N-Terminal Site-Specific Mono-PEGylation of Epidermal Growth Factor

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Purpose. N-terminal site-specific mono-PEGylation of recombinant human epidermal growth factor (EGF) was accomplished using polyethyleneglycol (PEG) derivatives (Mw = 2000 and 5000) through a reactive terminal aldehyde group.

Methods. The site-specific PEG conjugation was conducted at a slightly acidic pH condition (pH 5.5). The mono-PEGylation was targeted to an α-amine group at the N-terminal end of EGF to minimize reduction of biologic activity. Tryptic digestion mapping and MALDI-TOF MS techniques were applied to show the occurrence of mono-PEGylation at the N-terminus of EGF.

Results. The site-specific mono-PEGylated EGF, when compared with native EGF, fully retained its *in vitro* biologic activities such as cell proliferation and intracellular signal transduction. This revealed that although a synthetic polymer of a PEG was covalently conjugated to EGF, the internalized complex of PEGylated EGF-receptor within cells did not hamper the intracellular signal transduction events. The PEGylated EGF also exhibited a prolonged circulation in blood stream *in vivo* and markedly enhanced physical stability when incubated with tissue homogenate.

Conclusion. N-terminally mono-PEGylated EGF shows increased physical stability while retaining its biologic activity.

KEY WORDS: epidermal growth factor (EGF); poly(ethylene glycol) (PEG); site-specific PEGylation; biologic activity.

INTRODUCTION

Many therapeutic proteins have been conjugated with a water-soluble synthetic polymer, poly(ethylene glycol) (PEG), to enhance their pharmacological activities (1). Conjugation of PEG (PEGylation) to various proteins not only increases their half-life in the blood stream but also significantly reduces their immunogenicities (2,3). In particular, the prolonged circulation of PEGylated proteins reduces the necessity of multiple injections to patients. A wide range of therapeutic proteins have been PEGylated; for example, human growth hormone, interferon, insulin, granulocyte-colony stimulating factor (G-CSF), and interleukin II (1). Generally speaking, whereas PEGylation of proteins beneficially results in a longer circulation time in blood, it also causes the problem of reducing their intrinsic biologic activities. The steric hindrance effect of conjugated PEG significantly suppresses specific binding of PEGylated proteins to counterpart cellular receptors or substrates (4,5). However, the prolonged *in vivo* half-life of PEGylated proteins often compensates for the reduction of biologic activity, leading to an overall increase in their therapeutic effects *in vivo*. This is the so-called counteracting effect (6). Therefore, PEGylation of proteins at specific residues is necessary to exert minimal effect on receptor binding or substrate recognition. Protein engineering approaches have attempted to introduce a reactive amino acid, such as cysteine, at a desired site of a protein to achieve site-specific PEGylation (7). A PEG derivative having a thiol-selective reactivity could be conjugated to the mutation site in a site-specific manner (8). This method, however, resulted in the modification of the protein structure and thus could not warrant its original therapeutic efficacy and safety. Recently, site-specific PEGylation of proteins has been attempted using a special class of functionalized PEG derivatives under specific conjugation conditions. For example, N-terminal specific PEGylation of G-CSF was obtained by conjugating methoxy-PEG derivatives at acidic pH conditions (9). This strategy was based on the fact that primary amine residues in protein have different *pK*a values: *pK*a 7.8 for N-terminal α-amino group and 10.1 for ε-amino group in lysine residues (10). When the *N*-hydroxysuccinimide or propionaldehyde PEG was conjugated at lower pH conditions, PEGylation at the N-terminus site occurred preferentially due to the different pKa values between the two kinds of ε and α primary amines.

Recombinant human epidermal growth factor (EGF) is a 53-amino acid polypeptide, which exhibits potent mitogenic activities, such as cell proliferation and differentiation of various tissues and cells (11). It has the additional activity of inhibiting carcinoma cell growth where EGF receptors are overexpressed (12,13). EGF binds to the EGF receptor forming a dimerized complex, which is then internalized within cells. The internalized EGF receptor has a tyrosine kinase activity, thus initiating a series of signal transduction cascades. EGF has been used therapeutically in wound healing processes, especially in ulcer treatment (14,15). Moreover, it has been used as an effective targeting moiety for intracellular delivery of various anti-cancer drugs, peptides, and genes to specific cancer cells that overexpress EGF receptors (16,17). These studies have shown that EGF receptor-mediated endocytosis played a critical role in increasing the cellular uptake of various hydrophilic macromolecular drugs that have limited permeability across cell membranes. Previously, we have investigated PEGylated EGF in view of its stability in biodegradable microspheres and a PEG conjugation site-biologic activity relationship in mono-PEGylated EGF isomers (18,19). This study revealed that N-terminally PEGylated EGF fully retained its intrinsic activities in terms of cell proliferation, receptor binding, and internalization when compared to native EGF.

