Synthesis and Characterization of the Conjugate of Poly(ethylene glycol) with Human Epidermal Growth Factor(hEGF) and its Cell Proliferation Effect

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Abstract

3 Kinds of PEG-hEGF conjugate, PEG1-hEGF, PEG5-hEGF, PEG10-hEGF, were synthesized. Major fractions containing 2 chains of PEG were separated by preparative GPC. Molecular weight was estimated by GPC-MALLS, TOF-Mass and SDS-PAGE, and the data were well corresponding to calculated one. The cell proliferation effect of the conjugates was evaluated, indicating that the conjugate bound to longer chain PEG exhibits lower activity. Selective modification of hEGF and activity preservation of PEG-hEGF conjugate are undergoing.

Introduction

Human Epidermal growth factor(hEGF) is a small peptide of 53 amino acid residues(1,2) and its molecular weight is 6218 dalton. By using genetic engineering technology, production on large scale of mature hEGF has been possible(3,4), which made hEGF possible to be used in biological applications. Owing to its wide range of bioactivities, such as stimulation of cell proliferation(5) and inhibition of gastric acid secretion(6,7), many studies have been done to use it in the field of tissue engineering, pharmacological application(8,9), and even in biotechnological application such as biological wool harvesting(10). However, if hEGF is directly applied into biological systems, it is difficult to avoid the cleavage of the protein to inactive species by proteolytic enzymes and the rapid exclusion through the kidneys, which consequently causes the short half-life *in vivo*. It is also possible to induce undesired biological responses, such as immunogenecity and immunoreactivity, due to internalization of the exogenous protein.

One promising method to overcome above problems is to modify the exogenous protein with polymer chain. Several kinds of polymers, such as polyethylene glycol(11), polysaccharides(12), amino acid polymers(13), and anionic polymers(14,15), have been used as polymer chain. One of them, polyethylene glycol has been extensively studied for use as a chemical modifier in the synthesis of polymer-protein hybrid(11) owing to its good bioavailibility such as high water solubility, protein resistance, low toxicity and non-immunogenecity. Thus, the main advantage of PEG modification of proteins involves enhanced plasma half-life, improved water solubility, and decreased immunogenecity(16,17).

In this paper, we firstly report the synthesis and characterization of PEG-hEGF conjugates with different molecular weights of PEG and describe

their biological effect on cell proliferation *in vitro*.

Experimental

Materials

Human epidermal growth factor(hEGF) was kindly provided by Higeta Shoyu Co. Ltd., Chiba, Japan. Activated poly(ethylene glycol)s(ω -methoxy succinimidyl propionate-PEG) with different molecular weights(Mw 1,000, 5,000 and 10,000) were perchased from Shearwater Polymers, Inc., Huntsville, USA. 1-N-Methylpyrrolidone(NMP, Aldrich) was dried and distilled over calcium hydride under an argon atmosphere before use. All inorganic salts used for preparing buffer solutions were biological grade.

General Synthesis of PEG-hEGF Conjugates

To definite amount of borate buffer (0.1M, pH 8.6) solution containing calculated amount of hEGF(Table 1), NMP solution containing calculated amount of ω -hydroxy-PEG-succinimidyl propionate added and stirred for a period of time at room temperature. Any remaining activated PEG was removed by reaction for 30 minute with excess ε -amino caproic acid. After adding 100ml of distilled water to the solution, the precipitated solid was filtered off using membrane filter with 0.45um of pore size. The filtrate was desalted by ultrafiltration and lyophilized to give unfractionated conjugate.

Separation and Purification of PEG-hEGF Conjugates

Preparative GPC was performed by Shimadzu LC10 titanium liquid chromatography system, Shimadzu Corp., Kyoto, Japan, with a Pharmacia Superdex 75 preparative column(i.d. 16 x 600mm L), and with 0.05M phosphate buffer, pH 5.0, containing 0.5M sodium chloride as a eluate to minimize

hydrophobic interactions ; and the optical density was monitored at 280nm. After collecting the major fraction(PEG1-hEGF for PEG of Mw 1,000, PEG5-hEGF for 5,000, and PEG10-hEGF for 10,000, respectively) in elution pattern, the collected solution was desalted by utrafiltration and lyophilized to give fractionated PEG-hEGF conjugates.

Characterization of PEG-hEGF Conjugates

Molecular weight of PEG-hEGF conjugates was determined by using GPC-MALLS(multi angle laser light scattering) system with a DAWN DSP-F laser photometer of Wyatt Technology Corp. CA, USA, with Shodex KW-802.5 column, and with 0.05M phosphate buffer, pH 6.8, containing 0.15M sodium chloride as a eluate. To cross-check the molecular weights of the conjugates, TOF-Mass system of Shimadsu Corp(Kratos, Kompact Maldi III), Kyoto, Japan, was also employed, in which the matrix used was sinapinic acid. SDS-PAGE was carried out on Phast gredient gel(Pharmacia) according to the manufacturer's instruction. Protein content was determined by the Biuret method(18), with the optical density measured at 540㎚.

