unlike with tumors of 0.5 cm diameter, it failed to cause shrinkage or disappearance of these larger tumors. The level of EGF-Gen systemic exposure that was effective in SCID mice was achieved in cynomolgus monkeys without any significant side effects detectable by clinical observation, laboratory studies, or histopathological examination of multiple organs. EGF-Gen might be useful in treatment of breast cancer as well as other EGF-R positive malignancies.

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III. CONCLUSIONS

EGF-Gen conjugate inactivates the EGF-R tyrosine kinase as well as ErbB2, ErbB3, and Src protooncogene family PTK in breast cancer cells triggering apoptosis and clonogenic cell death. Our results indicate that the EGF-R-associated PTK complexes have vital anti-apoptotic functions in human breast cancer cells and may therefore be used as therapeutic targets.

In apoptosis assays, 10μg/ml (=37μM) Gen was not active against MDA-MB-231 and BT-20 breast cancer cells, whereas 1μg/ml (0.137 μM) EGF-Gen, which contains 270-fold less Gen was active. In clonogenic assays, the IC50 values for EGF-Gen against MDA-MB-231 and BT-20 breast cancer cells were >1,000-fold lower than those of unconjugated Gen (30 nM vs 112-119 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-Genistein 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 contacts 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 by covalent modification, reminiscent of the oxidative inactivation of CD19-associated Src family PTK by B43-Gen, an anti-CD19 antibody-Gen immunoconjugate.

EGF-Gen displays significant anti-tumor activity in a SCID mouse xenograft model of human breast cancer. EGF-Gen significantly improved tumor-free survival in a SCID mouse xenograft model of human breast cancer, when it was administered 24 hours after inoculation of tumor cells. Furthermore, treating SCID mice with established subcutaneous human breast cancer xenografts of 0.5 cm diameter

with EGF-Gen at this dose level resulted in disappearance of the tumors in 2 of 5 mice and >50% shrinkage in 3 of 5 mice within 10 days, whereas all of the control tumors in 5 PBS treated mice as well as 5 mice treated with unconjugated Gen (1 mg/kg/day x 10 days) showed >200% increase in diameter during the same observation period.

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subcutaneous tumors. Thus, EGF-Gen may be more effective as part of an adjuvant therapy regimen when the disease burden is not very large.

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V. APPENDICES

publications

CYTOTOXIC ACTIVITY OF EGF-GENISTEIN AGAINST BREAST CANCER CELLS

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[3]The abbreviations used are: EGF-R, epidermal growth factor receptor; Gen, Genistein; PTK, protein tyrosine kinases; EGF, epidermal growth factor.

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 protooncogene family protein tyrosine kinases (PTK) 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 with an IC50 value of 2.9 nM, whereas the IC50 value for unconjugated Gen was >100 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 receptor-specific because cells treated with the control granulocyte-colony stimulating factor (G-CSF)-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 IC50 values of EGF-Gen versus unconjugated Gen against MDA-MB-231 and BT-20 cells in clonogenic assays were 30± 3 nM versus 120 ± 18 μM ($P<0.001$) and 30 ± 10 nM versus 112 ± 17 μM ($P<0.001$), respectively. Thus, the EGF-Gen conjugate is a >1,000-fold more potent inhibitor of EGF-R tyrosine kinase activity in intact breast cancer cells than unconjugated Gen and a >1,000-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 anti-apoptotic 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 USA (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 epidermal growth factor (EGF) is a 53 amino acid, single-chain polypeptide (Mr 6216 daltons), which exerts biologic effects by binding to a specific 170 kDa cell membrane epidermal growth factor receptor (EGF-R/ErbB-1) (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 protein tyrosine kinase (PTK) activity. Binding of EGF to the 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, 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 Src protooncogene family PTK, including $p60^{src}$ (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 is a significant and independent indicator for recurrence and poor relapse-free survival (15-17).

Targeted delivery of genistein (Gen), a naturally occurring PTK inhibitory isoflavone (5,7,4'-trihydroxyisoflavone) 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, recombinant human EGF(rhEGF) was conjugated to Gen to construct an EGF-R-directed cytotoxic agent with PTK inhibitory activity. Here, we report the effects of the EGF-Gen conjugate on human breast cancer cells.

MATERIALS AND METHODS

Preparation of the EGF-Gen. rhEGF was produced in E. coli harboring a genetically engineered plasmid that contains a synthetic gene for human EGF fused at the N-terminus to a hexapeptide leader sequence for optimal protein expression and folding. 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 crosslinking agent, Sulfosuccinimidyl 6-[4'azido-2'-nitrophenylamino]hexanoate (Sulfo-SANPAH) (Pierce Chemical Co., Rockford, IL) (18) has been employed 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 dimethyl sulfoxide (DMSO)] and then irradiated 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 differentially hydroxyl-protected Gen resulted in the attachment of Gen via its available C7-hydroxyl 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 12 kDa EGF-EGF homoconjugates with or without conjugated Gen as well as higher molecular weight reaction products were removed by size-exclusion high-performance liquid chromatography (HPLC). Reverse phase HPLC using a Hewlett-Packard (HP) 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 (250x4 mm, Hewlett-Packard, Cat.# 79992402-584). Prior to the HPLC runs, a Beckman DU 7400 spectrophotometer was used to generate a UV spectrum for each of the samples to ascertain the λmax 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% to 100% eluent in a time interval of 0 to 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 (CH3CN), 20% H_2O and 0.1% TFA.

Electrospray ionization mass spectrometry (20, 21) was performed using a PE SCIEX API triple quadruple mass spectrometer (Norwalk, CT) to determine the 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% phosphate buffered saline [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 medium 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 incubated for 5-10 min at 37^{o}C until cells detached. Fresh medium was then added, cells 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^{8} cpm/μmol), ^{125}I-Gen (3.8×10^{8} cpm/μmol) and ^{125}I-EGF (2.2×10^{12} cpm/μmol; Amersham) were performed using standard procedures, as previously described (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 cells lines, NALM-6 (pre-B leukemia) and HL-60 (promyelocytic leukemia).

Immunocytochemistry. Immunocytochemistry was used to (i) examine the surface expression of EGF-R on breast cancer cells, (ii) evaluate the uptake of EGF-Gen by breast cancer cells and (iii) examine the morphologic 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 37oC for 5 min, 10 min, 15 min, 30 min, 60 min, and 24 hours. For EGF-R expression studies, cells were plated on poly-L-lysine coated glass-bottom 35 mm Petri dishes and maintained for 48 hr. At the end of the incubation, cells were washed with PBS and fixed in 2% paraformaldehyde. The cells were permeabilized and non-specific 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 Triton 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 hr at room temperature. After rinsing with PBS, cells were incubated for 1 hr 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 Labhophot upright microscope. Digital images were saved on a Jaz disk and processed with Adobe Photoshop software (Adobe Systems, Mountain View, CA).

In Vitro **Treatment of Cells with EGF-Genistein.** In order to determine the cytotoxic activity of EGF-Gen against breast cancer cells, cells in alpha-MEM supplemented with 10%(v/v) fetal calf serum were treated with various concentrations of EGF-Gen for 24 hours at 37°C, washed twice in alpha-MEM, and then used in either apoptosis assays or clonogenic assays, as described hereinafter. Controls included (a) cells treated with G-CSF-Gen (an irrelevant cytokine-Gen conjugate which 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 were 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 hours after treatment with EGF-Gen, cells were stimulated with 20 ng/mL EGF for 5 min, lysed in 1% Nonidet-P-40 buffer, and cell lysates were immunoprecipitated with an anti-EGF-R antibody reactive with the sequence Ala^{351}-Asp^{364} of the human EGF-R (Upstate Biotechnology

Inc. [UBI] Catalog # 05-104). EGF-R immune complexes were examined for tyrosine phosphorylation by Western blot analysis, as previously described (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 % inhibition value was determined by comparing the density ratios of the tyrosine phosphorylated EGF-R protein bands to those of the baseline sample and using the formula: % Inhibition = 100- 100x [Density of tyrosine phosphorylated EGF-R band]$_{\text{test sample}}$: [Density of tyrosine phosphorylated EGF-R band]$_{\text{baseline control sample}}$. The IC50 values 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 fragmentation in apoptotic cells, thereby providing MC540 binding as an early marker for apoptosis (24). Plasma membrane permeability to propidium iodide (PI, Sigma) develops at a later stage of apoptosis (24). MC540 binding and PI permeability were simultaneously measured in breast cancer cells 24 hours after exposure to EGF-Gen (either without any cytokine preincubation or following 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 $1x10^6$ cells were suspended in 5 μg/mL MC540 and 10 μg/mL PI 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 and a 575 nm band pass filter was placed in front of one photomultiplier tube to measure MC540 emission amd a 635 nm band pass filter was used for PI emission. To detect the DNA fragmentation in apoptotic cells, cells were harvested 24 hours 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 g. This protocol allows the recovery of fragmented DNA in the supernatant. To detect apoptosis-associated DNA fragmentation, supernatants were electropheresed on a 1.2% agarose gel, and the DNA fragments were visualized by ultraviolet 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 alpha-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 % inhibition of clonogenic cells at a particular concentration of the test agent using the formula: % Inhibition = (1 - Mean # of colonies [Test] / Mean # of colonies [Control]) x 100. Furthermore, the dosesurvival curves were constructed using the percent control survival (= Mean # of colonies[Test] / Mean # of colonies [Control] x 100) results for each drug concentration as the data points and the IC50 values were calculated The IC50 values were determined using an Prism Version II Inplot program (Graphpad Software, Inc., San Diego, CA). The mean IC50 values 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 ^{125}I-Genistein. The electrospray ionization mass spectrum of EGF-Gen also showed a single 7287 kDa EGF-Gen species containing one EGF molecule, three SANPAH molecules, and one Gen molecule. **Figure 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 (i) 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 EGF, (ii) a peak at 267 nm representing Gen, and (iii) a peak at 480 nm corresponding to the nitrobenzene substituted structure in the SANPAH moiety (**Figure 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-^{125}I-Gen, as well as quantitative anti-Gen Western blot analysis of non-radioactive 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-^{125}I-Gen, Final concentration: 260 nM = 1700 ng/ml) to EGF-R on these breast cancer cells in the presence and absence of 100-fold molar excess non-radioactive EGF using standard ligand binding assays (18, 19, 22). EGF-^{125}I-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 x10^6 EGF-Gen molecules/cell for MDA-MB-231 cells and 5.7x10^6 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-^{125}I-Gen did not bind to EGF-R negative HL60 or NALM-6 leukemia cell lines. EGF-Gen was as effective as unconjugated EGF in blocking the binding of ^{125}I-EGF to breast cancer cells, whereas GM-CSF did not block the binding of ^{125}I-EGF (**Table 1**). Thus, EGF-Gen was able to bind to EGF-R positive breast cancer cells via its EGF moiety. However, since (1) 35-44% of the EGF-Gen binding to breast cancer cells was not inhibitable by excess unconjugated EGF, (2) EGF-Gen binding not inhibitable by excess EGF was also observed with EGF-R negative leukemia cell lines NALM-6 and HL-60, and (3) unconjugated Gen showed binding to all cell lines, which was not inhibitable by EGF, the Gen moiety as well as non-specific 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 (**Figure 2**) and MDA-MB-231 (**Figure 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 morphologic changes in treated cells. EGF-Gen was very similar to unconjugated EGF with respect to its ability to bind to and induce internalization of EGF-R molecules. Within 5 min after exposure to EGF-Gen, the EGF-R/EGF-Gen complexes begin being internalized, as determined by co-localization of EGF-R (detected by anti-EGF-R antibody, green fluorescence) and EGF-Gen (detected by anti-Gen antibody, red fluorescence) in the cytoplasm of treated cells (**Figure 2 & Figure 3**). By 15-30 min, the EGF-R/EGF-Gen complexes were detected in the perinuclear region of the cells. The examination of the morphologic features of EGF-Gen-treated (but not EGF-treated) cells after 24 hours of exposure showed distinct changes consistent with apoptosis including marked shrinkage, nuclear fragmentation, and formation of apoptotic bodies (**Figure 2**).

Biologic Activity of EGF-Gen. EGF-Gen treatment resulted in decreased tyrosine phosphorylation of the EGF-R in a dose-dependent fashion (**Figure 4A**). Whereas EGF-Gen exhibited marked PTK-inhibitory activity in MDR-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 (**Figure 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 which does not react with EGF-R (**Figure 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 MBA-MB-231 cells. As shown in **Figure 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 PTK which are associated with the EGF-R.

Targeting Gen to vital PTK in leukemia cells results in apoptotic cell death (18, 19). Furthermore, the examination of the morphologic features of EGF-Gen treated BT-20 and MDA-MB-231 cells by immunocytohemistry 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 propidium iodide (PI) permeability of MDA-MB-231 breast cancer cells were simultaneously measured before and after treatment with 1μg/ml EGF-Gen (=0.1 μM), 10μg/ml EGF (1 μM) plus 10μg/ml unconjugated Gen (=37 μM), or 1μg/ml G-CSF-Gen. Whereas less than 10% 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 hours after EGF-Gen treatment (95.1% = 57.9% $MC540^+$ early stage apoptosis plus 37.2% $MC540^+/PI^+$ advanced stage apoptosis at 24 hours) (**Figure 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 **Figure 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 (**Figure 6**).

We compared the ability of equimolar concentrations of 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 (**Figure 7).**

We next tested the anti-cancer activity of EGF-Genistein against MDA-B-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 hour 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 which 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 **Figure 8,** EGF-Gen inhibited in each of 3 independent experiments the clonogenic growth of MDA-MB-231 as well as BT-20 cells at nanomolar concentrations with mean IC50 values 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 >1,000 fold higher mean IC50 values (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 P-values for the Student's t-test comparisons of the IC50 values for EGF-Gen vs Gen were <0.001 for both cell lines. The IC50 values derived from the composite MDA-MB-31 clonogenic cell survival curves were 39 nM for EGF-Gen and 160 μM for unconjugated Gen. The IC50 values derived from the composite BT-20 clonogenic cell survival curves were 20 nM for EGF-Gen and 147 μM for unconjugated Gen (**Figure 8**).