In this study, we PEGylated the N-terminus of EGF in a site-specific manner using PEG-propionaldehyde derivatives with two different molecular weights (Mw 2000 and 5000). Various characterization methods, such as tryptic digestion, reversed phase-high-performance liquid chromatography (RP-HPLC) mapping, and matrix-assisted laser desorption ionization time of flight (MALDI-TOF) techniques were used to reveal that the mono-PEGylation reaction occurred site-specifically at the N-terminal α-amine group. *In vitro* biologic activities were evaluated by cell proliferation, anti-phospho-

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tyrosine assays, and anti-phospho-ERK immunoblot assays. An *in vitro* stability experiment in tissue homogenate was performed to examine the therapeutic potential of mono-PEGylated EGF for wound healing. The *in vivo* circulation half-life in blood was also determined using a mouse model system.

MATERIALS AND METHODS

Materials

Recombinant human EGF was donated from Daewoong Pharmaceutical Co. (Seoul, Korea). Sequencing grade trypsin (from bovine pancreas) was purchased from Roche Diagnostics Co. (Indianapolis, IN, USA). PEG-propionaldehyde (methoxy-poly(ethylene glycol)-propionaldehyde) (MW 2000 and 5000) was obtained from Shearwater Polymers (Huntsville, AL, USA). NRK 47F cell line was a product of Korea Cell Line Bank (Seoul, Korea). [^{3}H] thymidine and [γ^{32}P] ATP were purchased from Amersham Pharmaceutical Biotech. (Buckinghamshire, UK). Dialysis membranes (MW cutoff 10,000) were purchased from Spectrum (Houston, TX, USA). Phosphate-buffered saline, HEPES-buffered saline, Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum, L-glutamine, penicillin, streptomycin, and trypsin-EDTA were obtained from Gibco-BRL (Grand Island, NY, USA). A micro-BCA protein assay kit was purchased from Pierce (Rockford, IL, USA). Sodium iodoacetate was provided by Sigma (St. Louis, MO). An enhanced chemiluminescence kit was obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). Bovine calf serum was obtained from HyClone (Logan, UT, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG, anti-mouse IgG, IgM, and IgA came from Kirkegaard and Perry Laboratories, Inc. (Gaitherburg, MD, USA). Anti-phosphotyrosine monoclonal antibody was prepared as described previously (20). Anti-actin monoclonal antibody was purchased from ICN Pharmaceuticals (Costa Mesa, CA, USA). Rabbit anti-phospho-ERK antibody was from New England Biolabs (Beverly, MA, USA).

Methods

PEGylation of EGF with PEG-Propionaldehyde Derivatives

Freeze-dried EGF (10 mg) and PEG-propionaldehyde derivatives (Mw = 2000, 10 mg; or 5000, 25 mg) were dissolved in 5 mL of sodium acetate buffer (50 mM, pH 5.5) in the presence of sodium borohydride (2.5 mM) as a reducing agent. The conjugation reaction was conducted for 1 day at room temperature. Dialysis (MW cutoff = 10,000) was performed extensively using a Tris buffer (Tris-HCl, 5 mM, pH 8.0) to terminate the PEGylation reaction and to remove unconjugated PEG-propionaldehyde derivatives, native EGF, and other chemical reagents. The final concentration, determined by a BCA assay after dialysis, was 1.32 mg/mL for PEG2k-EGF and 0.95 mg/mL for PEG5k-EGF.

Characterization of PEGylated EGF by Size-Exclusion Chromatography

EGF conjugates (PEG2k-EGF and PEG5k-EGF) were analyzed by size-exclusion HPLC chromatography (SEC-HPLC, Agilent 1100 series) equipped with an auto-sampler on a Shodex protein KW-800 column (Showa Denko, Japan); 20 μL of the sample was loaded into the column; an isocratic mobile phase composed of 50 mM NaCl and 5 mM Tris-HCl, pH 8.0; a flow rate of 1.0 mL/min; UV detection at 280 nm.

Mass Analysis of PEGylated EGF by MALDI-TOF MS

PEGylated EGF was analyzed by MALDI-TOF (Voyager DE-STR Perkin-Elmer PerSeptive Biosystem) mass spectrometry. The sample crystals were prepared by a rapid solvent evaporation method, and sinapinic acid was used as a matrix. The solvent composition was composed of 70% acetonitrile and 30% distilled water. One microliter of conjugated solution was mixed with 10 μL of the solvent, which was subsequently applied to the ZipTip (Bedford, MA, USA) to remove the salts. MALDI-TOF operating conditions were set as follows: mode of operation was linear, polarity was positive, an acceleration voltage was 25,000 V, and delayed extraction time was 160 ns.

RP-HPLC for Tryptic Digestion Mapping

PEGylated EGF was mixed with dithiothreitol solution at a final concentration of 5 mM to reduce the disulfide bonds into free thiol groups. The reduction lasted for 4 h. Iodoacetate solution (final concentration of 5 mM) was added to prevent any re-oxidation of the reduced free thiol groups, and was incubated for 6 h at room temperature. Dialysis (Mw cutoff: 3500) was performed twice in a 5 mM Tris-HCl buffer, pH 8.0, and was concentrated until the volume reached 1 mL (0.98 mg/mL for PEG2k-EGF and 0.54 mg/mL for PEG5k-EGF). Tryptic digestion was conducted in 50 mM Tris-HCl (pH 8.0), 1 mM $CaCl_2$, at 37°C for 12 h with an EGF/trypsin weight ratio of 100:1. Twenty-five microliters of the tryptic digestion solution was injected in a Waters Spherisorb C-18 column (4.6 × 250 mm) equipped with a guard column. Gradient elution was carried out at a flow-rate of 1.0 ml/min with solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile). A gradient program was set as follows: 2% solvent B for 10 min, 60% solvent B for 30 min, and 100% solvent B for 10 min.

