Preparation and Characterization of Mono-PEGylated Epidermal Growth Factor: Evaluation of *in Vitro* Biologic Activity

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Purpose. To isolate mono-PEGylated epidermal growth factor (EGF) isoforms, identify the site of PEGylation, and evaluate the biologic activity of each isoform.

Methods. EGF was PEGylated with an NHS-PEG derivative (Mw 3,400). Mono-PEGylated EGF fraction was separated by gel-filtration HPLC and three mono-PEGylated EGF isoforms were purified by RP-HPLC. Tryptic digestion mapping of both EGF and mono-PEGylated EGF isoforms was performed to identify the PEGylation sites using RP-HPLC. The digested fragments were also analyzed by matrix-assisted laser desorption and ionization time of flight (MALDI-TOF) mass spectroscopy for further verification of the three PEG conjugation sites. The biologic activity of positional isoforms was evaluated by a cell proliferation assay and a receptor tyrosine kinase activity assay to determine the effect of PEGylation site on its activity.

Results. Mono-PEGylated EGF was composed of three positional isomers. Tryptic digestion mapping and MALDI-TOF analysis permitted the identification of the PEGylated site of the three isoforms at N-terminus, Lysine 28, and Lysine 48. PEG-N-terminus EGF, among the three positional isomers, showed the highest activity in a cell proliferation assay and in a receptor-binding assay.

Conclusion. This study demonstrates that biologic activities of mono-PEGylated EGF isomers are highly dependent upon the site of PEGylation in EGF.

KEY WORDS: epidermal growth factor (EGF); polyethylene glycol (PEG); peptide mapping; biologic activity.

INTRODUCTION

Epidermal growth factor (EGF) is a polypeptide composed of 53 amino acid residues, which demonstrates a potent mitogenic effect in various tissues and cells such as mouse fibroblasts, human fibroblasts, rabbit lens epithelial cells and rat hepatocytes (1–3). EGF has been popularly used for targeting specific cells that express EGF receptors on cell membrane. In addition, various anti-cancer drugs and macromolecular therapeutic agents could be delivered in a site-specific manner by utilizing EGF as a targeting moiety (4,5). Conjugation of EGF to drugs can facilitate the cellular uptake of the drugs by enhanced extent of receptor-mediated endocytosis. Recently, it was also reported that EGF has an additional effect on inhibiting carcinoma cell growth on which EGF receptors are over-expressed (6,7).

PEGylation has been extensively applied for enhancing pharmacological effectiveness of therapeutic proteins (8). The conjugation of PEG on the surface of proteins prolonged the circulation time in the blood, increased the resistance against proteolytic digestion in serum, reduced the immunogenicity and antigenicity, and lowered the cytotoxicity (9–11). A variety of PEG conjugated proteins such as interferon α (12), human growth hormone (13), insulin (14), and salmon calcitonin (15) have shown superior pharmacological properties to unmodified ones. It has been demonstrated that the site of PEGylation was very important and that it had major effects on their biologic activities. EGF is another well-suited therapeutic protein for PEGylation because of its stability, small size, and versatile therapeutic applications (16). The PEG-EGF conjugates were applied to enhance the pharmacological properties for treating human brain tumors in a site-specific manner (17), for tissue engineering (18,19), and for the encapsulation within biodegradable polymers (20). However, no studies have been reported about the activity change of PEGylated EGF as a function of the PEGylation site.

In this study, EGF was PEGylated and three mono-PEGylated EGF isoforms were separated and their bioactivities were characterized as a function of PEGylation site. This investigation extends our previous report of EGF receptor-mediated non-viral gene delivery system based on mono-PEGylated human epidermal growth factor (21). Three positional isomers of mono-PEGylated EGF were collectively used as a ligand molecule in the previous report. In the present study, the three positional isomers were separated and the site of PEGylation was identified through tryptic peptide mapping and MALDI-TOF techniques. Biologic activity of each isomer was evaluated by measuring the extent of growth stimulation and inhibition of cells expressing EGF receptors and by estimating the *in vitro* phosphorylation of EGF receptors.