DISCUSSION

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 protooncogene family PTK in breast cancer cells triggering apoptosis and clonogenic cell death. These results indicate that the EGF-R-associated PTK complexes have vital anti-apoptotic 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 PTK rather than inhibition of the EGF-R family PTK since several studies have previously demonstrated that EGF-R tyrosine kinase activity is not essential for survival of cancer cells (25-29). For example, Fry et al. reported that the PTK inhibitor PD153035 inhibited EGF-R tyrosine kinase with an IC50 value of 29 pM but failed to kill EGF-R positive cells (25). Tanaka et al. reported that BE-23372M which inhibits the EGF-R with an IC50 value of 30 nM results only in 50% inhibition of target cell growth (26). RG-13022, a potent inhibitor of EGF-R tyrosine kinase, also elicited only a transient cytostatic effect on breast cancer cells even at 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, 10μg/ml (=37μM) Gen was not active against MDA-MB-231 and BT-20 breast cancer cells, whereas 1μg/ml (0.137 μM) EGF-Gen, which contains 270-fold less Gen was active. In clonogenic assays, the IC50 values for EGF-Gen against MDA-MB-231 and BT-20

breast cancer cells were >1,000-fold lower than those of unconjugated Gen (30 nM vs 112-119 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-Genistein 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 contacts 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 by covalent modification, reminiscent of the oxidative inactivation of CD19-associated Src family PTK by B43-Gen, an anti-CD19 antibody-Gen immunoconjugate (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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FIGURE LEGENDS

Figure 1. (A). HPLC Chromatogram of EGF-Gen. A sample of the EGF-Gen conjugate was analyzed on a Spherisorb ODS, 250x4 mm reverse-phase column using a 0.1% TFA-H2O2/0.1% TFA-80% acetonitrile-20% H2O2 gradient as described in the Methods. The retention time of EGF-Gen was 18.84 min. The solid line represents the absorbance at 265 nm and the dotted line reepresents the absorbance at 480 nm. **(B). UV Spectrum of the EGF-Gen.** The EGF-Gen peak obtained from the HPLC run shown in (B) was further analyzed by the diodide array multiple wavelength detector.

Figure 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, A'; 5 min: B, B'; 30 min: C, C'; 24 hr: D, 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 represents the nuclei stained with toto-3. A, A': BT-20 cells showed high level EGF-R expression; no red fluorescent staining was observed in untreated cells incubated with the anti-Gen antibody. B, B': Following 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. C, C': By 30 min, most of the EGF-R/EGF-Gen complexes were internalized and deposited in the perinuclear region (arrows). D, D': Following 24 hr exposure, the cells lost their adherent features and showed morphologic changes consistent with apoptosis (open arrow).

Figure 3. Binding and Internalization of EGF-Gen in MDA-MB-231 Cells. Cells were incubated with either unconjugated EGF (10 μg/ml) (A, A') or EGF-Gen (10 μg/ml) (B, B') for 15 min and processed for the detection of EGF-R (green fluorescence; left panel) and EGF-Gen (red fluorescence; right panel) by immunocytochemistry. EGF-Gen was very similar to 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.

Figure 4. Inhibitory Activity of EGF-Gen on EGF-R-associated PTKs of Human Breast Cancer Cells. **[A]** After a 24-hour incubation with EGF-Gen (0.1 μM, 1.0 μM, or 10.0 μM) or unconjugated Gen (10 μM), MDA-MB-231 cells were lysed in 1% Nonidet-P-40 buffer, and cell lysates were immunoprecipitated with an anti-EGF-R antibody reactive with the sequence Ala^{351}-Asp^{364} of the human EGF-R. The EGF-R immune complexes were then subjected to APT immunoblotting. **[B]** After a 24-hour incubation with 10.0 μg/mL (=1.3 μM) EGF-Gen, MDA-MB-231 cells were lysed in 1% Nonidet-P-40 buffer, cell lysates were immunoprecipitated with an anti-EGF-R antibody, and the EGF-R immune complexes were subjected to APT immunoblotting as in [A]. Controls included untreated cells as well as cells pretreated with 10-fold molar excess of unconjugated EGF (=13 μM) or unconjugated G-CSF (13 μM) prior to EGF-Gen incubation. **(C)** Src immune complex kinase assays in the presence of [γ-^{32}P]ATP (50 μCi/μmol) were performed on the lysates of MDA-MB-231 cells treated with 1 μg/mL EGF-Gen (0.1 μM), 1 μg/mL G-CSF-Gen, or 1 μg/mL EGF (0.1 μM) + 1 μg/mL Gen (3.7 μM). Controls included untreated cells.

Figure 5. 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 hours 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, 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.

Figure 6. Internucleosomal DNA Fragmentation in EGF-Gen-Treated Breast Cancer Cells. Cells were harvested 24 hours after treatment with PBS (CON), 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 base pairs.

Figure 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 hours after treatment with PBS, EGF-Gen (0.1 μM, 1.0 μM, 10 μM), or unconjugated Gen (1.0 μM, 10 μM, 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) (= % MC540 single fluorescent + % MC540/PI double fluorescent) is also provided.

Figure 8. EGF-Gen is more cytotoxic against clonogenic MDA-MB-231 and BT-20 human breast cancer cells than unconjugated Gen. MDA-MB-231 (shown in [A]) and BT-20 (shown in [B] cells were treated with 0.1, 0.3, 1.0, 3.0, 10, 30, or 100 μM EGF-Gen or equimolar concentrations of unconjugated Gen for 24 hours. Subsequently, cells were assayed for clonogenic growth in vitro, as described in Materials and Methods. . The colony numbers for MDA-MB-231 cells ranged from $385/10^5$ cells to 510 colonies/10^5 cells (mean ± SE = 450 ± 36 colonies/10^5 cells). The colony numbers for BT-20 cells ranged from $193/10^5$ cells to 276 colonies/10^5 cells (mean ± SE = 224 ± 26 colonies/10^5 cells). Composite clonogenic cell survival curves were generated using the dose response data from 3 independent experiments, each performed in duplicate. Each data point on the composite survival curve represents the mean % control clonogenic cell survival at a given drug concentration. The error bars for each data point are the standard error to the mean. The individual IC50 values for EGF-Gen from the 3 experiments ranged from 21nM to 42nM for MDA-MB-231 cells (mean ± SE = 30 ± 3 nM), and 17nM to 64 nM for BT-20 cells (mean ± SE = 30 ± 10 nM). The corresponding IC50 values for Gen ranged from 99 μM to 154 μM for MDA-MB-231 cells (mean ± SE = 120 ± 18 μM), and from 80 μM to 139 μM for BT-20 cells (mean ± SE = 112 ± 17 μM). The EGF-Gen IC50

values derived from the composite MDA-MB-31 and BT-20 clonogenic cell survival curves were 39 nM and 20 nM, respectively. By comparison, the IC50 values of the composite clonogenic survival curves for unconjugated Gen were 160 μM and 147, respectively.

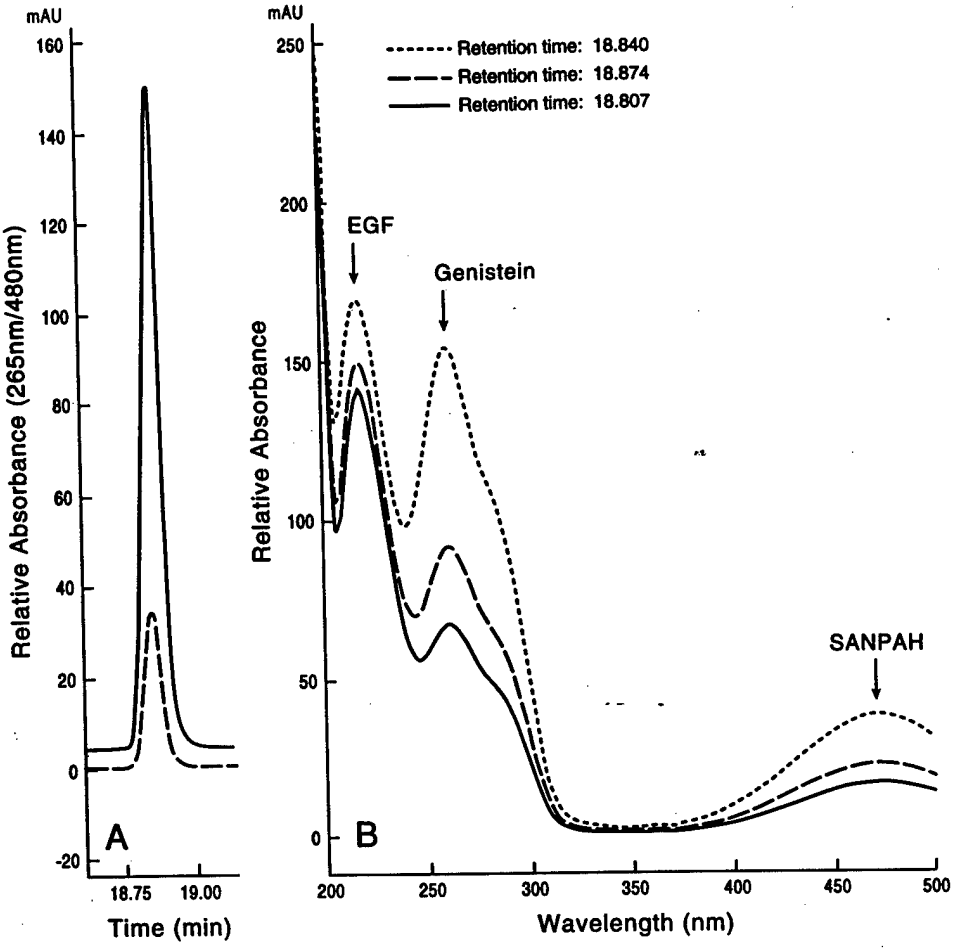
mAU
160
140
120
100
80
60
40
20
0
-20
Relative Absorbance (265nm/480nm)
A
18.75
19.00
Time (min)
mAU
250
200
150
100
50
0
Relative Absorbance
Retention time: 18.840
Retention time: 18.874
Retention time: 18.807
EGF
Genistein
SANPAH
B
200
250
300
350
400
450
500
Wavelength (nm)