Cell Proliferation Assay

NRK 47F cells were cultured at 37°C in 5% CO_2 in DMEM with 10% fetal bovine serum. They were seeded in each well at a density of 5×10^3 cells per cm^2 in a 24-well plate in 2 mL of DMEM containing 10% fetal bovine serum. After 24 h, serum-free DMEM was added to fast cells to synchronize the cell cycles. The NRK47F cells were incubated for 12 h before replacing the media with new media containing native EGF or mono-PEGylated species (aldehyde-PEG2k and 5k) at the concentration range from 1 pM to 100 nM.

The cells were incubated further for 8 h. Thymidine [^{3}H] (3 mCi/well) was subsequently added to each well and was incubated for another 8 h to incorporate the radioactive agent into newly synthesized chromosomes during cell proliferation. The cells were treated with 20% cold trichloroacetic acid and subsequently precipitated. The precipitates were collected on a nitrocellulose paper and counted with a scintillation counter (Beckman LS 6500).

Immunoblots for Detection of Signals from EGF Receptors

COS-7 cells were cultured at 37°C in 5% CO_2 in high glucose DMEM with 10% bovine calf serum. Proteins were denatured by boiling for 5 min at 95°C in a Laemmli sample buffer (21), separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred to nitrocellulose membrane by electro-blotting using the Bio-Rad wet transfer system. After blocking in TTBS buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) containing 5% skim milk powder, the membrane was incubated with individual monoclonal or polyclonal antibodies followed by further incubation with anti-mouse or anti-rabbit IgG coupled with horseradish peroxidase. Detection was performed using an enhanced chemiluminescence kit.

Circulation Half-Life of EGF and PEGylated EGF in Vivo

Native EGF or mono-PEGylated EGF (2 μg/0.1 mL) was administered to ICR mice (male, average weight 28.0 g, 7 weeks old) via a lateral tail vein. Blood samples were obtained by cardiac puncture from 5 to 240 min after injection. The blood (100 ~400 μL) was mixed with 2 μL of heparin solution and centrifuged at 17000 g for 5 min to obtain plasma. The supernatant solution was collected and diluted by 10^4~10^5 fold for adjusting the concentration within the standard linear range (0.6~20.7 pM) of the human recombinant EGF ELISA kit. A standard calibration curve was constructed using native or mono-PEGylated EGF for the ELISA assay. Five mice were used to determine the remaining EGF amount in plasma at each time point.

Stability of PEGylated EGF in Kidney Homogenate

Mouse kidney (1.5 g) was isolated and washed with phosphate-buffered saline. The kidney homogenate was prepared using a manual-type homogenizer in 0.1× phosphate-buffered saline and was subsequently centrifuged at 18,000g for 30 min. It was kept under –20°C before use. The protein concentration was determined by a Bradford assay (5.3 mg/mL). PEGylated EGF (0.5 μg/20 μL) was incubated with the kidney homogenate solution (2 mL) at 37°C, and samples (0.1 mL) were taken at predetermined time points (10, 30, 60, 120, 240, and 550 min). The remaining amount of EGF or PEGylated EGF in the tissue homogenate was determined by an EGF ELISA kit. The supernatant of kidney homogenate was used as a baseline standard in this ELISA system.

RESULTS

Preparation and Characterization of PEG2k-EGF and PEG5k-EGF Conjugates

Recombinant human EGF has 53 amino acid acids and three disulfide bonds. Three potential PEGylation sites are the two ε-amine groups of Lys28 and Lys 48, and one α-amine group at the N-terminus. Two PEG-propionaldehyde derivatives (Mw = 2000 and 5000) were conjugated in acidic conditions (pH 5.5) for about 1 day, and the unreacted EGF fraction and the reducing agent, $NaCNBH_3$, were removed by extensive dialysis (Mw cutoff = 10,000). In acidic conditions, PEG-propionaldehyde derivatives were selectively reacted with the N-terminal α-amine group of EGF to produce an alkylated linkage of secondary amine between PEG and EGF as a result of the different pKa values between the ε-amine group and the α-amine group.

Figure 1 shows SEC-HPLC chromatograms of (A) PEG2k-EGF, (B) PEG5k-EGF, and (C) PEG3.4k-EGF before and after dialysis. The first two PEGylated EGF samples (A, B) were prepared by using PEG-propionaldehyde derivatives in an acidic condition, and the other PEG3.4k-EGF (C) was prepared by using a PEG-N-hydroxysuccinimide derivative in a basic condition. It can be seen in the chromatograms that mono-PEGylated EGF was predominantly produced when using PEG-propionaldehyde derivatives, while a mixture of multi-PEGylated EGF species was formed when using a PEG-N-hydroxysuccinimide derivative. It was also noted that mono-PEGylated EGF could be readily separated from the unPEGylated EGF fraction by a simple dialysis process. Using a dialysis membrane with MW cutoff of 10,000 was sufficient in successfully purifying the mono-PEGylated EGF fraction without undergoing tedious chromatographic separation processes. However, it should be noted that a small fraction of unconjugated and inactivated PEG derivatives (PEG MW 5000) was possibly present in the PEG-EGF conjugate samples due to the limitation of the dialysis process.