MATERIALS AND METHOD

Materials

Recombinant human epidermal growth factor (rhEGF) was donated from Daewoong Pharmaceutical Co. (Seoul, Korea). Sequencing grade typsin (from bovine pancreas) was purchased by Roche Diagnostics Co. (Indianapolis, Indiana). mPEG-CO_2-NHS (methoxy-poly(ethylene glycol)-N-hydroxysuccinimide) (MW 3,400) was obtained from Shearwater Polymers (Huntsville, AL). An EGF ELISA kit was from R&D Systems (Minneapolis, Minnesota). EGF receptor was purchased from Promega (Madison, Wisconsin). The A431 (human epidermoid carcinoma) cell line was from Korea Cell Line Bank (Seoul, Korea) and NRK 47F (normal rat kidney) was a kind gift from Daewoong Pharmaceutical Co. (Seoul, Korea). [^{3}H] thymidine and [γ–^{32}P] ATP were purchased from Amersham Pharmaceutical Biotech. (Buckinghamshire, England). Dialysis membranes (MW cutoff, 3,500 and 10,000) were purchased from Spectrum (Houston, Texas). Whatman P81 paper was a product of Whatman Bioscience (Newton, Massachusetts). Phosphate-buffered saline (PBS), HEPES-buffered saline (HBS), Dulbecco's Modified Eagle's

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ABBREVIATIONS: DMEM, Dulbecco's Modified Eagle's Medium; EGF, epidermal growth factor; FBS, fetal bovine serum; HBS, HEPES-buffered saline; MALDI-TOF, matrix-assisted laser desorption and ionization time of flight, PBS, Phosphate-buffered saline.

Medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin, and trypsin-EDTA were obtained from Gibco-BRL (Grand Island, New York). A micro-BCA protein assay kit was purchased from Pierce (Rockford, Illinois). Sodium iodoacetate, angiotensin II, and sodium vanadate were provided by Sigma (St. Louis, Missouri).

Methods

Preparation and Isolation of Mono-PEGylated EGF Isoforms

Freeze-dried EGF (8 mg) and mPEG-CO_2-NHS (30-fold molar excess, 140 mg) were co-dissolved in 8 ml of a phosphate buffer (50 mM, pH 8.0). The conjugation reaction was carried out for 12 h at room temperature. The solution was diluted by addition of 0.1 N HCl (adjusted to pH 4) to terminate the reaction. To remove unconjugated mPEG-NHS molecules and unreacted EGF, the reaction solution was dialyzed twice (MW cutoff = 10,000) against 2.5 liter of the same phosphate buffer.

Characterization and Purification of Mono-PEGylated EGF Isoforms

PEGylated EGF species were analyzed by size-exclusion chromatography HPLC (SEC-HPLC) system (Waters 660E) on Shodex protein KW-800 column; 250 μl of the sample was loaded into the column; an isocratic mobile phase composed of 50 mM NaCl and 50 mM phosphate buffer, pH 6 was used at a flow rate of 1.0 ml/min; UV detection was carried out at 280 nm. To purify the mono-PEGylated mPEG-EGF conjugate from the multi-PEGylated species, the mono-PEGylated fraction eluted in the SEC-HPLC system was pooled and concentrated using a rotatory vacuum drier. The concentrated mono-PEGylated EGF mixture (4.5 ml) was further separated into three different fractions in a reversed-phase HPLC system. One hundred μl of the mixture was injected in a Waters Spherisorb C-18 column (4.6 × 250 mm) equipped with a guard column. Gradient elution was performed at a flow rate of 1.0 ml/min with a solvent A (0.1% TFA in water) and a solvent B (0.1% TFA in acetotitrile). A gradient program was set as follows: 30% solvent B for 10 min, 60% solvent B over 30 min, 100% solvent B over 5 min.

Tryptic Peptide Mapping of EGF for an Analysis of PEGylation Site

Tryptic digestion was performed to identify the PEGylation sites of EGF. Five milligrams of EGF was dissolved in 2 ml of 0.1 M Tris-HCl, pH 8.0, 6 M GuHCl, to fully denature EGF molecules and then 20 μl of dithiothreitol (DTT) solution (final concentration of 10 mM) was added to reduce the disulfide bonds in EGF. To prevent re-oxidation of the reduced free thiol groups, 200 μl of iodoacetate solution (0.1 M) was added to alkylate the free thiol groups, and incubated for 6 h at room temperature. Dialysis (MW cutoff: 3,500) was performed twice in a 5 mM Tris-HCl buffer, pH 8.0. The dialyzed solution was concentrated to 1 ml by a rotatory-vacuum drier. Tryptic digestion was carried out 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. Mono-PEGylated EGF isoforms (about 100 μg) were also digested with trypsin by the same procedure described above. The fraction amount of each mono-PEGylated EGF isoform was quantified by using a SEC-HPLC system.