Cell Proliferation Test

BHK-21 baby hamster kidney cell line was maintained as stock in DF and ITS+ medium. The rapidly growing cells were harvested, counted, and inoculated at a concentration of $2x10^3$ cells/㎖ into 96 well microtiter plates. After incubation for 24hrs, the PEG-hEGF conjugates dissolved in culture medium were applied to the culture wells in triplicated followed by incubating for 68hrs at 37℃ under 5% CO_2 atmosphere. After adding 10㎕ WST-1 reagent to the culture medium, it was incubated for 4hrs at 37℃. The absorbance at 450㎚ was measured with a microplate reader(Dynatech Model MR 700).

Results and Discussion

The amino acid sequence of hEGF has already been established(1,2) and its three amino groups, derived from two lysine and one terminal asparagine residue, can react with activated PEG to produce PEG-hEGF conjugate. To facilitate the conjugation reaction, N-1-methyl-2-pyrrolidone was used, controlling not exceed 20%(v/v) compared with the volume of borate buffer. The synthetic data of the conjugates are shown in Table 1. The molar ratios of activated PEG added to the reaction were 2 times to the mole of three amino groups of hEGF. GPC elution pattern of the conjugation product with different molecular weights(Mw 1,000, 5,000, and 10,000) are shown in Figure 1. It was found that one major component in the elution pattern is produced under the conjugation condition employed(Figure 1). The major fraction was separated by preparative GPC to determine its molecular weight and protein content and to investigate its cell proliferation effect. The yield of the major fraction depended on the initial molar ratio of PEG to hEGF and on the volume of the buffer and NMP added to the reaction(data not shown). Increasing the concentration of PEG(maximum 6 mole-times to hEGF) and decreasing the volume of the mixed solvent resulted in a higher yield of major fraction. In Figure 1, it was observed that elution time of the fraction C in all elution patterns was overlapped with that of the unreacted PEG under the used separation conditions. The fraction C, therefore, was not collected. Fraction A was also not collected due to its very low yield. In the case that excess amount of activated PEG more than 6 mole-times was added to the hEGF, the peak of the major fraction in all elution patterns was overlapped with that of unreacted PEG, which was confirmed by measuring elution time of hydrolyzed activated-PEG monitored at 230㎚. Complete purification of the major fraction, thus, was impossible. Molecular weight of the PEG-hEGF

conjugates separated by preparative GPC(PEG1-hEGF, PEG5-hEGF, and PEG10-hEGF) was measured by gel permeation chromatography with multi angle laser light scattering(MALLS) photometer and TOF-Mass system, respectively. The data are shown in Table 2. Estimation of the exact molecular weight of PEG-protein conjugates is one method to determine the degree of modification of protein(18,19). Molecular weight of PEG-hEGF conjugates should be decided very carefully because hEGF has only three amino groups in its amino acid residues, capable of reacting with PEG. It is also known that covalent attachment of the hydrophilic polymer PEG to proteins significantly affects its size(hydrodynamic radius)(20). Therefore, the use of universal calibration method for determining molecular weights of PEG-hEGF conjugates can not exactly give the degree of modification of the PEG-hEGF conjugates. Molecular weights of the conjugates measured by GPC-MALLS and TOF-Mass, respectively, were found to be very corresponding to the theoritical values, calculated assuming that two chains of PEG were bound to one molecule of hEGF(Table 2). Low rectivity of one amino group compared with other two amino groups in three amino group-containing residues of hEGF might result in such an elution patterns in GPC, showing that one major fraction is predominently synthesized(Figure 1). It is in general to react preferentially with amino group of lysine in conjugation of protein with PEG because the neucleophilicity of amino group of lysine is higher than that of other amino acid residues. Activated PEG, therefore, might be bound to two lysine residues of hEGF. Protein content of the conjugates was determined by the Biuret reaction(17), and the results are shown in Table 2. The low protein contents of the conjugates compared with calculated values are attributed to the hydration of the conjugates due to very high hydrophilic properties of PEG.

Cell proliferation effect of the conjugates are invesigated against BHK-21

cell and the results are shown in Figure 2 and 3. The higher molecular weight of PEG was bound to hEGF, the lower cell proliferation effect was exhibited, indicating that long chain PEG interferes active site of hEGF. The activity of PEG1-hEGF(ED_{50} : 40ng/㎖) on cell proliferation is decreased compared with unmodified hEGF(ED_{50} : 0.25ng/㎖). In order to preserve the activity of hEGF, selective modification of lysine residues with other activated PEGs is under progress.