To estimate the molecular weight distribution of PEGylated EGF, MALDI-TOF mass spectrometry was used to further confirm the SEC-HPLC results (Fig. 2). Mass value stated in this figure is expressed as Mp (the most probable mass peak) for the PEG2k-EGF and PEG5k-EGF conjugates (22). The measured mass was 8223.6 m/z for PEG2k-EGF and 11636.6 m/z for PEG5k-EGF without showing any other mass peaks. Considering the molecular weights of EGF (6045 Da) and two PEGs (2000 and 5000), the obtained mass of the conjugates was very consistent with the calculated mass sum of individual molecules.

To identify the PEGylation site of the mono-PEGylated EGF, trypsin digestion and RP-HPLC peptide mapping were carried out. EGF has four possible tryptic digestion sites: two lysine residues (Lys28 and Lys48) and two arginine residues (Arg41 and Arg45), thus tryptic digestion results in a mixture of five peptide fragments. Based on the previously reported peptide mapping patterns of PEGylated EGF conjugated with a PEG-N-hydroxysuccinimide derivative, it was found that PEG-propionaldehyde derivatives were site-specifically conjugated at the N-terminal α-amine group (19). SEC-HPLC, MALDI-TOF, and trypsin-digested peptide mapping results manifested the evidence that the site of PEGylation was the N-terminal α-amine of EGF.

Evaluation of Biologic Activities of N-Terminus Mono-PEGylated EGF

A NRK 47F cell line, a well-known EGF receptor expressed cell, was used to determine the biologic activities of N-terminus mono-PEGylated EGF (23). To measure the extent of the proliferation of NRK 47F cells, [^{3}H]-thymidine uptake was measured as a function of concentration (Fig. 3). The effective concentration (EC_{50}) for cell proliferation of native EGF was slightly lower that those of PEG2k-EGF and PEG5k-EGF. Although the ability to stimulate cell growth slightly decreased upon PEGylation, it could be said that PEGylated EGF retained the biologic activities of native EGF. The cell proliferation activities of N-terminal mono-

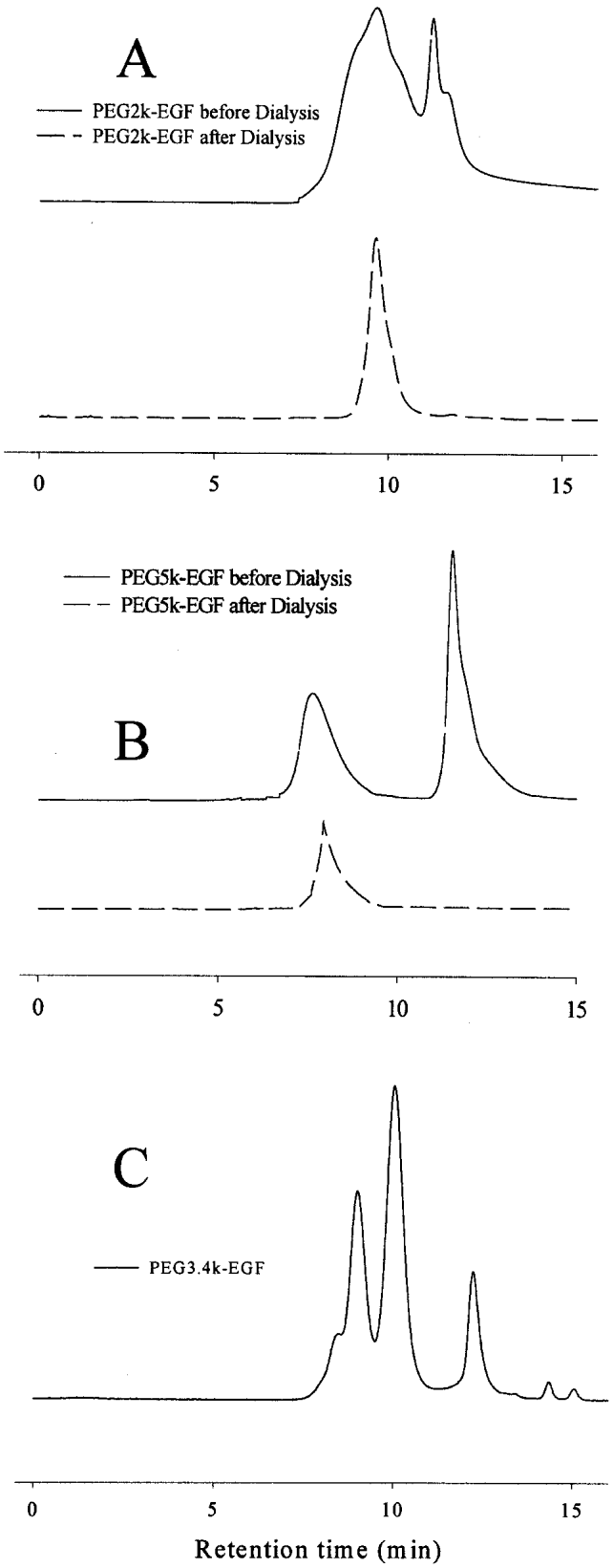

Fig. 1. Size exclusion high-performance liquid chromatograms of (A) PEG2k-EGF, (B) PEG5k-EGF before and after dialysis, and (C) PEG3.4k-EGF before dialysis. PEG derivatives used in A and B were PEG-propionaldehyde with MW of 2000 and 5000. PEG-N-hydroxysuccinimide derivative (MW 3400) was used in C. The native EGF was successfully removed after dialysis (Mw cutoff = 10 k).