Reversed-Phase HPLC for Tryptic Digestion Solution

Fifty micro liters 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 a solvent A (0.1% TFA in water) and a solvent B (0.1% TFA in acetonitrile). A gradient program was set as follows: 2% solvent B for 10 min, 60% solvent B over 30 min, and 100% solvent B over 10 min.

MALDI TOF Spectrometry

The tryptic digested fragments were purified and subsequently analyzed by MALDI-TOF (Voyager DE-STR Perkin-Elmer PerSeptive Biosystem) mass spectrometry. The sample crystals were prepared with sinapinic acid as a matrix. MALDI-TOF operation conditions were set as follows: mode of operation was linear, polarity was positive, the acceleration voltage was 25000 V, and delayed extraction time was 160 nsec.

Cell Proliferation Assay

NRK 47 and A431 cells were seeded at a density of 5×10^3 cells per cm^2 in 24-well plates in 2 ml of DMEM containing 10% FBS. After 24 h, the medium was removed and washed with PBS before the addition of serum free DMEM. NRK47F and A431 cells were pre-incubated for 12 h and for 8 h respectively. After different amounts of EGF and PEGylated EGF were added into each well at time (t) = 0, new media containing [^{3}H] thymidine were then added at specific time points. The duration of thymidine incubation was 8 h (from t = 12 to t = 20) for NRK 47F and 19 h (from t = 8 to t = 27) for A431. The cells were treated with 20% cold trichloroacetic acid and precipitated. The precipitates were collected on nitrocellulose papers and counted with a scintillation counter (Beckman LS 6500).

Tyrosine Kinase Activity of EGF Receptor

Upon EGF binding, the tyrosine kinase domain of EGF receptor becomes active initiating phosphorylation to its substrates and itself. The following four reagent solutions were prepared: 18 mM angiotensin II, 10 mg/ml of BSA, 1.5 M of ammonium sulfate, and 0.2 unit of EGF receptor in 20 mM HEPES (pH 7.4, 50 μM Na_3VO_4, 10 mM $MgCl_2$, 1 mM $MnCl_2$). The reagent mixture was composed of equal volumes of the four solutions described above (total 40 μl per reaction tube). The reagent mixture was incubated for 2 min at 30°C before the addition of 20 μl of the phosphorylation buffer (60 mM HEPES pH 7.4, 30 μM ATP, 150 μM Na_3VO_4, 30 mM $MgCl_2$, 3 mM $MnCl_2$ and 2 Ci per mM of [γ–^{32}P] ATP) containing either native EGF or mono-PEGylated EGF isoforms to a final concentration of 0.001 nM to 100 nM. The enzyme reaction was initiated by mixing the phosphorylation buffer and the reagent mixture. The reaction was left for 3 min and stopped by adding 20 μl of 20% trichloroacetic acid (TCA) solution to the reaction tube and placed in ice (total 80 μl). The solution was centrifuged at 16,000 rpm for 5 min and

the supernatant solution (60 μl) was transferred into a new tube containing 60 μl of dH_2O. The reaction mixture (100 μl) were spotted onto Whatman P81 paper, which was washed three times with a 0.5% phosphoric acid solution and placed in a scintillation vial to count the radioactivity.

The amount of transferred [γ–^{32}P] onto angiotensin II was calculated by the following equation:

$$[CPM]_{angiotensin} = \{[CPM]_{total} - [CPM]_{blank}\} - d[CPM]_{auto}$$

where, $[CPM]_{angiotensin}$ is the radioactivity of transferred phosphate molecules to angiotensin II, $[CPM]_{total}$ is the total radioactivity observed in a scintillation counter, $[CPM]_{blank}$ is the blank radioactivity, and $d[CPM]_{auto}$ is the cpm value of autophosphorylated on EGF receptor. More detailed procedures can be obtained from the reference (22).