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Captions of Figure

Figure 1. Comparison of the chromatograms of PEG-hEGF conjugates and PEGs

Figure 2. Effect of PEG-hEGF conjugates containing different Mw of PEG on the proliferation of BHK-21 cell(baby hamster kidney-derived cell)

Figure 3. Comparison of the effect of PEG1-hEGF conjugate on the proliferation of BHK-21 cell

Table 1. Synthetic conditions of PEG-hEGF conjugates at room temperature

conjugate	Mw of PEG	MeO-PEG[a](mg) (mol)	hEGF[b] (mg) (mol)	Buffer (mℓ)[c]	NMP (mℓ)[d]	time (h)	yield (%)
PEG1-hEGF	1,000	207.5 (1.93×10^{-4})	208.0 (3.22×10^{-5})	20	5	1	84.2
PEG5-hEGF	5,000	385.5 (7.71×10^{-5})	83.2 (1.29×10^{-5})	8	2	1	91.6
PEG10-hEGF	10,000	966 (9.66×10^{-5})	104 (1.61×10^{-5})	20	5	2	97.2

a) ω-Monomethoxy-PEG-succinimidyl propionate

b) Human epidermal growth factor (Mw=6,218)

c) 0.1 M, pH 8.6

d) 1-N-Methyl-2-pyrolidone

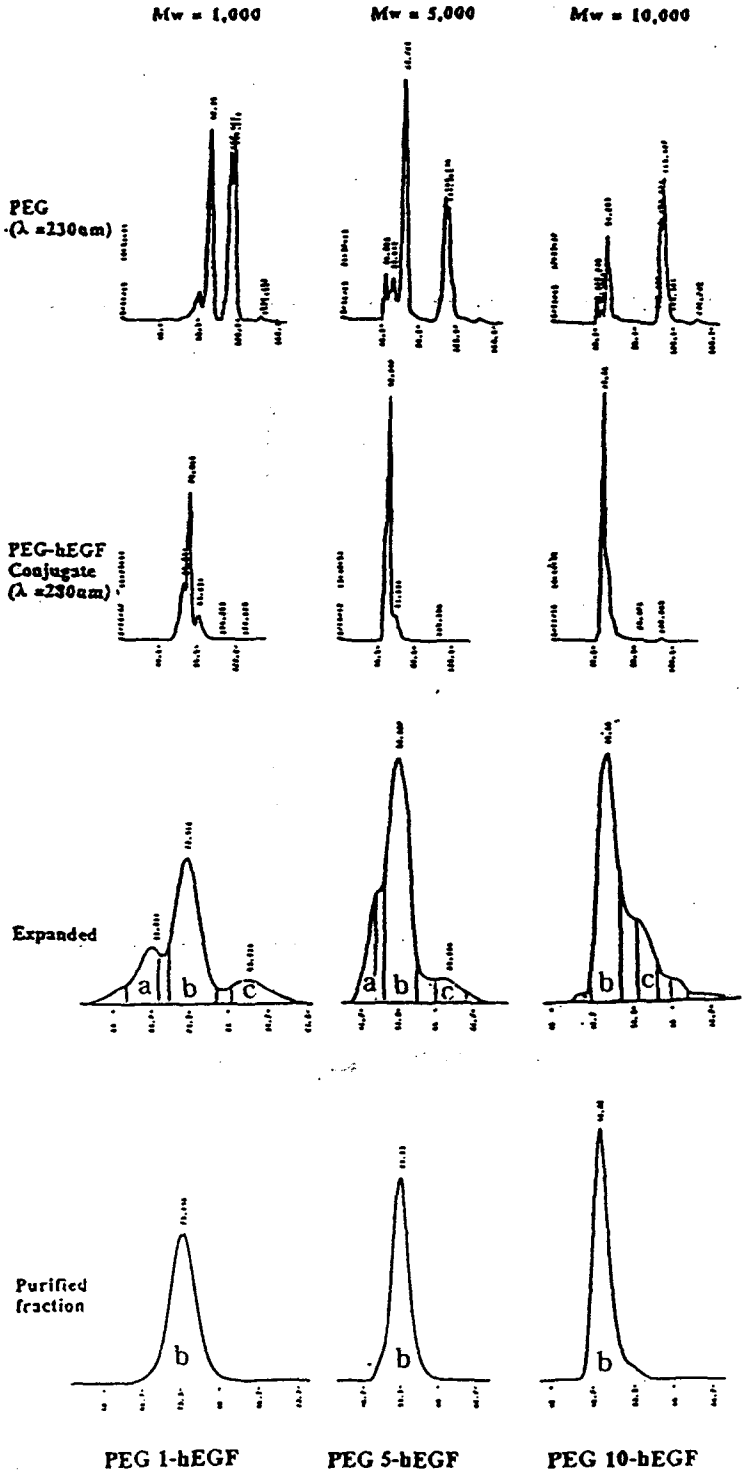
e) Calculated based on the chromatograms obtained by UV monitoring (280nm)