Figure 1

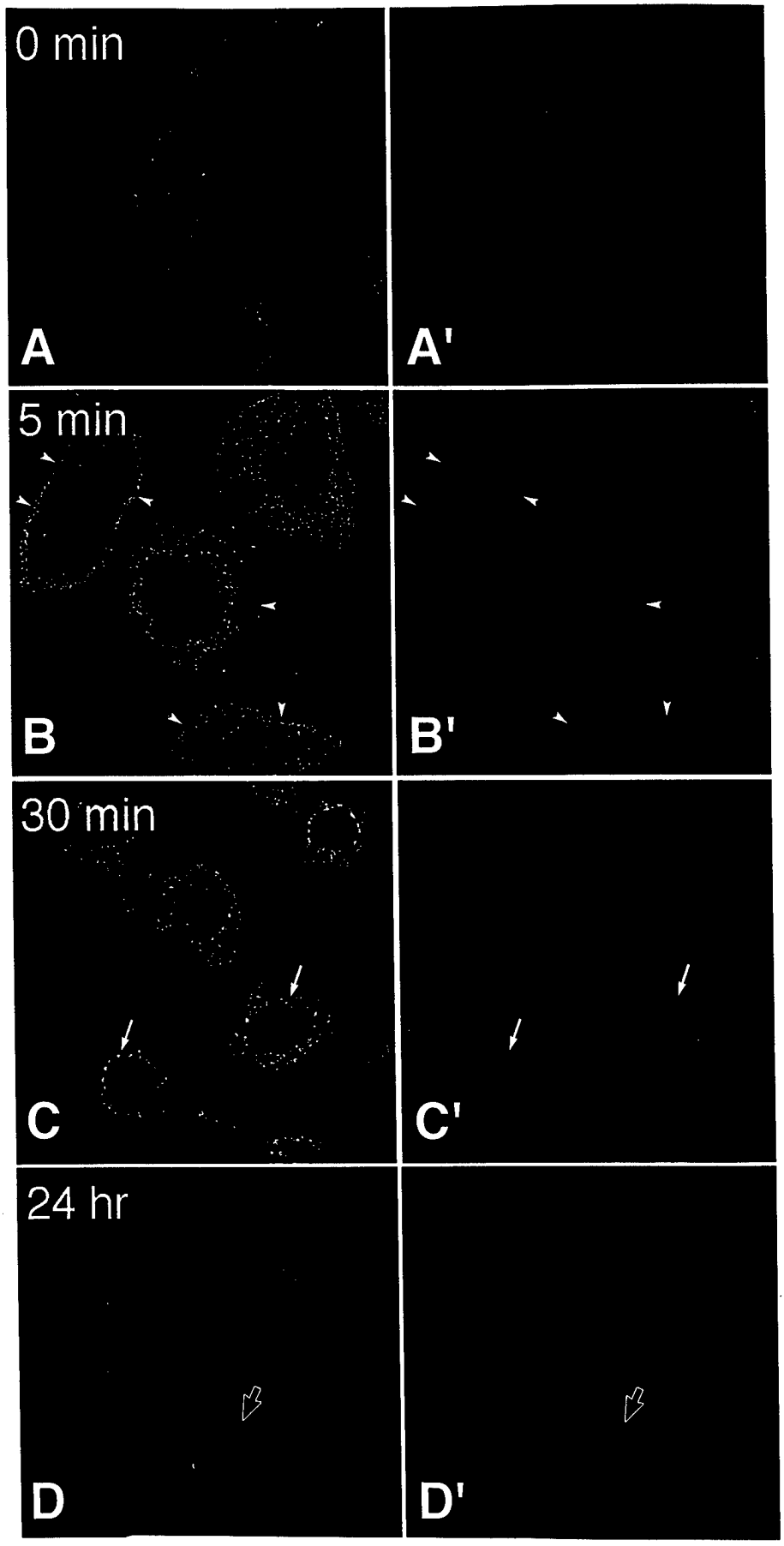

Figure 2

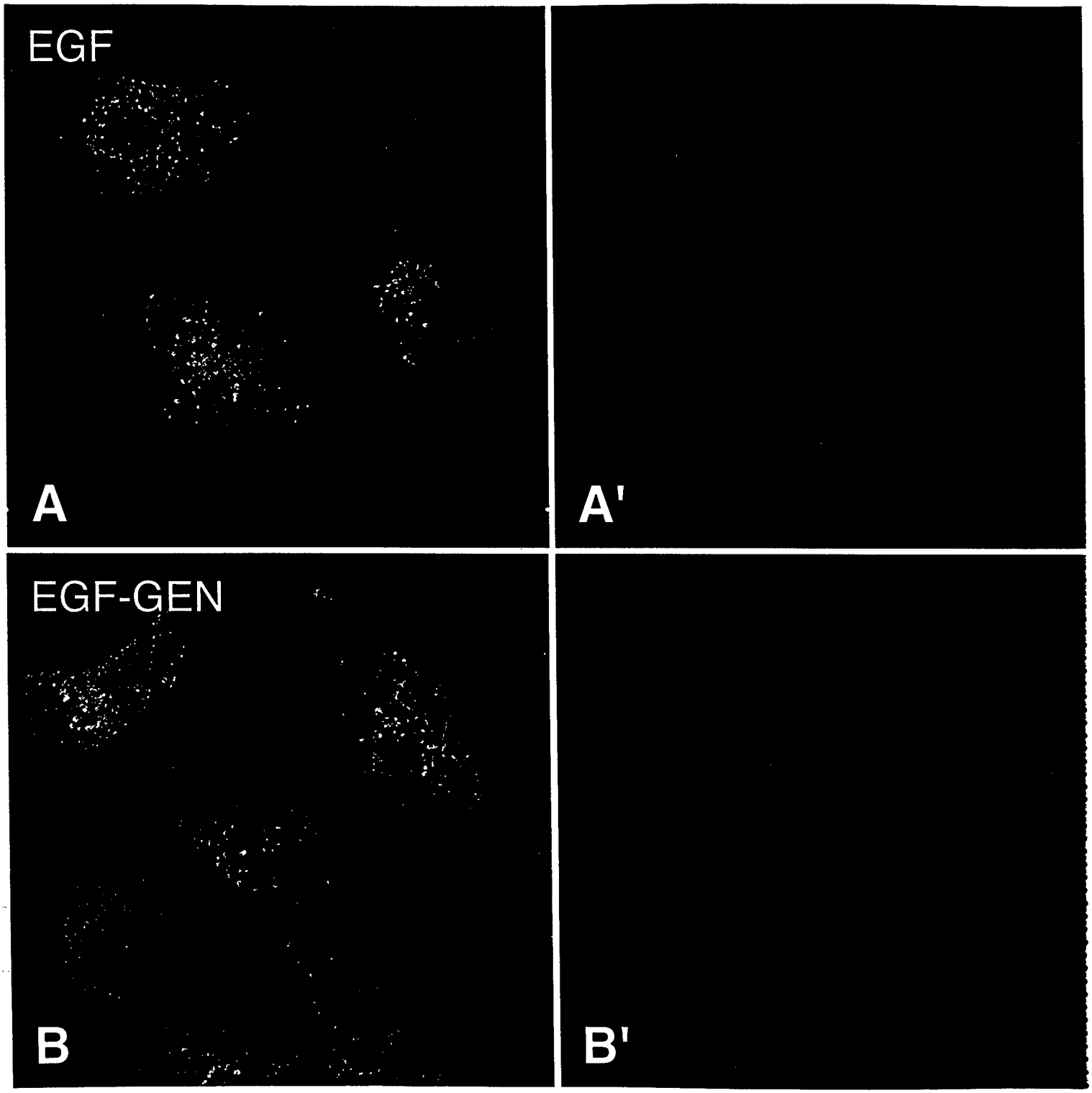

Figure 3

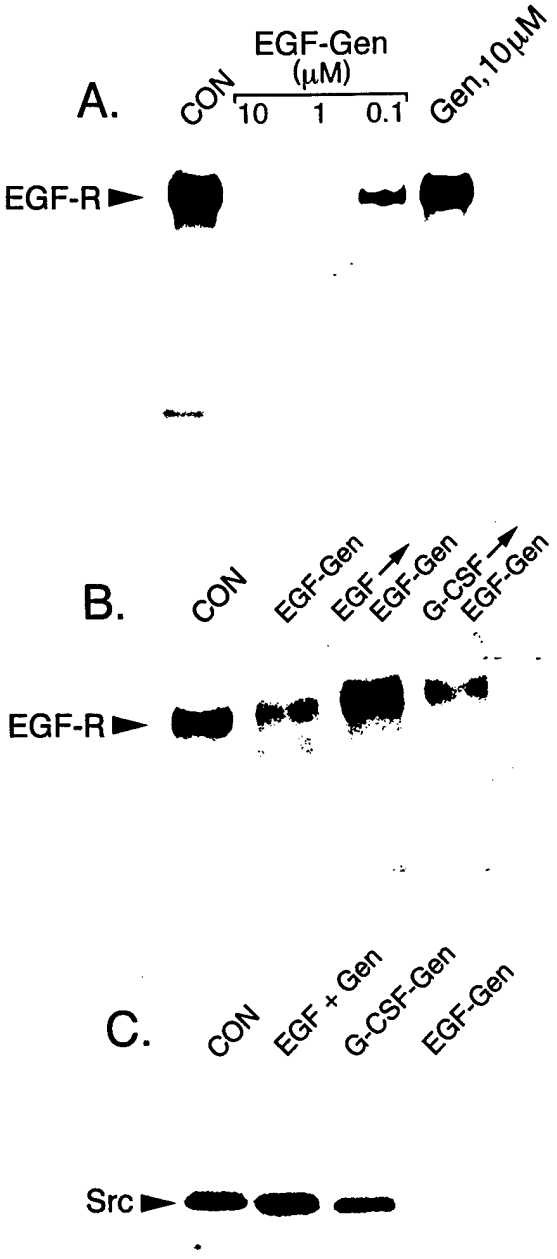
A.
EGF-Gen
(μM)
CON
10
1
0.1
Gen,10μM
EGF-R
B.
CON
EGF-Gen
EGF
EGF-Gen
G-CSF
EGF-Gen
EGF-R
C.
CON
EGF + Gen
G-CSF-Gen
EGF-Gen
Src

Figure 4

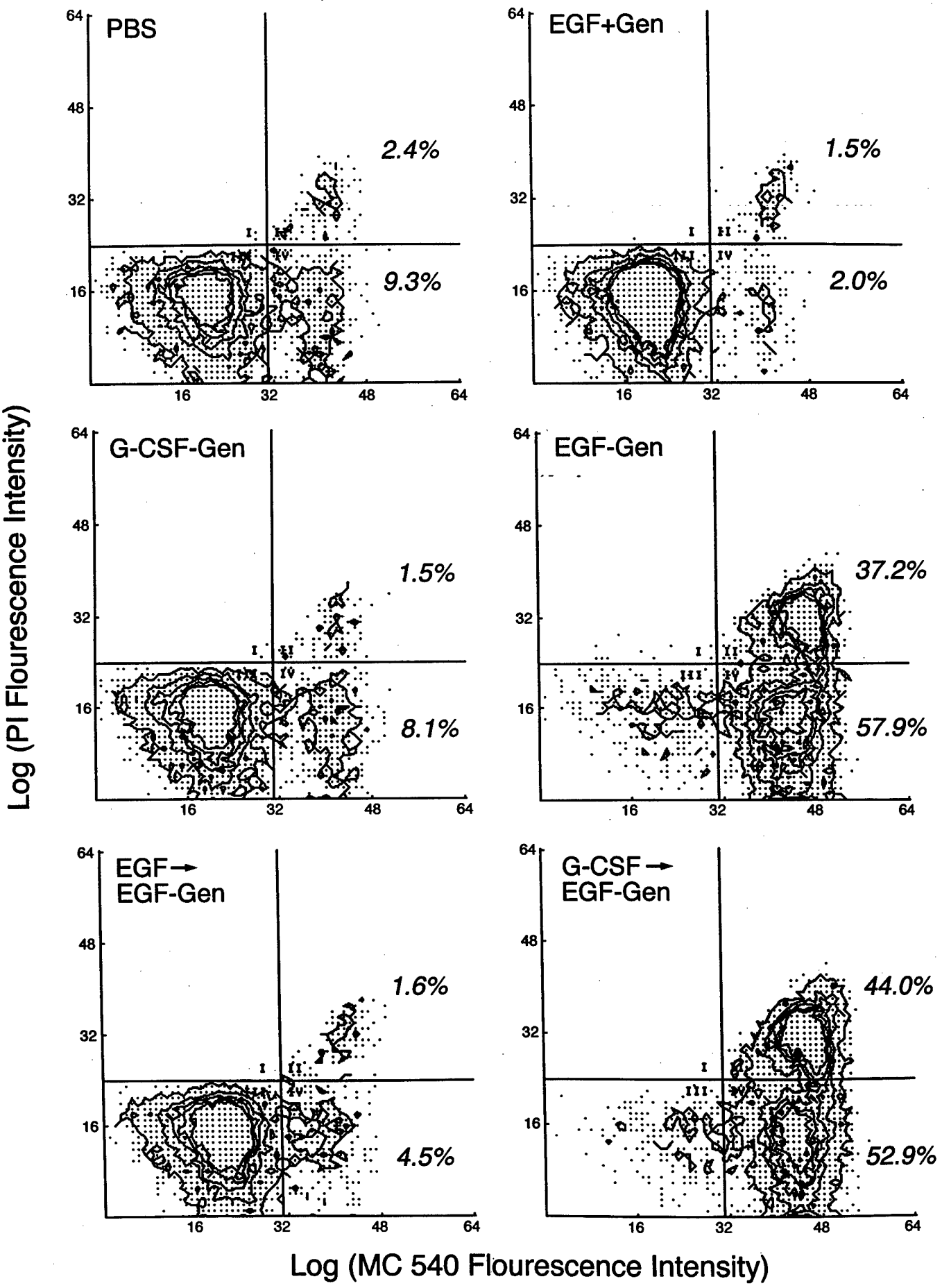
PBS
2.4%
9.3%
EGF+Gen
1.5%
2.0%
G-CSF-Gen
1.5%
8.1%
EGF-Gen
37.2%
57.9%
EGF→
EGF-Gen
1.6%
4.5%
G-CSF→
EGF-Gen
44.0%
52.9%
64
48
32
16
I
II
III
IV
Log (PI Flourescence Intensity)
Log (MC 540 Flourescence Intensity)

Figure 5

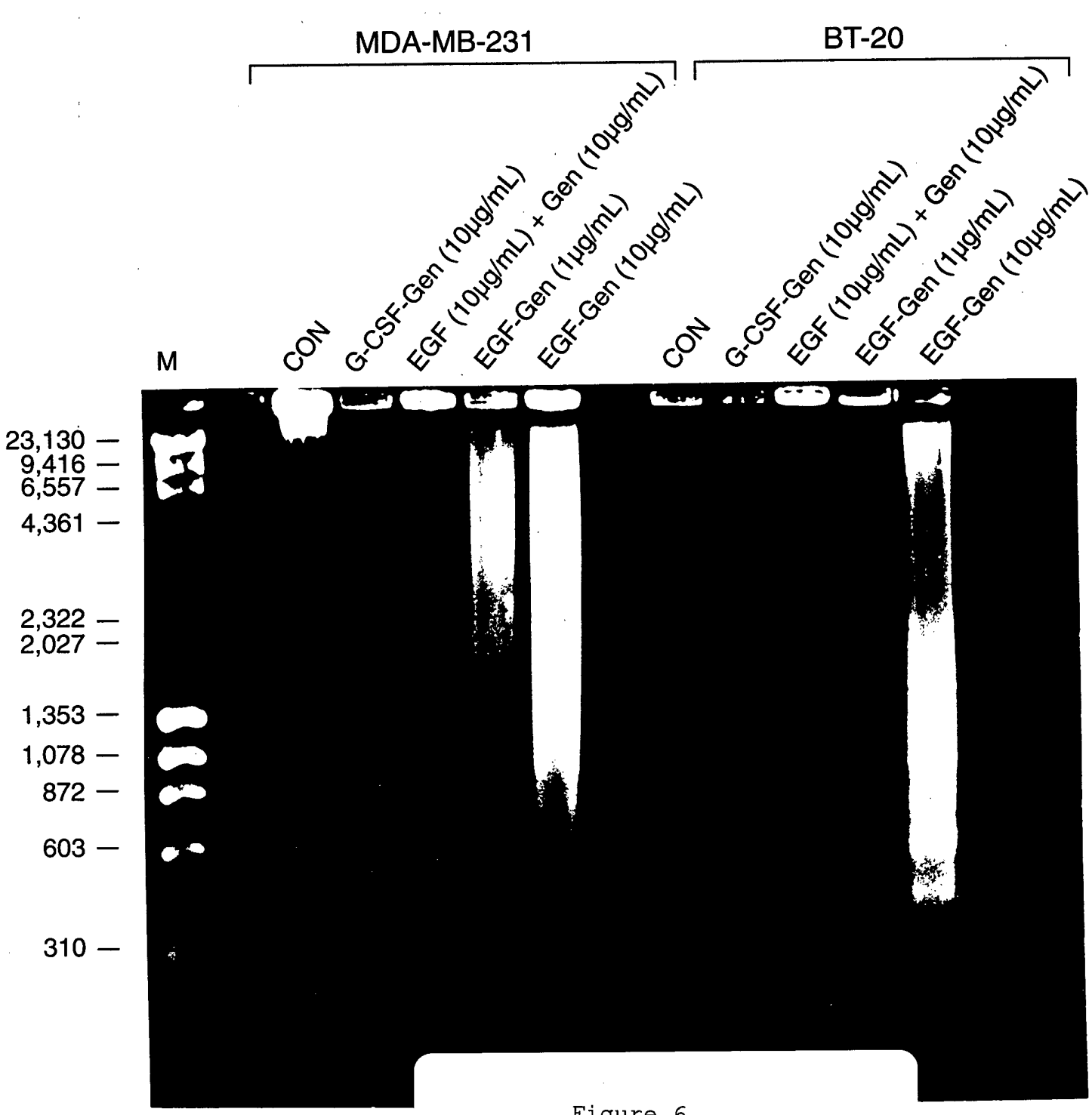
MDA-MB-231
BT-20
M
CON
G-CSF-Gen (10μg/mL)
EGF (10μg/mL) + Gen (10μg/mL)
EGF-Gen (1μg/mL)
EGF-Gen (10μg/mL)
CON
G-CSF-Gen (10μg/mL)
EGF (10μg/mL) + Gen (10μg/mL)
EGF-Gen (1μg/mL)
EGF-Gen (10μg/mL)
23,130
9,416
6,557
4,361
2,322
2,027
1,353
1,078
872
603
310