RESULTS AND DISCUSSION

EGF has three potential PEGylation sites: two primary ε-amines of lysine residues (Lys28, Lys48) and one primary α–amine of N-terminus. Figure 1 shows a 3-D solution structure of EGF (23,24). In this figure, the amino acid residues represented by space-filling balls are the three potential PEGylation sites (N-terminus, Lys28, and Lys48) and two critical receptor-binding residues (Arg41 and Leu47). It can be seen that the three primary amines are not structurally buried inside of EGF, but are exposed on the surface. This suggests that a mPEG-NHS derivative can have access to all the three primary amine groups of EGF, producing a heterogeneous mixture of tri-, di-, and mono-PEGylated EGF. It is also noted that the receptor binding residues are located far away from the PEG conjugation sites of N-terminus and Lys28 residues. After PEGylation reaction between EGF and mPEG-NHS derivative, mono-PEGylated EGF fraction was separated and pooled by using a SEC-HPLC system (21). The obtained mono-PEGylated EGF fraction was further separated into each positional isoform using reversed-phase HPLC (Fig. 2). Three peaks appeared in the chromatogram, which was thought to be as N-terminus PEGylated EGF, Lys28 PEGylated EGF, and Lys48 PEGylated EGF. The three peaks were denoted by A, B, and C in the order of elution time at 29.07, 30.17, and 34.60 min, respectively. The appearance of the three peaks with an almost similar peak area implies that all the available primary amine groups in EGF have almost equal reactivity to the mPEG-NHS derivative.

Figure 3A shows the RP-HPLC profile of fragmented nascent EGF after the treatment of trypsin. Trypsin cleaves the carboxyl-side of lysine and arginine residues producing five peptide fragments. The possible fragments generated by the trysin digestion are denoted as T1 (Asp1 – Lys28), T2 (Tyr29 – Arg41), T3 (Cys42 – Arg45), T4 (Asp46 – Lys48), and T5 (Trp49 – Arg53). In the chromatogram, the peaks were named as 1, 2, 3, 4, and 5 in the elution order. To identify each peak, each fraction was collected, concentrated, and then analyzed by MALDI-TOF mass spectrometry. The experimental MALDI-TOF mass results showed reasonable consistency with the theoretical mass of each fragment. Therefore, each peak can be assigned as follows: 1 – T3, 2 – T4, 3 – T5, 4 – T2, and 5 – T1 (Fig. 3B).

The sites of PEG conjugation in EGF were analyzed by tryptic digestion mapping of A, B, and C fractions obtained in Figure 2. Many previous studies reported that the amino acid residue conjugated with a polymer, especially PEG, showed

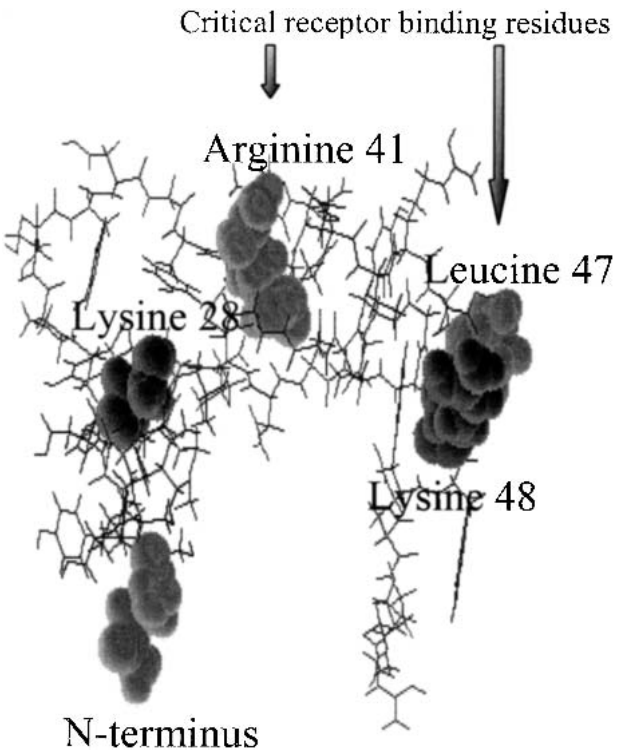


Fig. 1. A three dimensional structure of human EGF. Major PEGylation sites are primary amine of N-terminus, and ε-amines of Lys28 and Lys48. Critical residues involving EGF receptor binding are indicated by arrows: Arg 41 and Leu 47.