PEGylated EGF prepared from PEG-propionaldehyde derivatives were slightly higher than those prepared from PEG-N-hydroxysuccinimide derivatives (19). This may have been due to the different linkage chemistries; alkylation vs. acylation. The former had a positively charged amine group and the latter had a neutral amide group, similar to native EGF. It also revealed that the molecular weight of PEG might not be a major factor in affecting the biologic activities of EGF.

Upon binding of EGF to its receptor on the cell membrane, the EGF receptors dimerize and induce the autophosphorylation of tyrosine residues in their cytoplasmic domain. The dimeric EGF-receptor complexes with a tyrosine kinase activity are internalized within cells, and initiate the mitogen-activated protein (MAP) kinase signal cascade (24). Among the many signal molecules involved in the MAP kinase signaling pathway, extracellular signal-regulated protein kinase (ERK) was chosen as a downstream phosphorylation marker to demonstrate the full activation of cellular signals from the endosomes where PEGylated EGF-receptor complexes were located (25). Immunoblots were performed to estimate the phosphorylation levels of EGF receptors and ERK molecules by the treatment of agonists (native EGF, PEG2k-EGF or PEG5k-EGF) in a dose dependent fashion (Fig. 4). The first line is the immunoblot of anti-phospho-tyrosine, the second is anti-phospho-ERK, the third is anti-ERK, and the last is anti-actin. The extent of tyrosine phosphorylation stimulated by native and PEGylated EGF increases as the dose of agonists increases. Similarly, the amount of phospho-ERK is also increased in spite of the same residual amount of ERK molecules in cytosol (the third line). The results reveal that the PEG chain that was conjugated to EGF N-terminus, regardless of their molecular weights, did not hamper the intracellular signal transduction events. Remarkably, the band intensities of phospho-tyrosine EGF receptors and phospho-ERK did not decrease when compared to those of native EGF.

Evaluation of *in Vivo* and *in Vitro* Stabilities of PEGylated EGF

PEGylation has been a useful method for enhancing pharmaceutical stability of therapeutic peptides and proteins by prolonging their circulation time in the blood (1,6–8). *In vivo* clearance rates of EGF, PEG2k-EGF, and PEG5k-EGF were analyzed by injecting them into the blood stream through a tail vein of mice (Fig. 5). The concentration of EGF was adjusted to 910 nM for the injection and the residual amounts of EGF were detected as a function of time by a human ELISA kit. PEGylation increased the circulation half-life ($t_{1/2}$) about 4-fold: 6.71 min for EGF, 15.59 min for PEG2k-EGF, and 24.36 min for PEG5k-EGF. PEG5K-EGF had a longer circulation time than PEG2K-EGF due to the effect of the chain length of PEG. The molecular weight of PEG plays a critical role in increasing the circulation time by decreasing the glomerular filtration rate and by resisting proteolytic digestion. Furthermore, *in vitro* stability was measured by incubating PEGylated EGF with kidney homogenate. Tissue homogenate contains many proteases that degrade therapeutic proteins. Figure 6 shows the residual amounts of native EGF, PEG2k-EGF, and PEG5k-EGF in the tissue homogenate as a function of time. Because the tissue homogenate showed a nonspecific colorimetric reaction with the rhEGF ELISA kit (8.4 ± 0.3 pg/mL), all the data