Figure 6

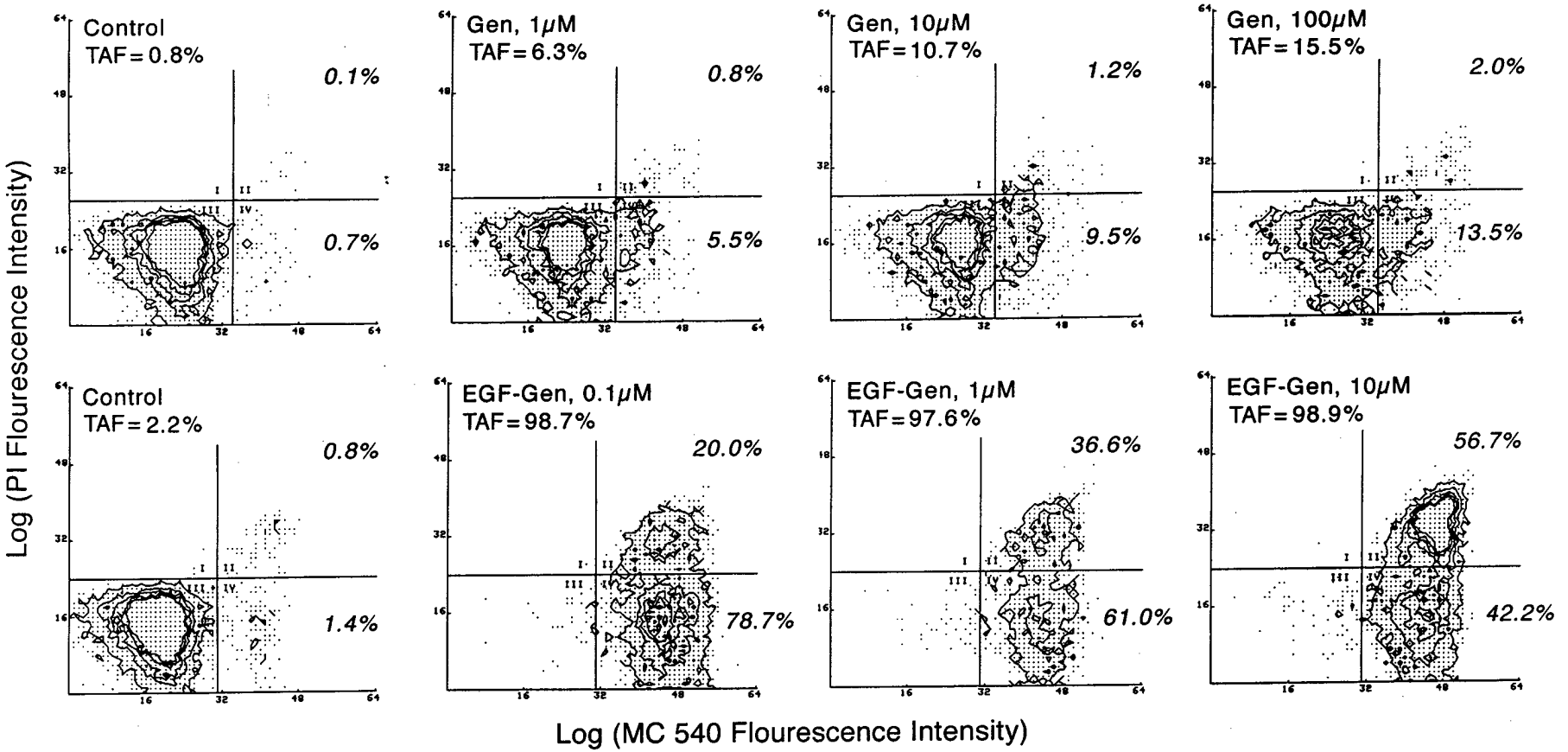
Control
TAF=0.8%
0.1%
0.7%
Gen, 1μM
TAF=6.3%
0.8%
5.5%
Gen, 10μM
TAF=10.7%
1.2%
9.5%
Gen, 100μM
TAF=15.5%
2.0%
13.5%
Control
TAF=2.2%
0.8%
1.4%
EGF-Gen, 0.1μM
TAF=98.7%
20.0%
78.7%
EGF-Gen, 1μM
TAF=97.6%
36.6%
61.0%
EGF-Gen, 10μM
TAF=98.9%
56.7%
42.2%
Log (PI Flourescence Intensity)
Log (MC 540 Flourescence Intensity)

Figure 7

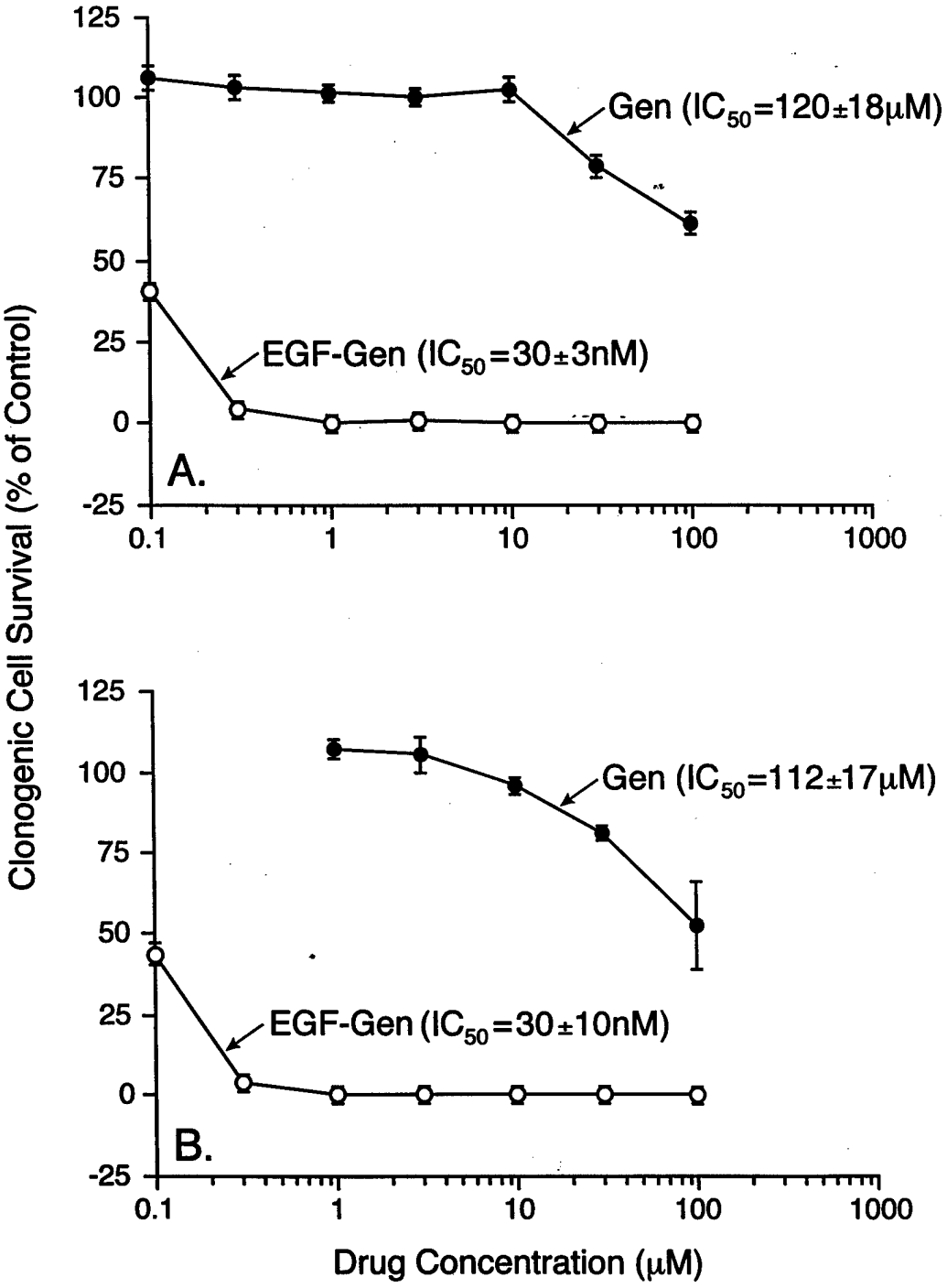
125
100
75
50
25
0
-25
Clonogenic Cell Survival (% of Control)
Gen (IC50=120±18μM)
EGF-Gen (IC50=30±3nM)
A.
0.1
1
10
100
1000
125
100
75
50
25
0
-25
Gen (IC50=112±17μM)
EGF-Gen (IC50=30±10nM)
B.
0.1
1
10
100
1000
Drug Concentration (μM)

Figure 8

Table 1. Specific binding of EGF-^{125}I-Gen to Breast Cancer Cells

EGF-^{125}I-Gen Binding to Breast Cancer Cells

Cell line	-cold EGF (cpm)	+cold EGF (cpm)	Specific binding	Inhibitable binding	p mol/10^6 cells	Molecules per cell
MDA-MB-231	4531	1983	2548	56%	7.5	$4.5x10_6$
BT-20	7511	2663	4848	65%	9.5	$5.7x10_6$
NALM-6	2708	3091	0	None	None	None
HL-60	788	1346	0	None	None	None

^{125}I-EGF Binding to Breast Cancer Cells

Cell line	-cold EGF (cpm)	+cold EGF (cpm)	Specific binding (cpm)	Inhibitable binding	+cold EGF-Gen (cpm)	Inhibition by EGF-Gen	+cold GM-CSF (cpm)	Inhibition by GM-CSF
MDA-MB-231	15,102	1,100	14,002	93%	1,398	91%	15,440	0%
BT-20	17,351	ND	ND	ND	1,624	91%	16,486	5%

^{125}I-Gen Binding to Breast Cancer Cells

Cell line	-cold EGF (cpm)	+cold EGF (cpm)	Specific binding	Inhibitable binding	p mol/10^6 cells	Molecules per cell
MDA-MB-231	852	860	0	None	None	None
BT-20	2439	2540	0	None	None	None
NALM-6	1098	N.D.	N.D.	N.D.	N.D.	N.D.
HL-60	814	N.D.	N.D.	N.D.	N.D.	N.D.

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.

Table 2. Cytotoxic Activity of EGF-Gen Against Clonogenic Breast Cancer Cells

Cell Line	Treatment	Mean No. Colonies Per 1 x 10^5 Cells	Percent Inhibition of Clonogenic Cells
MDA-MB-231	PBS	394 (368,420)	–
Breast Cancer	EGF, 10 µg/mL	524 (512,536)	0.0
	Gen, 10 µg/mL	275 (268,282)	30.2
	EGF-Gen, 10 µg/mL	0 (0,0)	>99.4
	GCSF-Gen, 10 µg/mL	395 (390,400)	0.4
BT-20	PBS	155 (143,167)	–
Breast Cancer	EGF, 10 µg/mL	161 (152,170)	0.0
	Gen, 10 µg/mL	117 (113,121)	24.5
	EGF-Gen, 10 µg/mL	0 (0,0)	>99.4
	GCSF-Gen, 10 µg/mL	154 (150,158)	0.6
PC-3	PBS	298 (287,309)	–
Prostate	EGF, 10 µg/mL	355 (307,403)	0.0
Cancer	Gen, 10 µg/mL	256 (253,259)	14.1
	EGF-Gen, 10 µg/mL	0 (0,0)	>99.7
	GCSF-Gen, 10 µg/mL	309 (298,320)	0.0
NALM-6	PBS	214 (209,219)	–
Pre-B ALL	Gen, 10 µg/mL	175 (162,188)	18.2
$EGFR_c(-)$	EGF-Gen, 10 µg/mL	210 (187,233)	0.0
	B43-Gen, 10 µg/mL	0 (0,0)	>99.5

Cancer cells were treated with the indicated agents for 24 hours at 37°C, washed twice, and then plated in duplicate Petri dishes at 10^5 cells/mL. The clonogenic medium was alpha-MEM supplemented with 0.9% methylcellulose, 30% fetal bovine serum, and 50 µM 2-mercaptoethanol. Colonies were enumerated on day 7 using an inverted phase microscope of high optical resolution.

IN VIVO TOXICITY, PHARMACOKINETICS, AND ANTI-CANCER ACTIVITY OF GENISTEIN LINKED TO RECOMBINANT HUMAN EPIDERMAL GROWTH FACTOR

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[3]The abbreviations used are: EGF-R, epidermal growth factor receptor; Gen, Genistein; PTK, protein tyrosine kinases; EGF, epidermal growth factor.

ABSTRACT

Epidermal growth factor-receptor (EGF-R)-associated protein tyrosine kinase (PTK) complexes have vital anti-apoptotic functions in human breast cancer cells. We have previously shown that targeting the naturally occurring PTK inhibitor genistein to the EGF-R-associated PTK complexes using the EGF-Genistein (Gen) conjugate triggers rapid apoptotic cell death in human breast cancer cells and abrogates their in vitro clonogenic growth. In the present study, we examined the *in vivo* toxicity profile, pharmacokinetics, and anti-cancer activity of EGF-Gen. No toxicities were observed in mice treated with EGF-Gen at dose levels as high as 40 mg/kg administered intraperitoneally (i.p.) as a single dose or 140 mg/kg administered i.p. over 28 consecutive days. EGF-Gen significantly improved tumor-free survival in a SCID mouse xenograft model of human breast cancer, when it was administered 24 hours after inoculation of tumor cells. At 100 μg/kg/d x 10 days (1 mg/kg total dose), which is >100-fold less than the highest tested and nontoxic cumulative dose (i.e., 140 mg/kg) in mice, EGF-Gen was more effective than cyclophosphamide (50 mg/kg/d x 2 days), adriamycin (2.5 mg/kg x 1) or methotrexate (0.5 mg/kg x 1), the most widely used standard chemotherapeutic drugs for breast cancer, and resulted in 60 % long-term tumor-free survival. Furthermore, treating SCID mice with established subcutaneous human breast cancer xenografts of 0.5 cm diameter with EGF-Gen at this dose level resulted in disappearance of the tumors in 2 of 5 mice and >50% shrinkage in 3 of 5 mice within 10 days, whereas all of the control tumors in 5 PBS treated mice as well as 5 mice treated with unconjugated Gen (1 mg/kg/day x 10 days) showed >200% increase in diameter during the same observation period. EGF-Gen treatment reduced the growth rate of breast cancer xenografts of 1.0 cm diameter, but unlike with tumors of 0.5 cm diameter, it failed to cause shrinkage or disappearance of these larger tumors. The level of EGF-Gen systemic exposure that was effective in SCID mice was achieved in cynomolgus monkeys without any significant side effects detectable by clinical observation, laboratory studies, or histopathological examination of multiple organs. EGF-Gen might be useful in treatment of breast cancer as well as other EGF-R positive malignancies.