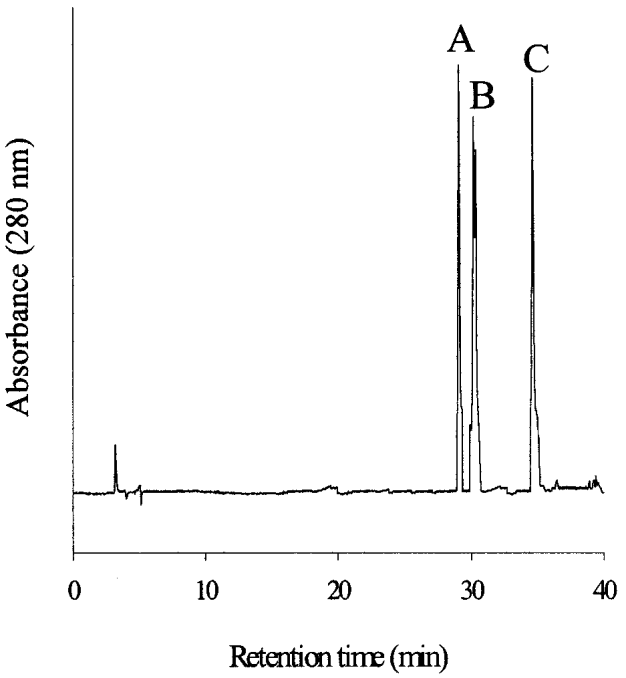
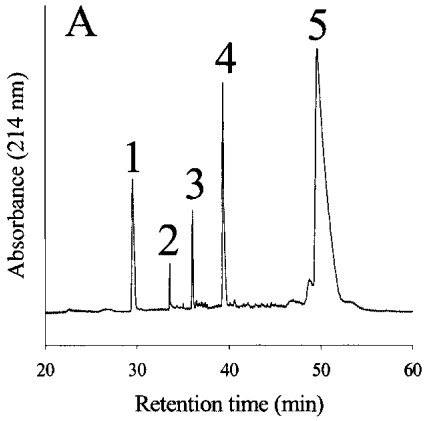


Fig. 2. Reversed-phase HPLC chromatogram of three positional isomers of mono-PEGylated EGF. A, B, and C represent the positional isomers of mono-PEGylated EGF conjugates purified from SEC-HPLC.



B

	Measured Mw	Expected Mw	Identity
1	627.6	626.6	T3
2	394.6	374.4	T4
3	789.4	788.9	T5
4	1498.5	1498.6	T2
5	3290.5	3289.3	T1

Fig. 3. (A) Reversed phase HPLC chromatogram of tryptic digestion of native EGF. (B) Identification of individual native EGF fragments generated by tryptic digestion by MALDI-TOF mass spectroscopy.

resistance to digestion by trypsin because of the steric repulsion effect of PEG (12–15). The tryptic digestion patterns of A, B, and C fraction are significantly different compared with that of native EGF, as shown in Fig. 4. For the fraction A, the peak denoted as 5 in native EGF is significantly reduced and a new peak appears over the retention time of 50 min. For the fraction B, peaks 4 and 5 disappear and for the fraction C, peaks 2 and 3 are significantly reduced. Moreover, new additional peaks can be detected in the chromatogram for A, B, and C fractions; they are noted as x, y, and z, respectively. These might be assigned as PEGylated polypeptide fragments, being resistant to the tryptic digestion. As summarized in Table I, the tryptic digestion mapping strongly indicates that PEG-N-terminus EGF could be assigned for the fraction A, PEG-Lys28 EGF for the fraction B, and PEG-Lys48 EGF for the fraction C. The new peak of x, y, and z, appearing in the chromatograms of A, B, and C, respectively, was separated and analyzed in MALDI-TOF mass spectrometry to further verify the PEGylation sites. As shown in the Figure 5, the mass spectroscopy gives semi-quantitative mass profiles of each PEG conjugated EGF fraction. The observed mass peak 'X' appears at 6700 m/z with a multi-distributed pattern, suggesting that it is a mass sum of T1 fragment (3290 Da) and PEG (3400 Da). Additionally, mass peaks of 'Y' and 'Z' are detected at 8200 and 4500 m/z, respectively, corresponding to a mass sum of T1-T2 fragment (4789 Da) and PEG (3400 Da), and of T4-T5 (1184 Da) and PEG (3400 Da). Therefore, we assigned the fraction of 'X' as PEG-N-term-T1 fragment, of 'Y' as PEG-Lys28-T1-T2, and of 'Z' as PEG-Lys48-T4-T5. These MALDI-TOF data support that the newly eluted peaks were digested PEG conjugated EGF fragments, which have reasonably consistent mass ranges as predicted by the result of RP-HPLC patterns (Figure 4).