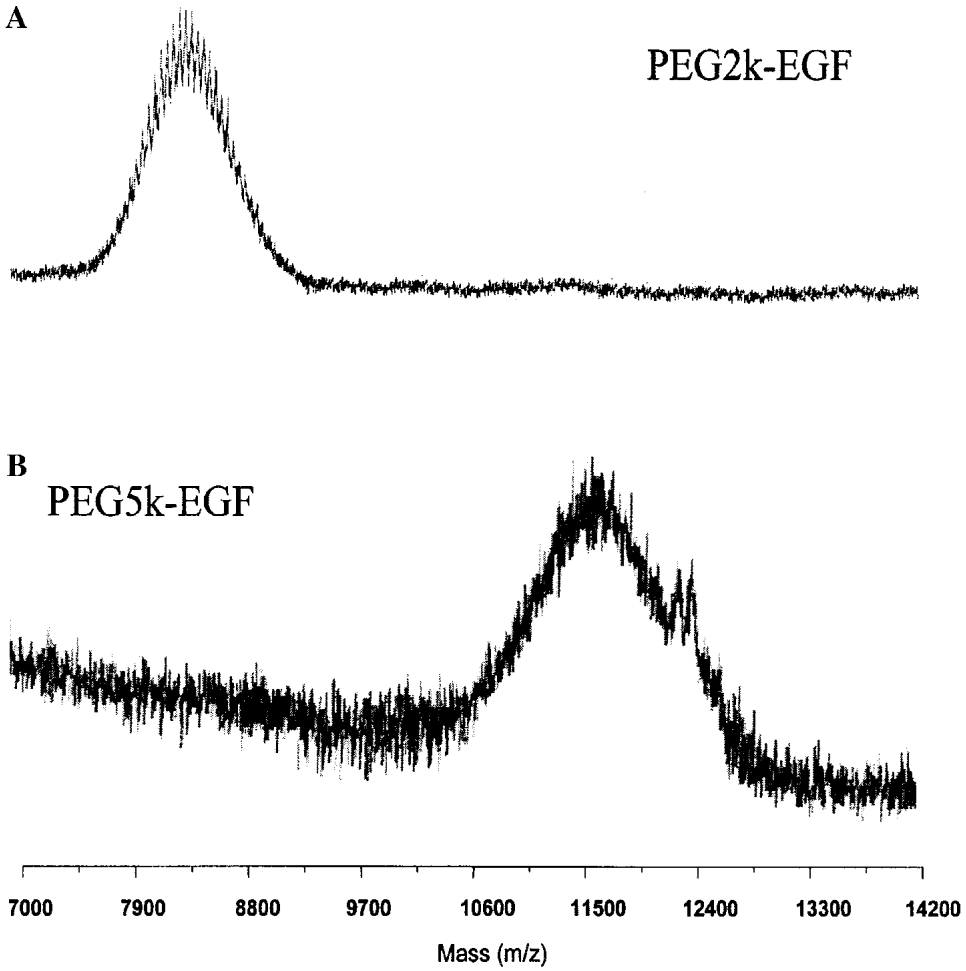

Fig. 2. MALDI-TOF spectra of (A) PEG2k-EGF and (B) PEG5k-EGF. The molecular weight of EGF is 6045 Da. The most probable mass (Mp) of PEG2k-EGF and PEG5k-EGF are 8223.6 Da and 11636.6 Da, respectively.

obtained in this figure were calculated by subtracting the background value caused by tissue homogenate. PEGylated EGF shows increased stability against protease attacks in the tissue homogenate. Upon incubation for 1 h, native EGF rapidly degraded (<20%). However, PEGylated EGF exhibited a dramatically increased stability: the remaining amount of PEG2k-EGF was 50% and PEG5k-EGF was 88% during the same period. It should be stressed that the residing PEG5k-rhEGF retained its original structure even after 10 h of incubation with kidney homogenate.

DISCUSSION

Preparation and Characterization of Mono-PEGylated EGF

A main goal of this study was to develop a simple preparation method for N-terminal site-specific mono-PEGylated EGF still retaining its full biologic activities. The current work was based on our previous report in which N-terminally mono-PEGylated EGF showed the highest *in vitro* biologic activities such as cell proliferation, cell inhibition, and tyrosine phosphorylation of EGF receptors (19). The PEG derivatives used in this study were two kinds of methoxy-PEG-propionaldehyde having average Mw of 2000 and 5000. In our previous study, PEGylated EGF (PEG3.4K-EGF) was prepared by using PEG-N-hydroxysuccinimide derivatives, which revealed that *in vitro* biologic activities were significantly reduced when PEGylation occurred at Lys 28 and Lys 48 residues. In contrast, N-terminal PEGylation did not hinder the binding capacity of EGF to its receptor, because the N-terminal residue is located far from the receptor-binding site.

SEC-HPLC chromatograms in Fig. 1 suggest that using PEG-propionaldehyde derivatives at acidic pH produced only a binary mixture of mono-PEGylated EGF and native EGF, whereas the use of PEG-N-hydroxysuccinimide at a basic pH resulted in up to tri-PEGylated EGF species. The conjugation of PEG derivatives to primary amine groups of EGF takes place primarily via a nucleophilic substitution reaction: the attack of unprotonated amine group to the carbonyl groups of aldehyde and N-hydroxysuccinimide. This indicated that all three primary amines (one α and two ε) were equally reactive to PEGylation in basic conditions thus producing a heterogeneous mixture of multi-PEGylated species. In contrast, in the acidic condition, selective unprotonation of the N-terminal α-amine group could occur making it more reactive than the ε-amine groups in lysine residues because of the difference of the *pK*a values: 7–8 for α-amine, and 10–11 for ε-amine (10).

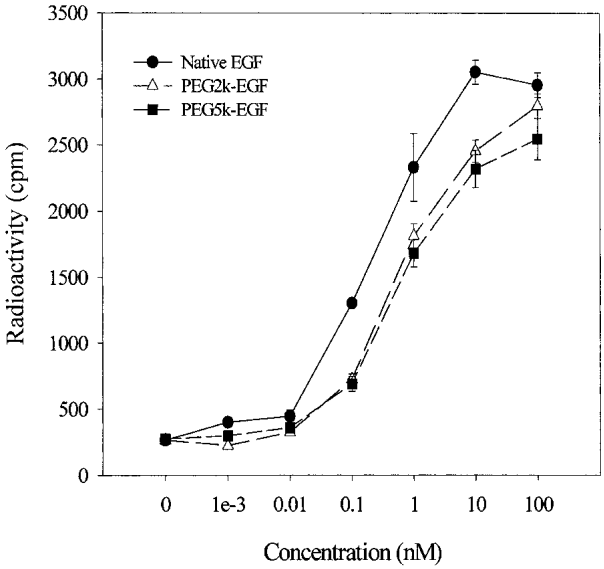

Fig. 3. Cell proliferation of NRK47F induced by EGF, PEG2k-EGF, and PEG5k-EGF, as measured by [^{3}H]-thymidine uptake (n = 3).