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 USA (1). Front-line cytotoxic chemotherapy of metastatic breast cancer with the most effective regimens offers a median duration of response of only 8 months and once patients progress after the front-line therapy, the response rate is only 20-35% for second-line combination chemotherapy (2-4). Thus, currently, the major challenge in the treatment of breast cancer is to cure patients who have metastatic disease (5).

Human epidermal growth factor (EGF) is a 53 amino acid, single-chain polypeptide (Mr 6216 daltons), which exerts biologic effects by binding to a specific 170 kDa cell membrane epidermal growth factor receptor (EGF-R/ErbB-1) (6-9). 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). In breast cancer, expression of the EGF-R is a significant and independent indicator for recurrence and poor relapse-free survival (10-12).

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 protein tyrosine kinase (PTK) activity. Binding of EGF to the 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, Erb-B3), resulting in activation with autophosphorylation of the PTK domain (13, 14). EGF-R is physically and functionally associated with Src protooncogene family PTK, including $p60^{src}$ (13-15). 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 (15-17).

Our recent studies provided evidence that the membrane-associated EGF-receptor (R)-protein tyrosine kinase (PTK) complexes serve as endogenous negative regulators of apoptosis in breast cancer cells (18). We therefore postulated that the EGF-R, similar to the CD19 receptor on leukemia and lymphoma cells

(19), may be a suitable target for biotherapy using tyrosine kinase inhibitors. Genistein (Gen), an isoflavone (5,7,4'-trihydroxyisoflavone) from fermentation broth of *Pseudomonas spp.*, is a naturally occurring tyrosine kinase inhibitor present in soybeans (20). We found that targeting Gen to the EGF-R-PTK complexes in breast cancer cells using the EGF-Gen conjugate triggers apoptotic cell death (18). The purpose of the present study was to further evaluate the clinical potential of this membrane-directed apoptosis induction strategy by examining the *in vivo* toxicity profile, pharmacokinetics, and efficacy of EGF-Gen in preclinical animal model systems.

MATERIALS AND METHODS

Preparation of the EGF-Genistein Conjugate. EGF-Gen was produced by conjugating recombinant human EGF to genistein (Gen) according to a recently published photochemical conjugation method using the hetero-bifunctional photoreactive crosslinking agent, Sulfosuccinimidyl 6-[4'azido-2'-nitrophenylamino]hexanoate (Sulfo-SANPAH) (Pierce Chemical Co., Rockford, IL), as previously described in detail (18). The chemical composition and *in vitro* biologic activity of EGF-Gen were previously reported (18).

Cross-reactivity of Human EGF and Anti-Human EGF-R Antibodies with Mouse EGF-R. Livers and thymus of BALB/c mice were frozen in liquid nitrogen and 5 μm thick tissue sections were prepared using a cryostat. The sections were fixed in 2% paraformaldehyde (pH 7.4) and processed for standard indirect immunofluorescence using a monoclonal antibody directed to the extracellular domain of the human EGF-R (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA) as the primary antibody and a goat anti-mouse IgG conjugated to FITC (Amersham Corp., Arlington Heights, IL) as the secondary antibody. In parallel, sections were also stained by direct immunofluorescence staining techniques with FITC-conjugated EGF (Molecular Probes, Inc., Eugene, OR) in Hank's Balanced Salt buffer containing BSA, 0.1% sodium azide, and 20 mM HEPES (pH 7.0) according to the manufacturer's recommendations. Coverslips were mounted with Vectashield containing propidium iodide (Vector Labs, Burlingame, CA) to stain the nuclei.

Mouse Toxicity Studies. The toxicity profile of EGF-Gen in BALB/c mice was examined, as previously reported for other biotherapeutic agents (19, 21). All BALB/c mice used in the toxicity studies were obtained from the specific pathogen free (SPF) breeding facilities of the National Institutes of Health (NIH; Bethesda, MD) at 6 - 8 weeks of age. The mice were housed in an American Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved specific pathogen-free mouse facility. All husbandry and experimental contact made with the mice maintained SPF conditions. The mice were kept in Micro-Isolator cages (Lab Products, Inc., Maywood, NY) containing autoclaved food, water and bedding. Female BALB/c mice were used and monitored daily for lethargy, cleanliness and morbidity. At the time of death, necropsies were performed and the toxic effects of immunoconjugate administration were assessed. For histopathologic studies, tissues were fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin by routine methods. Glass slides with affixed 6 micron tissue sections were prepared and stained with Hemotoxylin and Eosin (H&E).

In single dose toxicity studies, female BALB/c mice were administered an i.p. bolus injection of EGF-Gen in 0.2 ml PBS, or 0.2 ml PBS alone (control mice). In cumulative toxicity studies, mice received a total of 2800 μg (=140 mg/kg) EGF-Gen i.p. over 28 consecutive days. No sedation or anesthesia was used throughout the treatment period. Mice were monitored daily for mortality for determination of the day 30 LD50 values. Mice surviving until the end of the 30 days monitoring were sacrificed and the tissues were immediately collected from randomly selected mice, and preserved in 10% neutral buffered formalin. Standard tissues collected for histologic evaluation included: bone, bone marrow, brain, cecum, heart, kidney, large intestine, liver, lung, lymph node, ovary, pancreas, skeletal muscle, skin, small intestine, spleen, stomach, thymus, thyroid gland, urinary bladder, and uterus (as available).

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. MDA-MB-231 cell line was maintained in RPMI 1640 medium 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 incubated for 5-10 min at 37°C until cells detached. Fresh medium was then added, cells aspirated and dispensed into new flasks.

In some experiments, the cytotoxic activity of plasma samples from EGF-Gen treated cynomolgus monkeys was examined using a methylcellulose colony assay system (18, 22). In brief, MDA-MB-231 cells (10^7/mL in RPMI + 10% FBS) were treated overnight at 37°C with 1: 20 (v/v) PBS-diluted plasma samples from EGF-Gen-treated monkeys. After treatment, cells were washed twice, plated at 10^6 cells/mL in RPMI + 10% FBS + 0.9% methylcellulose in Petri dishes, and cultured for 7 days at 37°C in a humidified 5% CO2 incubator. Subsequently, MDA-MB-231 colonies containing > 20 cells were enumerated using a inverted phase-contrast microscope and the % inhibition of colony formation was calculated using the formula: % Inhibition = 1 - (Mean No. Colonies in Test Culture/Mean No. Colonies in Control Culture)x100. In some experiments, excess unconjugated EGF was added to the plasma samples to block the action of EGF-Gen by competing for the EGF-R on cancer cells. Excess G-CSF was used as a control.

Maintenance of SCID Mouse Colony. The SCID mice were housed in an AAALAC-approved specific pathogen-free facility. Animal housing was located in a secure indoor facility with controlled temperature, humidity, and noise levels. The SCID mice were housed in microisolater cages which were autoclaved with rodent chow. Water was also autoclaved and supplemented with trimethoprim/sulfomethoxazol 3 days/week.

SCID Mouse Xenograft Model of Human Breast Cancer. The left hind legs of the CB. 17 SCID mice were inoculated s.c. with $1x10^6$ MDA-MB-231 breast cancer cells in 0.2 mL PBS.
SCID mice inoculated with human breast cancer cells were treated with EGF-Gen (0.2 µg/dose = 10 µg/kg/dose or 2.0 µg/dose = 100 µg/kg/dose in 0.2 ml PBS) with daily i.p doses for 10 treatment days starting the day after inoculation of cancer cells. Daily treatments with PBS, 10 µg (=500 µg/kg) G-CSF-Gen, 10 µg Gen (=500 µg/kg) combined with 10 µg (500 µg/kg) EGF or 10 µg (500 µg/kg) Gen alone were used as controls. 50 µg (2.5 mg/kg) Adriamycin (Ben Venue Laboratories, Inc., Bedford, OH

44146) or 9.3 μg (= 465 μg/kg) methotrexate (Lederle Parenterals, Inc., Carolina, Puerto Rico 00630) were given as single dose i.p. bolus injections on the day after inoculation of cancer cells. 1 mg (50 mg/kg) cyclophosphamide (Bristol-Myers Squibb Co., Princeton, New Jersey 08543) was injected i.p. on two consecutive days starting the day after inoculation of cancer cells. Mice were monitored daily for health status and tumor growth, and were sacrificed if they became moribund, developed tumors which impeded their ability to attain food or water, or at the end of the 7-month observation period. For histopathologic studies, tissues were fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin by routine methods. Glass slides with affixed 6 micron tissue sections were prepared and stained with hematoxylin/eosin. Primary endpoints of interest were tumor growth and tumor -free survival outcome. Estimation of life table outcome and comparisons of outcome between groups were done, as previously reported (19-21). The efficacy of EGF-Gen against established tumors was examined by treating SCID mice with subcutaneous MDA-MB-231 xenografts of 0.5 cm or 1.0 diameter with 100 μg/kg/day EGF-Gen i.p on 10 consecutive days and determining the tumor diameter daily for 20 days from the start of therapy. Control mice were treated with 0.2 ml PBS for 10 consecutive days.

Cynomolgus Monkey Experiments. Female cynomolgus monkeys were obtained from BioMedical Resources Foundation of Houston, TX. The monkeys were housed in the AAALAC-accredited primate facility. The monkeys were singly housed in stainless steel cages and provided with toys and treats for enrichment. Prior to entering the study, the monkeys were housed in a quarantine-room in the same facility for 6 weeks. During this time, they were TB-tested three times, serologically screened for Herpes virus simiae, and screened for enteric bacterial, protozoal, and helminth pathogens. In pharmacodynamic studies, monkeys were fasted overnight prior to anesthesia and treatment. After induction of anesthesia (Ketamine hydrochloride 10-15 mg/kg), a catheter was placed percutaneously either into the right or left cephalic vein using a sterile disposable kit. This catheter was taped in place for administration of EGF-Gen or maintenance fluids (Normal saline at 4 ml/kg/hr via an infusion pump) and for drawing of blood samples. A Harvard infusion pump was used to administer EGF-Gen as a constant intravenous infusion over a 1 hour period.

Pharmacokinetic Studies. Tissue distribution studies in SCID mice were performed using EGF-^{125}I-Gen and ^{125}I-Gen, as described in detail in previous publications from our laboratory (19). A flow-limited physiological pharmacokinetic model was used to characterize the tissue disposition of EGF-Gen in non-tumor bearing as well as tumor bearing SCID mice (19, 23). Tissue volumes and plasma flow rates were those previously described for mice (24). A set of linear differential equations describing the mass balances of each model compartment was used to estimate tissue partition coefficients (i.e., the ratio of the drug concentration in the tissue of interest to the drug concentration in the plasma at equilibrium) for each organ. These differential equations were simultaneously solved using the ADAPT II software (25). Biliary excretion and gut reabsortion were incorporated into the physiological model in the form of saturable processes, based on previous studies establishing saturable biliary excretion of unconjugated recombinant human EGF (26). In plasma half-life studies in SCID mice and cynomolgus monkeys, the EGF-Gen conjugate concentrations were measured in the plasma samples using the EGF Quantikine ELISA kit from R&D Systems, a quantitative sandwich enzyme immunoassay, which allows the detection of the EGF-Gen conjugate via its EGF moiety. In these studies, EGF-Gen was administered to SCID mice by intraperitoneal injection at doses of 100 μg/kg and 1 mg/kg. Four mice were used at each dose level, and blood samples were obtained at six non-overlapping time points from each pair of mice. Mice were serially bled by retroorbital puncture at 0 min, 10 min, 30 min, 1 hour, 2 hours, 4 hours, and 12 hours following the administration of the first bolus dose of EGF-Gen. In cynomolgus monkeys, EGF-Gen was administered on each of the 10 treatment days intravenously over 1 hour and plasma samples were obtained at 0 min, 30 min, 45 min, 1 hour, 2 hours, 4 hours, 8 hours, and 12 hours post-infusion time points after the first dose of EGF-Gen. A two compartment first-order pharmacokinetic model was fit to the plasma concentration-versus-time data for EGF-Gen. Maximum likelihood estimation as implemented in ADAPT II software, was used to estimate the central compartment volume of distribution, elimination rate constant, and distribution rate constants for EGF-Gen, as previously described (19-21, 25).

RESULTS AND DISCUSSION

Biodistribution and Toxicity of EGF-Gen in Mice. Human EGF binds to murine EGF-R in mouse tissues, as determined by immunocytochemistry (**Figure 1**). Because of the crossreactivity of human EGF and EGF-Gen with murine EGF-R, we decided to use mice in the initial evaluation of the biodistribution and toxicity of EGF-Gen. Tissue distribution studies were performed using EGF-^{125}I-Gen and a flow-limited physiological pharmacokinetic model was used to characterize the *in vivo* tissue disposition of EGF-Gen in non-tumor bearing as well as tumor bearing SCID mice. For each organ, the partition coefficients (i.e., R values = the tissue-to-plasma equilibrium distribution ratios for linear binding) were determined, as previously described (19). When compared to unconjugated Gen, a much greater amount of EGF-Gen partitioned to bone marrow (R bone marrow: 0.00008 ml/g for Gen vs 0.53 ml/g for EGF-Gen), spleen (R spleen: 0.04 ml/g for Gen vs 5.28 ml/g for EGF-Gen), liver (R liver: 0.50 ml/g for Gen vs 7.40 ml/g for EGF-Gen), kidney (R kidney: 0.25 ml/g for Gen vs 0.93 ml/g for EGF-Gen), and lungs (R lungs: 0.53 ml/g for Gen vs 1.44 ml/g for EGF-Gen) (**Table 1**). In both tumor bearing and non-tumor bearing mice, EGF-Gen most extensively partitioned to the liver with tissue drug concentrations exceeding plasma concentrations more than seven times ($P<0.05$) (**Table 1**). By contrast, very little EGF-Gen partitioned to the subcutaneous xenografts in tumor bearing SCID mice. The partition coefficients for the liver and tumor were 8.2 and 0.2, respectively (**Table 1**).