One of the important functions of EGF is a potent mitogenic activity in several tissues and cell lines in *in vivo* and

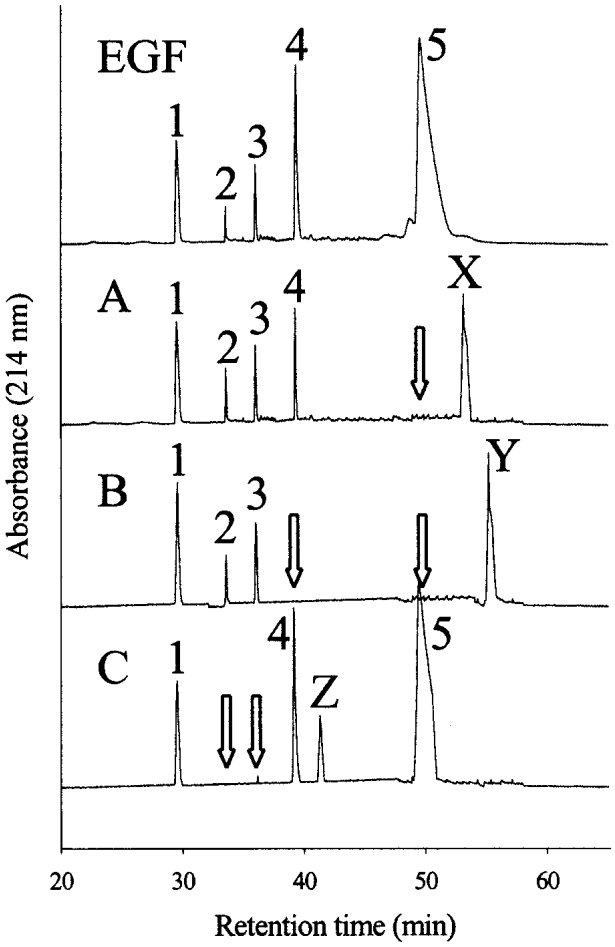


Fig. 4. Reverse-phase HPLC of trypsin digested native EGF and three positional isomers of mono-PEGylated EGF. A, B, and C are the fractions eluted from RP-HPLC in Figure 2. The newly produced peaks of X, Y, and Z were purified for further analysis of MALDI-TOF.

Table I. Identification of Three Positional Isomers of Mono-PEGylated EGF by Tryptic Digestion Analysis

	EGF Fragments					
	T3	T4	T5	T2	T1	Identity
Native EGF	+	+	+	+	+	
A	+	+	+	+	–	PEG-N-term-EGF
B	+	+	+	–	–	PEG-Lys28 EGF
C	+	–	–	+	+	PEG-Lys48 EGF

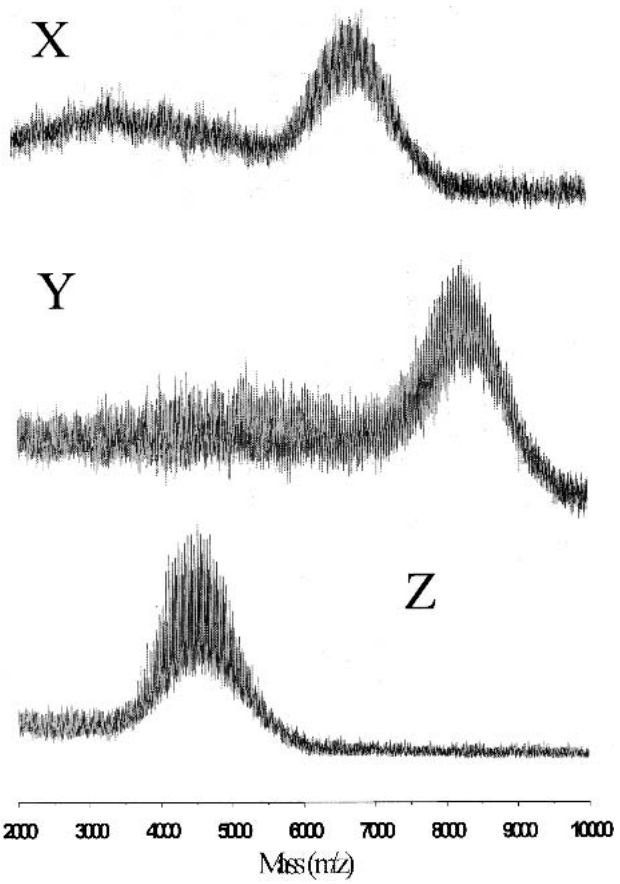


Fig. 5. MALDI-TOF mass spectra of digested mono-PEGylated EGF fragments. X, Y, and Z mean the newly eluted peaks detected in Figure 4. The 6700 m/z peak assigned for X is PEG-N-term-T, 8200 m/z for Y is PEG-Lys28-T1-T2, and 4500 m/z for Z is PEG-Lys48-T4-T5.