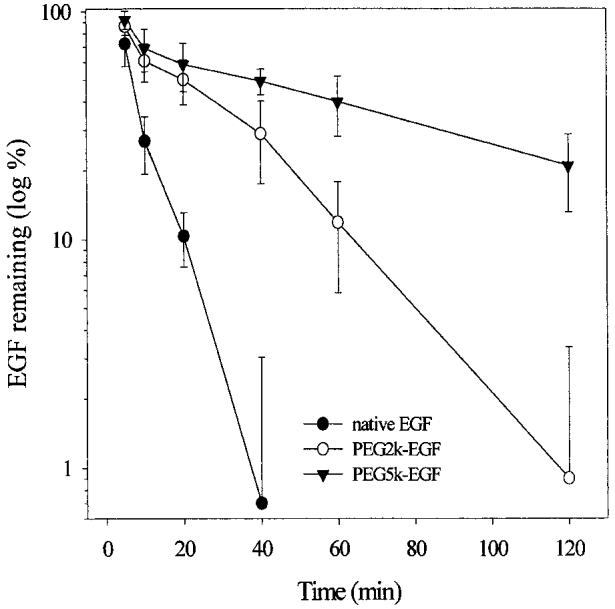

Fig. 5. *In vivo* circulation profiles of native EGF and PEGylated EGF when injected in the blood stream (n = 5).

This reductive amination reaction with the N-terminal α-amine group, in the presence of sodium cyanoborohydride as a reducing agent, possibly resulted in the site-specific mono-PEGylated EGF (9). Mono-PEGylated protein conjugates, such as G-CSF, were similarly obtained through a selective conjugation at the N-terminal α-amine residue by varying reaction pH. After the simple purification step of dialysis, nearly homogeneous mono-PEGylated EGF conjugates could be obtained in the absence of EGF as evidenced by the SEC and MALDI-TOF analysis. The one-step purification used in this study would be a very advantageous factor for future pharmaceutical applications of mono-PEGylated EGF, because time-consuming and tedious chromatographic separations, often required for the purification of mono-PEGylated proteins, would not be necessary. To prove that the PEGylation site was indeed the N-terminal α-amine group, tryptic digestion peptide mapping was performed. It confirmed that the mono-PEGylation occurred site-specifically at the N-terminal α-amine group.

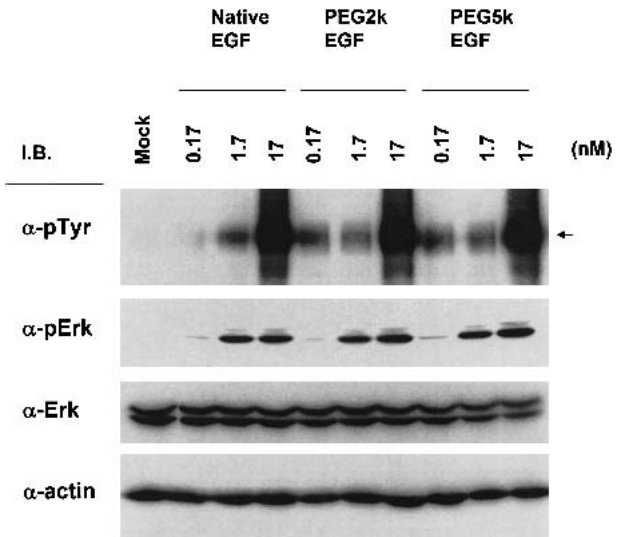

Fig. 4. Immunoblots for anti-phosphotyrosine, anti-phospho-ERK, anti-ERK, and anti-actin for various concentrations of native EGF and PEGylated EGF. Cell lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by immunoblot analysis. The arrow represents the region corresponding to the molecular weight of EGF receptor.

Evaluation of Biologic Activities of N-Terminal Mono-PEGylated EGF

For PEGylated therapeutic proteins, the "counter-acting effect" is defined as two opposite effects of PEGylation on *in vitro* and *in vivo* biologic activities. Because of the steric effect of surface tethered hydrophilic PEG chains on receptor bindings or protein-protein interactions, PEGylated proteins exhibit much reduced biologic activities *in vitro,* when compared with unPEGylated native proteins. However, *in vivo*, they show enhanced physical stabilities such as increased half-life in circulation and improved resistance against proteolysis. Thus the observed overall biologic activities of PEGylated proteins *in vivo* appear to be superior to those of unPEGylated proteins. This occurs because the prolonged circulation effect compensates for the loss of activity (6,26,27). N-terminal site-specific mono-PEGylated EGF did not lose its cell proliferation activity *in vitro*, compared to native EGF, presumably because the receptor-binding site was located far from the PEGylation site (28,29). It has been demonstrated in previous studies that the critical EGF residues involved in recognition by its receptor were arginine 41 and tyrosine 37, located in the vicinity of the C-terminus.