In toxicity studies, 28 female BALB/c mice were injected intraperitoneally with a single bolus dose of 2 μg (=100 μg/kg) - 800 μg (=40 mg/kg) EGF-Gen in 0.2 ml PBS. EGF-Gen was not toxic to mice at any of these dose levels; none of the mice experienced any side effects or died of toxicity during the 30 day observation period. Even at the highest doses of 400 μg or 800 μg (= 40 mg/kg) EGF-Gen, mice did not become weak or lethargic, lose weight, or develop diarrhea or a scruffy skin. When mice were treated with multiple doses of EGF-Gen at a total dose level of 2.8 mg (=140 mg/kg) according to a 100 μg/mouse/day (= 5 mg/kg/day) x 28 days schedule, no significant toxicity was observed and none of the

10 mice died. No histopathologic lesions were found in any of the organs of EGF-Gen treated mice receiving a single dose or multiple doses of EGF-Gen, including the liver which had the highest partition coefficient in tissue distribution studies. Thus, the maximum tolerated dose (MTD ~ LD10) of EGF-Gen was not reached at the 40 mg/kg single dose level or the 140 mg/kg cumulative dose level.

***In Vivo* Anti-tumor Activity of EGF-Gen in a SCID Mouse Xenograft Model of Human Breast Cancer.** CB.17 SCID mice developed rapidly growing tumors after subcutaneous inoculation of $1x10^6$ MDA-MB-231 cells. We examined the *in vivo* anti-tumor activity of EGF-Gen in this SCID mouse xenograft model of human breast cancer. EGF-Gen significantly improved tumor-free survival in a dose-dependent fashion, when it was administered 24 hours after inoculation of tumor cells. At a dose level of 100 μg/kg/d x 10 days (1 mg/kg total dose), which is >100-fold less than the highest tested and nontoxic cumulative dose (i.e., 140 mg/kg) in mice, EGF-Gen was more effective than cyclophosphamide (50 mg/kg/d x 2 days), adriamycin (2.5 mg/kg x 1) or methotrexate (0.5 mg/kg x 1), the most widely used standard chemotherapeutic drugs for breast cancer. **Figure 2** shows the tumor-free survival outcome of SCID mice treated with EGF-Gen, chemotherapeutic drugs (CHEMO = cyclophosphamide, adriamycin, or methotrexate), or control agents after inoculation with MDA-MB-231 breast cancer cells. None of the 60 control mice (CON) treated with PBS (N=40), G-CSF-Gen (100 μg/kg/day) (N=10), or unconjugated Gen (100 μg/kg/day) (N=10) remained alive tumor-free beyond 108 days (median tumor-free survival = 52 days) (**Figure 2A**). All of the 10 mice treated with EGF plus Gen developed tumors within 45 days with a median tumor-free survival of only 39 days. Tumors reached a size of 0.5 cm^3 by 84±3 days in PBS, G-CSF-Gen, or Gen treated CON mice, 62± 1 days in EGF plus Gen treated mice, and 77 ± 16 days in the CHEMO group (**Figure 2B**). Within the CHEMO group, all of the adriamycin-treated mice developed rapidly growing tumors with a median tumor-free survival time of only 42 days. Tumors reached a size of 0.5 cm^3 by 92 ± 6 days in cyclophosphamide-treated mice, 72 ± 5 days in adriamycin treated mice, and 71 ± 5 days in methotrexate-treated mice. By comparison, 40 ± 16% of mice treated for 10 consecutive days with 10 μg/kg/day EGF-Gen survived tumor-free beyond 3 months and 20 ± 13% were still alive tumor-free at 7 months (median tumor-free survival time = 97 days; CON vs 10 μg/kg/day EGF-Gen, $P<0.0001$ by log-rank test). Remarkably, 60

± 16% of mice treated for 10 consecutive days with 100 μg/kg/day EGF-Gen remained alive free of detectable tumors for more than 7 months (CON vs 100 μg/kgday EGF-Gen, P<0.00001 by log-rank test) (**Figure 2**). Tumors developing in EGF-Gen-treated mice reached the 0.5 cm^3 tumor size much later than control mice (114 ± 8 days [10 μg/kg/day dose level] and 129 ± 14 days [100 μg/kg/day dose level] vs 84± 3 days, P=0.007 (10 μg/kg/day dose level) and P<0.001 (100 μg/kg/day dose level)). The average size (mean ± SE) of tumors at 90 days and 120 days were 0.257±0.059 cm^3 and 0.822±0.146 cm^3, respectively for mice in the CON group. By comparison, the average size (mean ± SE) of tumors at 90 days and 120 days were significantly smaller at 0.013± 0.007 cm^3 (P=0.009) and 0.166 ± 0.083 cm^3 (P=0.006) for mice treated with EGF-Gen at 100 μg/kg/day dose level. Thus, EGF-Gen elicited significant *in vivo* anti-tumor activity at non-toxic doses. The inability of 10 μg (=500 μg/kg)/day x 10 days of unconjugated Gen (= 37,000 pmols) in combination with unconjugated EGF to confer tumor-free survival in this SCID mouse model in contrast to the potency of 2 μg (=100 μg/kg)/day x 10 days EGF-Gen containing 309 pmols of Gen demonstrates that (a) the in vivo anti-tumor activity of EGF-Gen cannot be attributed to its EGF moiety alone and (b) conjugation to EGF enhances the anti-tumor activity of Gen against breast cancer cells by >100-fold.

In contrast to EGF-Gen, cyclophosphamide (50 mg/kg/day x 2 days; N=5), adriamycin (2.5 mg/kg, N=10), or methotrexate (0.5 mg/kg, N=5) did not significantly affect tumor development in this SCID mouse model. Of the 20 mice treated with one of these chemotherapeutic drugs, only 10±7 % remained tumor-free beyond 3 months, which indicates no improvement over the control group and a worse tumor-free survival outcome compared to the 2.0 μg/day EGF-Gen group (median tumor-free survival = 48 days; CON vs CHEMO, P= 0.32; EGF-Gen, 2.0 μg/day vs CHEMO, P<0.05) (**Figure 2B**)

Furthermore, treating SCID mice with established subcutaneous human breast cancer xenografts of 0.5 cm diameter with EGF-Gen at this dose level resulted in eradication of the tumors in 2 of 5 mice and >50% shrinkage in 3 of 5 mice within 10 days. The day 10 diameters of tumors in the EGF-Gen treated group were 0.1 cm, 0.0 cm, 0.0 cm, 0.2 cm, and 0.1 cm with a mean (± SE) diameter of 0.08 ± 0.04 cm

(**Figure 3A**). There was no significant tumor progression between days 10 and 20 in this group of mice. The day 20 tumor diameters were 0.3 cm, 0.0 cm, 0.0 cm, 0.1 cm, and 0.2 cm with a mean (±SE) diameter of 0.12 ± 0.06 cm. In contrast to the tumors in EGF-Gen-treated mice, all of the control tumors in 5 PBS treated mice as well as 5 mice treated with unconjugated Gen (1 mg/kg/day x 10 days) showed >200% increase in diameter within 10 days: The day 10 tumor diameters ranged from 1.0 to 1.4 cm (mean ±SE = 1.24 ± 0.08 cm) in PBS-treated mice and from 1.2 to 1.4 cm (mean ±SE= 1.28 ±0.04 cm) in Gen-treated mice (P values <0.0001 for EGF-Gen vs PBS as well as EGF-Gen vs Gen). These tumors continued their rapidly progressive growth and the day 20 dimaters ranged from 1.8 to 2.6 cm (mean ±SE= 2.20±0.16 cm) in PBS-treated mice and from 1.9 to 2.4 (mean ±SE = 2.12 ± 0.10 cm) in Gen-treated mice (**Figure 3A**).

EGF-Gen treatment significantly reduced the growth rate of breast cancer xenografts of 1.0 cm diameter during the 20-day observation period but unlike with tumors of 0.5 cm diameter, it failed to cause shrinkage or disappearance of these larger tumors (**Figure 3B**). The day 10 tumor diameters ranged from 2.4 to 3.3 cm (mean ± SE = 2.90 ± 0.21 cm) in PBS-treated mice and from 2.3 to 3.5 cm (mean ± SE = 2.90 ± 0.25 cm) in Gen-treated mice. By comparison, the tumor diameters in EGF-Gen-treated mice ranged from 1.3 to 2.3 cm (mean ± SE = 1.70 ± 0.23 cm) (P valuues: EGF-Gen vs PBS = 0.008; EGF-Gen vs Gen = 0.009) (**Figure 3B**).

In Vivo **Pharmacokinetic Features of EGF-Gen in SCID mice.** We sought to determine the therapeutic systemic exposure levels of EGF-Gen by examining its pharmacokinetics when administered at dose levels which were effective in the SCID mouse xenograft model of human breast cancer. Thus, SCID mice were treated with daily intraperitoneal (i.p) bolus injections of 10 μg/kg or 100 μg/kg EGF-Gen for 10 consecutive days. The differences between various pharmacokinetic parameters of healthy mice versus mice with breast cancer xenografts were calculated based on 95% confidence intervals provided by ADAPT II software. EGF-Gen was cleared rapidly from blood with an elimination half-life of 1.3 (± 0.2)-1.6 (± 0.4) hours (**Table 2, Figure 4A**). At the lower dose level, EGF-Gen was cleared more rapidly from blood [13.1 (± 2.4) vs 6.4 (± 0.6) ml/hr/g], had a larger central volume of distribution

[30.0 (± 3.6) vs 11.6 (± 1.3) ml/g], had a lower measured maximum plasma concentration (0.33 vs 0.45 ng/ml), and yielded a lower systemic exposure level (i.e., area under the concentration-time curve) [7.6 (± 1.4) vs 16.0 (± 1.5) μgxhr/L] in SCID mice with human breast cancer xenografts than in healthy SCID mice that were not inoculated with breast cancer cells ($P<0.05$) (**Table 2, Figure 4A**). The differences between these parameters were calculated based on 95% confidence intervals provided by ADAPT II software. These results suggested that EGF-Gen likely binds to EGF-R^+ human breast cancer cells infiltrating SCID mouse tissues, resulting in more rapid removal from plasma in mice with metastatic human breast cancer. The systemic exposure level, as measured by the area under the serum concentration-time curve (AUC), achieved by the therapeutically effective 10 μg/kg/day dose level of EGF-Gen was 16.0 (± 1.5) μg*hr/L in non-tumor bearing healthy SCID mice and 7.6 (± 1.4) μg*hr/L in SCID mice bearing 1 cm^3 MDA-MB-231 tumors (**Table 2**). By comparison, treatment with 100 μg/kg/day x 10 days of EGF-Gen yielded an AUC of 2564 (± 231) μgxhr/L in non-tumor bearing mice and an AUC of 3125 (±281) μgxhr/L in tumor-bearing mice. Thus, the AUC showed a dramatic 160-fold (2564 μgxhr/L vs 16 μgxhr/L, $P<0.001$) to 411-fold (3125 μgxhr/L vs 7.6 μgxhr/L, $P<0.001$) increase as the dose of EGF-Gen was increased 10-fold. This dramatic increase in AUC which was accompanied by a dramatic 232-fold [11.6 (± 1.3) ml/g vs 0.05 (± 0.01) ml/g, $P<0.001$] to 600-fold [30.0 (± 3.6) ml/g vs 0.05 (±0.01) ml/g, $P<0.001$) decrease of the volume of distribution is most likely due to a saturable receptor-dependent binding and uptake of EGF-Gen, that has been reported to occur with unconjugated human EGF in rats at a dose level of 100 μg/kg (27). As a result of the increase in AUC, the clearance (i.e., Dose/AUC) of EGF-Gen significantly decreased with this dose escalation (**Table 2, Figure 4A**). The dose-dependent decrease in clearance was not associated with significant differences in $t1/2\beta$ values [1.3 ± 0.2 vs 2.1 ± 0.3 for non-tumor bearing mice, and 1.6 ± 0.4 vs 1.0 ± 0.1 for tumor bearing mice], which is in accord with the published observations of Kim et al. (26, 27). These results taken together with previous reports regarding the pharmacokinetics of unconjugated EGF are consistent with the notion that the initial redistribution of EGF-Gen from plasma to EGF-R^+ cells in various tissues determining the $t1/2\alpha$ values is affected by factors influencing the binding of EGF-Gen to EGF-R^+ cells (e.g. affinity of the EGF-Gen conjugate for EGF-R, number of EGF-R^+ targets in the extravascular

compartments), while the later phase of removal from plasma determining the t1/2β values is likely affected by the EGF-R turnover rates and dose-independent disassociation of EGF-Gen from surface EGF-R molecules.

Pharmacodynamic Features and Toxicity of EGF-Gen in Cynomolgus Monkeys. Because EGF-Gen was not toxic to healthy mice even at doses as high as 40 mg/kg given as a single dose or 140 mg/kg given in multiple doses despite the cross-reactivity of human EGF with murine EGF-R, we postulated that such systemic exposure levels could also be achieved in cynomolgus monkeys without excessive toxicity. To test this hypothesis, we measured in cynomolgus monkeys the systemic exposure levels achieved after treatment with 50 μg/kg/day x 10 days and 100 μg/kg/day x 10 days. The plasma concentration-time curves of EGF-Gen in monkeys were also biphasic (**Figure 4B**). The volume of distribution and clearance tended to decrease as the daily dose increased from 50 μg/kg to 100 μg/kg, similar to what was observed in mice. As shown in **Table 2,** treatment with 100 μg/kg/day EGF-Gen yielded an AUC of 1400 μg*hr/L. This systemic exposure level is much higher than the target AUC of 16 μg*hr/L, which was found to be effective in the SCID mouse model of human breast cancer.

Notably, no clinical or laboratory evidence of significant toxicity was observed in these monkeys, except for a transient alopecia in two of the monkeys (**Table 3**). In particular, we observed no gastrointestinal or hepatic toxicity. No histopathologic lesions were found in the organs of EGF-Gen treated monkeys that were electively euthanized. Thus, EGF-Gen concentrations higher than those which are required to elicit therapeutic efficacy against human breast cancer cells in the SCID mouse xenograft model of human breast cancer were achieved in cynomolgus monkeys without significant systemic toxicity.

We also examined the anti-cancer activity of plasma samples from EGF-Gen-treated monkeys by determining their ability to inhibit the in vitro clonogenic growth of the human breast cancer cell lines MDA-MB-231 and BT-20. As detailed in **Table 4**, 1: 50 PBS-diluted plasma samples obtained at 1 hour after treatment with 1 mg/kg EGF-Gen (but not 1:50 diluted pretreatment plasma samples from the same monkeys) abrogated the in vitro colony formation by these breast cancer cell lines. Notably, excess

unconjugated EGF (but not excess G-CSF) could competitively block the cytotoxicity of the EGF-Gen-containing monkey plasma samples (**Table 4**). These results confirmed the biologic activity and stability as well as EGF-R-specificity of the circulating EGF-Gen molecules in cynomolgus monkeys.