in vitro (2). Although the PEGylation of EGF is expected to prolong the circulation time in blood, enhance the physical stability, and reduce the immunogenicity, the conjugates might inevitably have reduced biologic activities because of the steric hindrance effect of PEG chains. The three separated mono-PEGylated EGF isoforms with full identification of their PEGylation sites were tested for their biologic activities using two kinds of cell lines, NRK 49F and A431. Figure 6A shows the cell proliferation profiles of NRK 49F cells, a well-known EGF receptor expressing cell line, upon incubation with different amounts of EGF and three mono-PEGylated EGF isoforms. The cell proliferation of NRK 49F cells incubated with the PEG-N-terminus EGF conjugate was stimulated at the effective concentration of >1 nM as measured by [^{3}H] thymidine uptake. The level of DNA synthesis was about 60% compared to that of native EGF at a higher concentration range. However, the DNA synthesis level was substantially reduced for the PEG-Lys28 EGF and the PEG-Lys48 EGF conjugates, suggesting that the conjugated PEG in the middle of the polypeptide backbone of EGF significantly suppressed the interaction between EGF and its receptor, possibly due to the aforementioned steric effect of PEG. It has been known that EGF also has a growth inhibition effect on EGF receptor over-expressing cells such as A431 (6,7). Since EGF receptor over-expression is considered an indication of non-small cell lung cancer or epithelial carcinomas, EGF and related cytokines have been applied as ligands for the target-specific treatment of human cancers (4,17). Figure 6B shows that the DNA synthesis was inhibited upon incubation of EGF and mono-PEGylated EGF isoforms in A431 cells. PEG-N-terminus EGF conjugates exhibited reduced inhibition activity at sub-nanomolar ranges (<1nM) when compared with native EGF. However, at higher concentrations (>1 nM), the conjugate showed similar levels of inhibiting DNA synthesis. From cell growth and inhibition studies, PEG-Lys28-EGF and PEG-Lys48-EGF conjugates showed significant reduction of biologic activities, while PEG-N-term EGF showed only a slight reduction. The critical EGF receptor binding residues were reported to be Arg41 and Leu47, which were confirmed by a genetic point mutation analysis (25–28). Considering the previously reported data and the 3-D solution structure of EGF (Fig. 1), the conjugated PEG at the N-terminus residue of EGF is likely to provide a negligible effect on biological activities. Lys28 is located

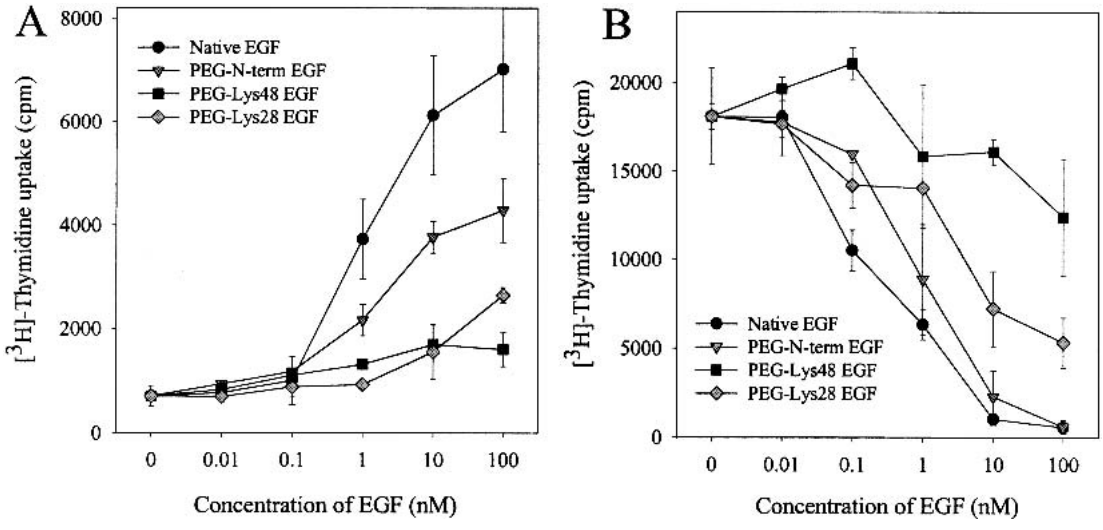


Fig. 6. (A) Cell proliferation assay ([^{3}H] thymidine uptake) initiated by native EGF and three positional isomers of mono-PEGylated EGFs in NRK-49F cells (n = 3). (B) Growth inhibition of A431 cells by incubation of native EGF and three positional isomers of mono-PEGylated EGFs (n = 3).