Upon binding of EGF to its receptor, EGF receptors are dimerized and internalized within cells activating a series of signal transduction pathways (24). The major destination of EGF-receptor complexes after endocytosis is late endosomes and lysosomes, where some of EGF receptors are dissociated and subsequently recycled from the early endosomal compartments to cell membranes. Intracellular trafficking of EGF-receptor complexes plays a critical role in defining the physiologic responses of individual cells or tissues such as cell

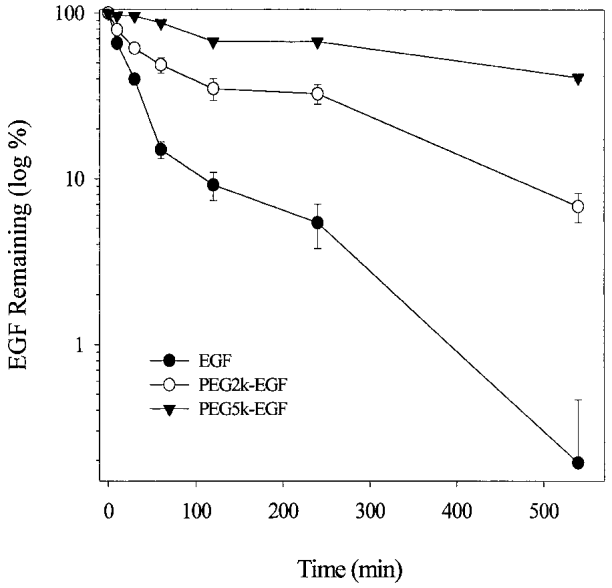

Fig. 6. *In vitro* stability of EGF, PEG2k-EGF, and PEG5k-EGF in kidney homogenate (n = 3).

proliferation, differentiation, and functions. EGF induced cellular responses could be fully achieved only when EGF-receptor complexes are normally internalized (30–33). From this point of view, internalized EGF-receptor complexes are important in activating multiple cascade events of phosphorylation activities in downstream signal transduction pathways (28). N-terminal mono-PEGylated EGF complexed with EGF receptors in a dimeric form was likely to be involved in many phosphorylation cascade events that were mostly kinase enzymatic reactions. Hence two PEG chains tethered to the dimeric complex of EGF-EGF receptors must be accompanied in each step of the MAP kinase cascade. It would be of interest to see whether high molecular weight and long-chain PEG chains conjugated to the complex interfere with the multiple kinase reactions. Extracellular signal-regulated proteins kinase (ERK) was chosen as an intracellular marker protein because of the close relationship between the internalization of EGF-receptor complex and ERK phosphorylation (25,31,34). The clathrin-mediated endocytosis of the complex is closely related to the subsequent phosphorylation of ERK. Figure 4 shows that both PEG2k-EGF and PEG5k-EGF similarly activated ERK phosphorylation in comparison to nascent EGF. The PEG molecular weight did not seem to be a major factor in the phosphorylation of ERK, suggesting that the MAP kinase cascade was not disrupted by the PEG chain conjugated to the dimeric complex. These data clearly demonstrated that N-terminal specific mono-PEGylated EGF retained full receptor-binding capacity at the cell membrane without restraining the intracellular trafficking events of signal molecules. To our knowledge, the effect of synthetic polymers conjugated to cytokine molecules on the intracellular downstream signal transduction pathway has never been reported.

Evaluation of *in Vivo* and *in Vitro* Stabilities of PEGylated EGF

In general, factors determining pharmacokinetic properties of therapeutic proteins *in vivo* are renal excretion, endocytosis by receptors, recycling of proteins, immune complex formation, and proteolysis by proteases during blood circulation (35). Smaller proteins, such as G-CSF and various peptide hormones, are easily cleared by glomerular filtration by the kidney (36). EGF and PEGylated EGF are likely to be cleared by such processes. The increased circulation time due to PEGylation is about 4-fold: $t_{1/2}$ was 6.71 min for EGF and 24.36 min for PEG5k-EGF. As expected, using higher molecular weight PEG resulted in longer circulation time primarily due to decreased renal clearance rate and higher resistance against plasma proteases. PEG chains conjugated to therapeutic peptides and proteins have also played a critical role in preventing proteolytic degradation by various proteases present in blood and tissues (37,38). Figure 6 shows the dramatically enhanced resistance of PEGylated EGF against protease degradation in kidney tissue homogenate. The enhanced stability of EGF by PEGylation suggests that N-terminal mono-PEGylated EGF could be formulated as a topical ointment for the effective treatment of various wounds (14,15).

CONCLUSIONS

In this study, we demonstrated that PEG-propionaldehyde derivatives were specifically conjugated at the N-terminal α-amine group of EGF at a slightly acidic pH environment. The biologic activities of PEGylated EGF, analyzed by cell proliferation and immunoblot analysis of EGF receptor and ERK phosphorylations, were similar to those of native EGF. N-terminal specific mono-PEGylated EGF exhibited prolonged circulation in the blood stream and enhanced stability in tissue homogenate. The PEGylated EGF has a potential to be applied in wound healing treatments, sustained release, tissue engineering, and gene delivery.

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