Protein tyrosine kinases have long been suspected to play pivotal roles in regulation of cell survival in cancer cells (19, 28-37). Our recent studies provided experimental evidence that the EGF-R-associated PTK complexes are of vital importance for the survival of breast cancer cells and therefore EGF-R may serve as a suitable target for biotherapy of breast cancer using PTK inhibitors (18). EGF-Gen is an experimental anti-cancer drug which targets the naturally occurring PTK inhibitory isoflavone Gen to the membrane-associated anti-apoptotic EGF-R/PTK complexes and triggers apoptotic cell death (18). In the present study, we examined the in vivo anti-cancer activity, pharmacokinetic features, as well as toxicity profile of EGF-Gen. Here, we presented experimental evidence that EGF-Gen displays significant anti-tumor activity in a SCID mouse xenograft model of human breast cancer. EGF-Gen significantly improved tumor-free survival in a SCID mouse xenograft model of human breast cancer, when it was administered 24 hours after inoculation of tumor cells. Furthermore, treating SCID mice with established subcutaneous human breast cancer xenografts of 0.5 cm diameter with EGF-Gen at this dose level resulted in disappearance of the tumors in 2 of 5 mice and >50% shrinkage in 3 of 5 mice within 10 days, whereas all of the control tumors in 5 PBS treated mice as well as 5 mice treated with unconjugated Gen (1 mg/kg/day x 10 days) showed >200% increase in diameter during the same observation period.

The inability of unconjugated Gen or unconjugated EGF plus unconjugated Gen to exhibit significant anti-tumor activity in this SCID mouse model of metastatic human breast cancer demonstrates that the anti-cancer activity of EGF-Gen cannot be attributed to either the EGF or Gen moieties alone. Daily administration of 2 μg EGF-Gen, which contains 309 pmols of Gen in conjugated form, for a total of 10 days was a highly effective treatment regimen, whereas daily administration of 10 μg Gen, which corresponds to 37,000 pmols, alone or in combination with 10 μg unconjugated EGF (5-fold higher dose of EGF than what is contained in 2 μg EGF-Gen) for 10 days was not effective at all. Thus, the conjugation of Gen to the targeting EGF molecule enhanced its *in vivo* activity against breast cancer

cells more than 100-fold. These findings confirm and extend our in vitro data demonstrating that, compared to unconjugated Gen, EGF-Gen is >1,000-fold more potent cytotoxic agent against EGF-R^+ human breast cancer cells (18).

EGF-Gen improved tumor-free survival in a SCID mouse model of human breast cancer at systemic exposure levels non-toxic to mice or cynomolgus monkeys. Therefore, therapeutic levels of EGF-Gen may also be achievable in women with metastatic breast cancer without excessive toxicity. Notably, EGF-Gen was more effective than cyclophosphamide, adriamycin, or methotrexate in our MDA-MB-231 SCID mouse xenograft model of human breast cancer. Furthermore, plasma samples from EGF-Gen treated cynomolgus monkeys elicited potent and EGF-R-specific *in vitro* anti-tumor activity against EGF-R^+ human breast cancer cell lines. These promising preclinical results obtained with EGF-Gen indicate that further clinical development of this promising new anti-breast cancer agent is warranted. EGF-Gen treatment reduced the growth rate of breast cancer xenografts of 1.0 cm diameter, but unlike with tumors of 0.5 cm diameter, it failed to cause shrinkage or disappearance of these larger tumors. This is not surprising especially with the liver having a >40-fold higher partition coefficient than the subcutaneous tumors. Thus, EGF-Gen may be more effective as part of an adjuvant therapy regimen when the disease burden is not very large.

EGF-R overexpression is found in many types of cancer besides breast cancer (39). Thus, EGF-Gen could potentially be used in several different types of cancer. The EGF-R on cancer cells represents a potential target for other forms of biotherapy as well (9, 31,40,41). Anti-EGF-R antibodies may be useful in the treatment of EGF-R positive malignancies by disrupting EGF-mediated signal transduction events (40, 41). Whether EGF-Gen is superior to such anti-EGF-R antibodies should be examined in appropriate preclinical and clinical settings.

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FIGURE LEGENDS

Figure 1. Binding of Anti-Human EGF-R Antibody and Human EGF to EGF-R on Murine Hepatocytes. [A] Expression of EGF-R (green fluorescence) on the hepatocytes as shown by crossreactivity of anti-human EGF-R antibody. [B] Lack of binding of FITC-conjugated human EGF (green fluorescence) to murine thymocytes. [C] Binding of FITC-conjugated human EGF (green fluorescence) to murine hepatocytes. Red fluorescence represents the propidium iodide staining of the nuclei.

Figure 2. Antitumor Activity of EGF-Gen Against Human Breast Cancer in SCID Mice. Tumor free survival curves (shown in A) and life-table analysis of tumor-free survival outcome (shown in B) of SCID mice challenged with $1x10^6$ MDA-MB-231 cells. Twenty-four hours after s.c. inoculation of cancer cells, mice received EGF-Gen (10 μg/kg/day x 10 days, N=10, or 100 μg/kg/day x 10 days, N=10), EGF (500 μg/kg/day x 10 days) + Gen (500 μg/kg/day x 10 days) (N=15), or chemotherapy (N=20) (i.e., cyclophosphamide , 50 mg/kg/day x 2 days; adriamycin, 2.5 mg/kg single bolus dose; or methotrexate, 0.5 mg/kg single bolus dose), as described in Materials and Methods. Controls (N=60) were treated with PBS, G-CSF-Gen (500 μg/kg/day x 10 days), or Gen (500 μg/kg/day x 10 days). [1] The P-values for tumor-free survival comparisons were determined using the log-rank test, whereas the P-values for the average time to 0.5 cm^3 tumor size were determined using student t-tests.

Figure 3. *In vivo* activity of EGF-Gen against established tumors. The efficacy of EGF-Gen against established tumors was examined by treating SCID mice with subcutaneous MDA-MB-231 human breast cancer xenografts of 0.5 cm (shown in [A]) or 1.0 diameter (shown in [B]) with 100 μg/kg/day EGF-Gen i.p for 10 consecutive days and determining the tumor diameter daily for 20 days from the start of therapy. Control mice were treated with 0.2 ml PBS for 10 consecutive days or unconjugated Gen at 500 μg/kg/day x 10 days. P-values were determined using student t-tests.

Figure 4. Pharmacokinetic Features of EGF-Gen in SCID Mice (Panel A) and Cynomolgus Monkeys (Panel B). Plasma concentration-time curves of EGF-Gen after intraperitoneal bolus injection into tumor-free SCID mice at doses of 0.1 μg/g (□) and 1.0 μg/g (Δ), and into MDA-MB-231 tumor xenograft-bearing SCID mice at 0.1 μg/g (■) and 1.0 μg/g (▲). Monkeys received a 1-hour intravenous infusion of EGF-Gen at 0.05 μg/g (○) and 0.1 μg/g (□) dose levels. Lines represent pharmacokinetic model simulations for tumor-free (solid line) and tumor-bearing (dashed line) animals; symbols depict measured plasma concentrations of EGF-Gen.

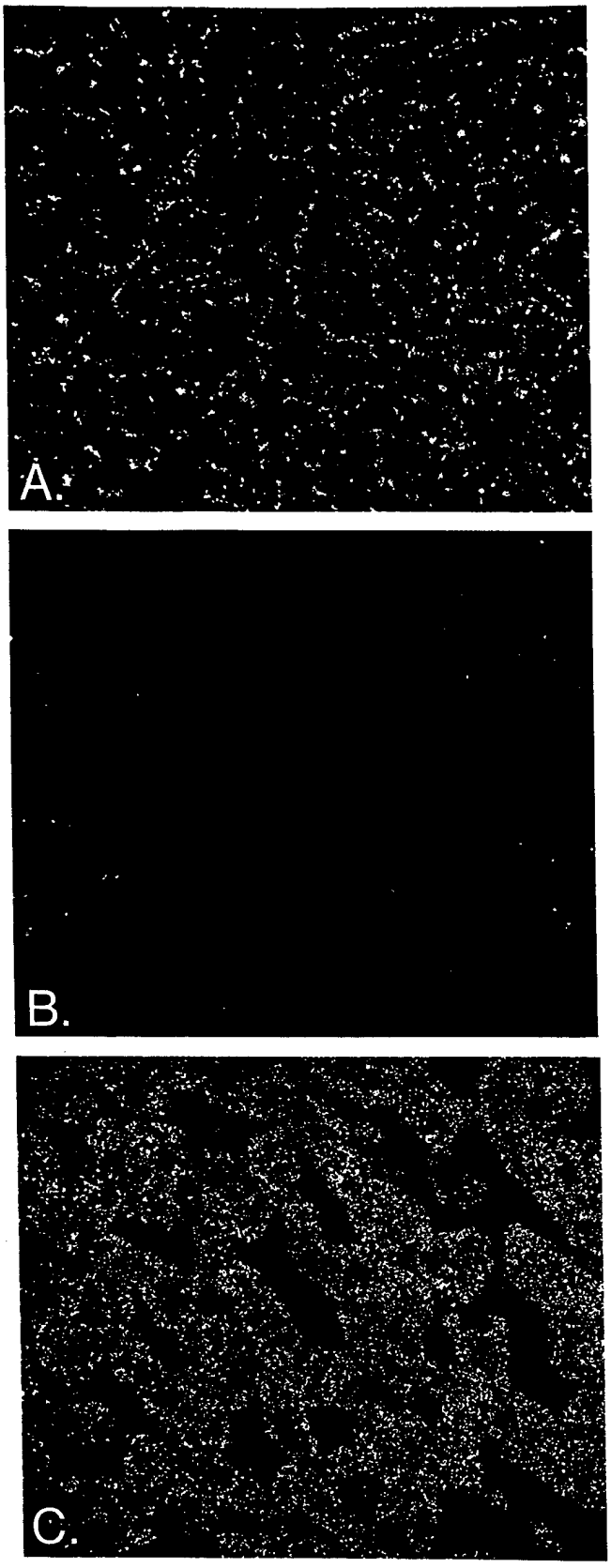
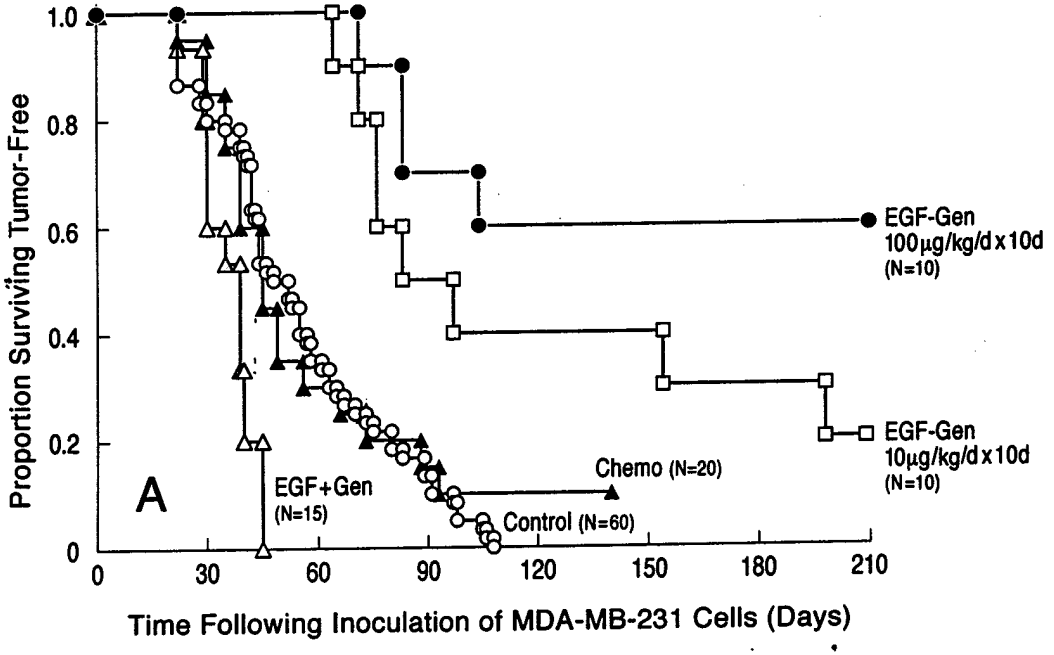


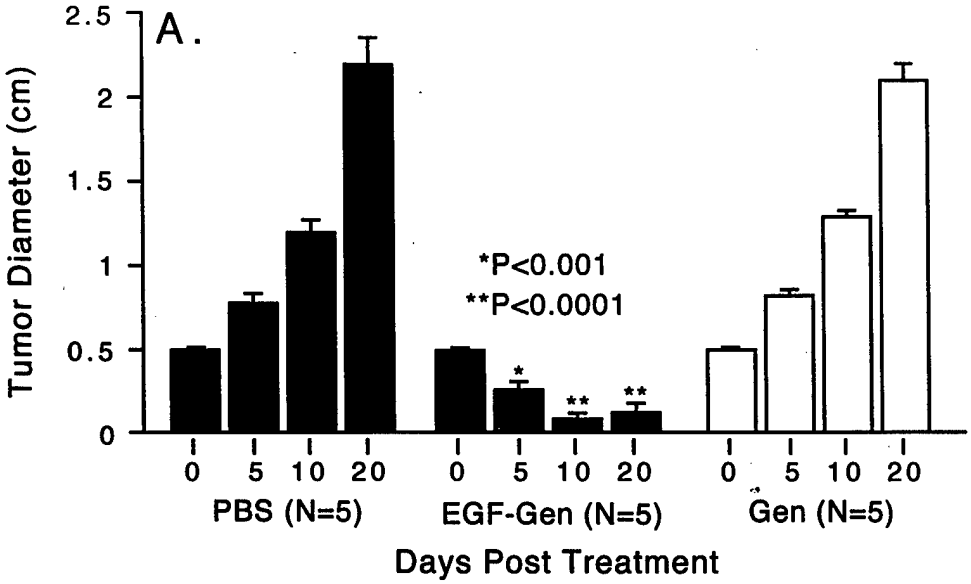
Figure 1



B. Life Table Analysis

Treatment Group	# of Mice	Proportion Surviving Tumor-Free (%) 30 days	60 days	90 days	180 days	Median Tumor-Free Survival (days)	P-value (log rank) vs PBS	vs EGF-Gen 2 µg/day	Time to 0.5cm³ tumor size	P-value vs PBS
CON	60	83±5	35±6	13±4	0±0	52	—	<0.00001	84±3	—
EGF+Gen	15	80±10	0±0	0±0	0±0	39	0.003[1]	<0.0001	62±1	0.02[1]
EGF-Gen, 10µg/kg/day	10	100±0	100±0	50±16	30±15	97	<0.00001	0.2	114±8	0.007
EGF-Gen, 100µg/kg/day	10	100±0	100±0	70±15	60±16	>210	<0.00001	—	129±14	<0.001
CHEMO	20	95±5	30±10	15±8	N.D.	48	NS	<0.0001	77±16	NS