closer to the receptor binding sites than the N-terminus residue; the conjugated PEG to this site would have imposed more steric effect in receptor binding than that of N-terminal residue. PEG conjugation to Lys48 resulted in very low biologic activities because it directly restricts the binding of EGF to the receptor due to its very proximal position to the EGF receptor binding sites.

To confirm whether the binding of PEGylated EGF to receptor is specific enough to initiate cytosolic signals by phosphorylation of messenger proteins, *in vitro* receptor tyrosine kinase activity assay was carried out (Fig. 7). EGF receptor has an intrinsic tyrosine kinase activity which phosphorylates many signal proteins in cytosol such as phospholipase C-γ and GTPase activating protein (29,30). The degree of phosphorylation agreed well with the cell growth results (Fig. 6 and 7): native EGF > PEG-N-terminus EGF > PEG-Lys28 EGF > PEG-Lys48 EGF. This result reveals that site-specific PEGylation to the N-terminus of EGF is the most promising and desirable way to maximize the biologic activity of PEGylated EGF.

Mouse EGF has only one terminal primary amine without having lysine groups in its backbone. Thus, many research groups have been using it because of an easy way to obtain mono-PEGylated EGF. However, mouse EGF has a critical problem in many clinical applications due to its origin. Therefore, acquisition of mono-PEGylated human EGF retaining its biologic activity is highly required for many therapeutic applications such as EGF receptor targeted cancer treatments and target-specific drug delivery systems. Mono-PEGylated EGF could be used as a targeting moiety to deliver plasmid DNA and various drugs to specific cells and tissues expressing EGF receptors on their membrane.

CONCLUSION

EGF was conjugated with mPEG-NHS and three positional mono-PEGylated isomers were separated. Tryptic digestion mapping followed by RP-HPLC identified the site of PEGylation. The mass of digested fragments conjugated with PEG was also measured by MALDI-TOF MS for the further confirmation of the PEGylation sites. Cell growth stimulation or inhibition experiments revealed that PEG-N-terminus EGF showed the highest biologic activity and PEG-Lys48 EGF demonstrated the lowest one. The activation of tyrosine kinase function of EGF receptor by mono-PEGylated EGF also exhibited the highest phosphorylation degree for the PEG-N-terminus EGF.

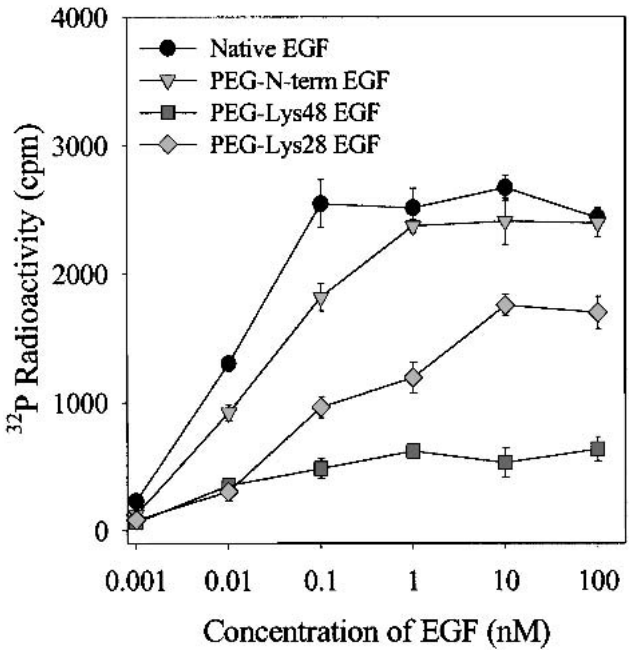


Fig. 7. *In vitro* assay of tyrosine kinase activity of EGF receptor. The amount of transferred γ–^{32}P molecules from ATP to angiotensin, used as a substrate, was measured by a scintillation counter (n = 3).

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