Figure 2


A.
Tumor Diameter (cm)
2.5
2
1.5
1
0.5
0
*P<0.001
**P<0.0001
0 5 10 20
0 5 10 20
0 5 10 20
PBS (N=5)
EGF-Gen (N=5)
Gen (N=5)
Days Post Treatment

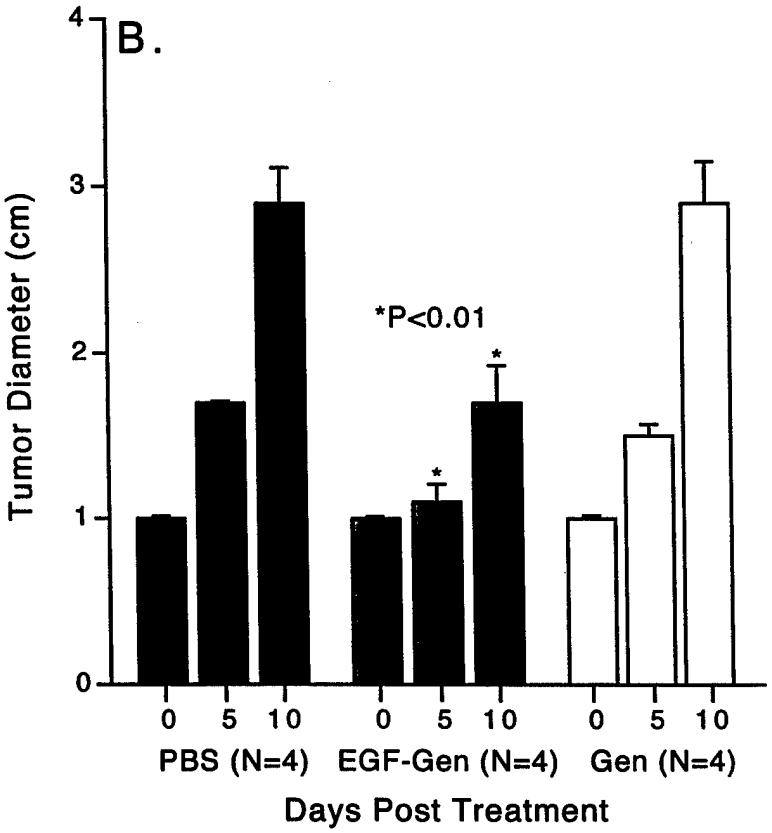

B.
Tumor Diameter (cm)
4
3
2
1
0
*P<0.01
0 5 10
0 5 10
0 5 10
PBS (N=4)
EGF-Gen (N=4)
Gen (N=4)
Days Post Treatment

Figure 3

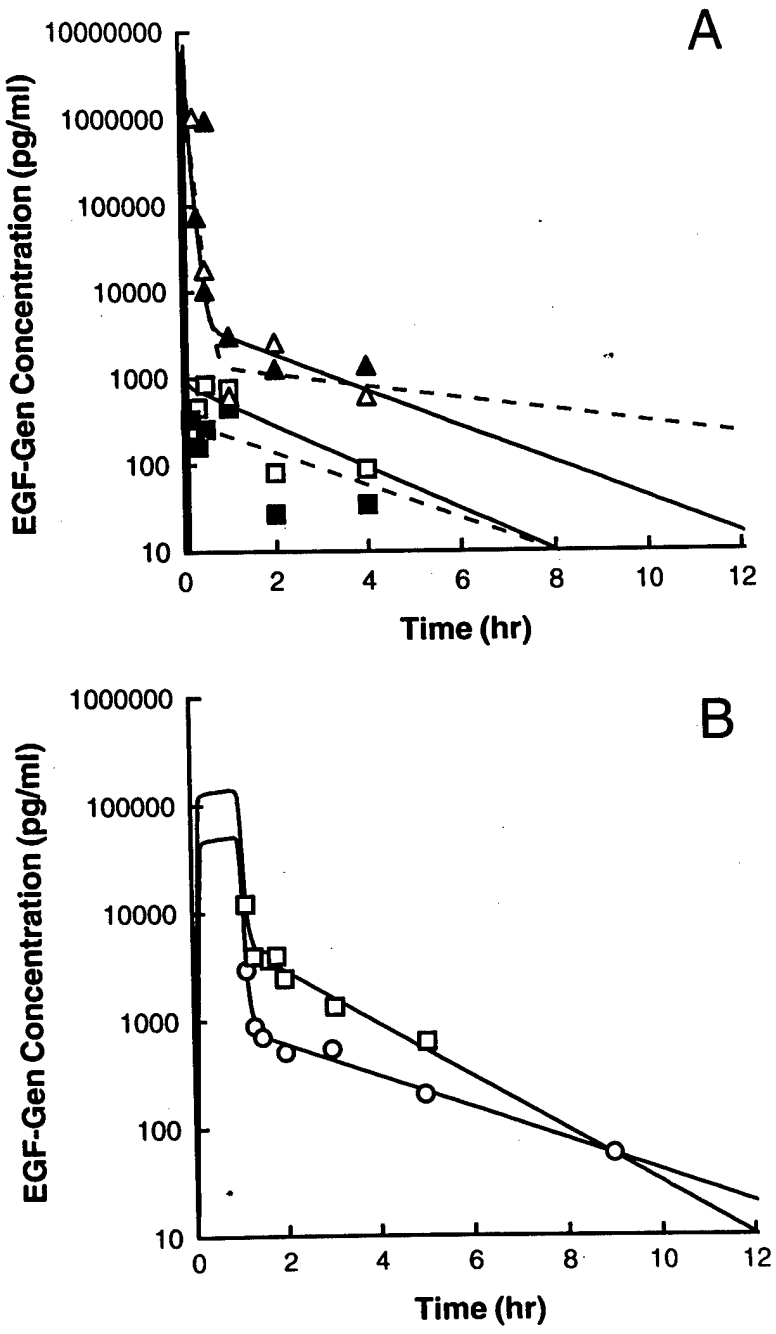

A
10000000
1000000
100000
10000
1000
100
10
EGF-Gen Concentration (pg/ml)
0
2
4
6
8
10
12
Time (hr)
B
1000000
100000
10000
1000
100
10
EGF-Gen Concentration (pg/ml)
0
2
4
6
8
10
12
Time (hr)

Figure 4

Table 1. Tissue Distribution Parameters for EGF-Genistein and Genistein in SCID Mice.

Tissue	EGF-Gen		Gen
	No Tumor	Tumor	No Tumor
	Linear binding constant (R)		
Brain	0.05	0.05	0.03
Heart	0.38	0.35	0.34
Skin	0.20	0.15	0.21
Muscle	0.11	0.09	0.09
Bone marrow	0.53	0.42	0.00008
Stomach	1.00	1.13	N.D.
Spleen	5.28	3.87	0.04
Lungs	1.44	0.84	0.53
Kidney	0.93	0.86	0.25
Liver	7.40	8.15	0.50
Intestine	0.86	1.08	N.D.
Tumor	N.A.	0.16	N.A.

A flow-limited physiological pharmacokinetic model was used to characterize the tissue disposition of both drugs in mice. Volume terms and flow rates were those previously described for mice (*23*). A set of differential equations describing the mass balances of each model compartment was used to estimate linear binding constants for each organ. These differential equations were simultaneously solved with the use of ADAPT II software (*25*). R, tissue-to-plasma equilibrium distribution ratio for linear binding in ml/g. N.A., not applicable.

Table 2. Pharmacokinetic Parameters of EGF-Gen in Mice and Monkeys

		Mice without Tumor		Mice with 1 cm^3 Tumor		Monkeys without Tumor	
Parameter	(units)	100 µg/kg	1,000 µg/kg	100 µg/kg	1,000 µg/kg	500 µg/kg	1,000 µg/kg
Vc	(ml/g)	11.6	0.05	30.0	0.05	0.06	0.04
Ke	(1/hr)	0.6	7.8	0.4	6.5	17.3	16.0
Kcp	(1/hr)	0	0.3	0	0.2	1.0	1.4
Kpc	(1/hr)	0	0.3	0	0.7	0.4	0.6
$T_{1/2\alpha}$	(hr)	0	0.09	0	0.1	0.04	0.04
$T_{1/2\beta}$	(hr)	1.3	2.1	1.6	1.0	2.1	1.2
CL	(ml/hr/g)	6.4	0.39	13.1	0.32	1.0	0.7
Vdss	(ml/g)	11.6	0.1	30	0.07	0.2	0.7
Cmax	(ng/ml)	0.45	960	0.33	1100	2.9	12.0
AUC	(µg x hr/L)	16	2564	7.6	3125	500	1400

Vc = central volume of distribution; Ke = elimination rate constant; Kcp, Kpc = distribution rate constants, $T_{1/2\alpha}$ = distribution half-life, $T_{1/2\beta}$ = elimination half-life; CL = systemic clearance from plasma, Vdss = volume of distribution at steady state, Cmax = measured maximum plasma concentration, AUC = area under the concentration-time curve. Dose 1 µg/g was used for efficacy study.

Table 3. Toxicity of EGF-Genistein in Cynomolgus Monkeys

System	Grade of Maximum Toxicity (Time)				
	52 B 0.025 mg/kg/day x 10d	52 A 0.050 mg/kg/d x 10d	52 I 0.100 mg/kg/d x 10d	63 A 0.100 mg/kg/d x 10d	63 F 0.1 mg/kg/d x 10d
Activity/Feeding	0	2 (d7–9)	2 (d5–8)	1	0
Fever	0	0	0	0	0
Weight loss	0	0	2 (d12)	0	0
Skin (Alopecia)	0	2 (d13–50)	1	0	0
Cardiac					
Tachycardia	1	1	1	2 (d8)	1
Hypotension	0	0	0	0	0
Pulmonary					
Clinical	0	0	2 (d10)	0	0
Respiratory rate	0	2 (d3-7)	0	0	0
Renal					
Creatinine	0	0	0	0	1
Electrolytes	0	0	0	0	0
Proteinuria	0	1	0	1	0
Hematuria	0	0	0	0	0
Liver					
ALT	2 (d14–18)	2 (d2-3)	1	1	1
Bili	0	0	0	0	0
Gastrointestinal					
Nausea/Vomiting	0	0	0	0	0
Diarrhea	1	0	1	0	0
Constipation	0	0	0	0	0
Nervous System					
Central	0	0	0	0	0
Peripheral	0	0	0	0	0
Coagulation					
PT	0	0	1	0	0
PTT	0	0	0	0	0
Infection	0	0	1	0	0
Blood					
Neutropenia	0	0	0	0	0
Anemia	2 (d7–10)	2 (d9-10)	1	2 (d5-6)	1
Thrombocytopenia	0	0	0	0	0
Metabolic	0	0	1	0	1

Cynomolgus monkeys were treated with intravenous infusions of EFG-Gen or Gen, as described in the Methods. For each >Grade I toxicity the onset and duration of toxicity are indicated in parentheses. ALT, alanine aminotransferase: Bili, bilirubin. Monkeys were electively euthanized at the following time points after initiation of therapy: 52 B, 182days; 52 A 186days; 52 I, 127days; 63 A, 15days; 63 F, 30days; 52 F, 135days; 52 G, 135days. No test substance related histopathologic lesions were found in any of the monkeys.

Table 4. In Vitro Anti-tumor Activity of Plasma from EGF-Genistein-treated Cynomolgus Monkeys Against EGF-R$^+$ MDA-MB231 Human Breast Cancer Cell Lines

Plasma Samples	Mean No. Colonies/10^5 Cells	% Inhibition
None	348.5 (344, 353)	—
Monkey 52A (EGF-Gen Dose = 50 µg/kg)		
Pretreatment	354.5 (321, 388)	0
1 hour posttreatment	28.5 (21, 36)	91.8
1 hour posttreatment + 10 µg/ml EGF	301.5 (289, 314)	13.5
1 hour posttreatment + 10 µg/ml G-CSF	21.5 (16, 27)	93.8
Monkey 52I (EGF-Gen Dose = 100 µg/kg)		
Pretreatment	323.5 (320, 327)	7.2
1 hour posttreatment	0 (0, 0)	>99.7
1 hour posttreatment + 10 µg/ml EGF	293.0 (278, 308)	15.9
1 hour posttreatment + 10 µg/ml G-CSF	0 (0, 0)	>99.7
Monkey 63A (EGF-Gen Dose = 100 µg/kg)		
Pretreatment	395.0 (391, 399)	0
1 hour posttreatment	4.5 (2, 7)	98.7
1 hour posttreatment + 10 µg/ml EGF	279.5 (276, 283)	19.8
1 hour posttreatment + 10 µg/ml G-CSF	2.5 (1, 4)	99.3
Monkey 63F (EGF-Gen Dose = 100 µg/kg)		
Pretreatment	323 (306, 340)	7.3
1 hour posttreatment	0 (0, 0)	>99.7
1 hour posttreatment + 10 µg/ml EGF	340 (309, 371)	2.4
1 hour posttreatment + 10 µg/ml G-CSF	0 (0, 0)	>99.7

Cells (10^6/mL RPMI+10% FCS) were incubated overnight at 37°C with 1:50 (v/v) PBS-diluted plasma samples from EGF-Genistein-treated cynomolgus monkeys. After treatment, cells were washed twice, plated at 10^5 cells/ml in RPMI+10% FCS+ 0.9% methylcellulose in Petri dishes, and cultured for 7 days at 37°C in a humidified 5% CO_2 atmosphere. Subsequently, colonies were enumerated and the % inhibition was calculated using the formula:

$$\% \text{ Inhibition} = 1 - \frac{\text{mean no. colonies in test culture}}{\text{mean no. colonies in control culture}} \times 100.$$

Excess EGF was added to some of the plasma samples to block the activity of EGF-Genistein by competing for the EGF-R molecules on MDA-MB231 cells. Excess G-CSF was used as a control for comparison.