Table 2. Charaterization data of the PEG-hEGF conjugates

compound	Mw			Protein content (%)	
	Calcd.[a]	Found		Calcd.[a]	Found
		GPC-MALLS	TOF-Mass		
hEGF[b]	6,218	6,200	6,200	-	-
PEG1-hEGF	8,200	7,600	8,500	75.7	65.2
PEG5-hEGF	16,200	16,000	15,900	38.3	28.0
PEG10-hEGF	26,200	26,000	26,300	23.7	20.0

a) It was calculated assuming that two chains of PEG were bound to one molecule of hEGF

b) Mw of the human epidermal growth factor(hEGF) is 6,218, and was calculated based on its amino acid residues.


Mw = 1,000
Mw = 5,000
Mw = 10,000
PEG
(λ =230nm)
PEG-hEGF
Conjugate
(λ =230nm)
Expanded
a
b
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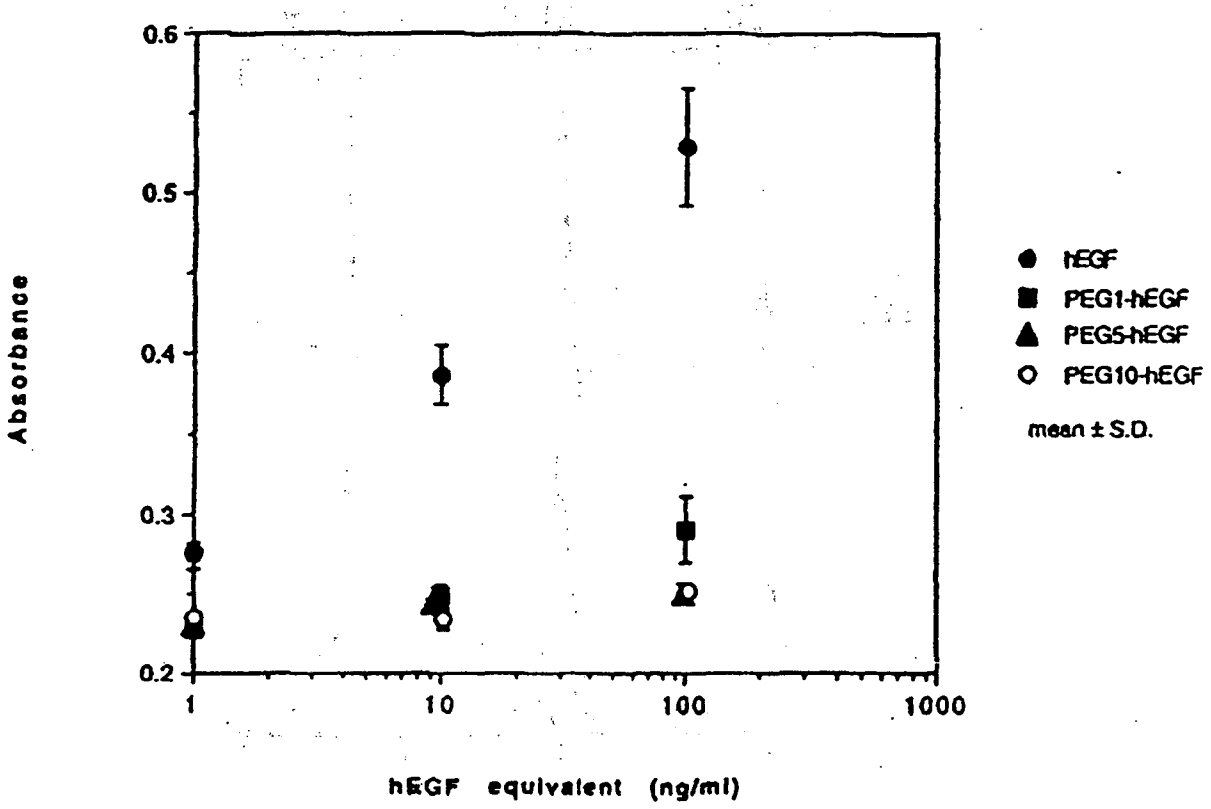
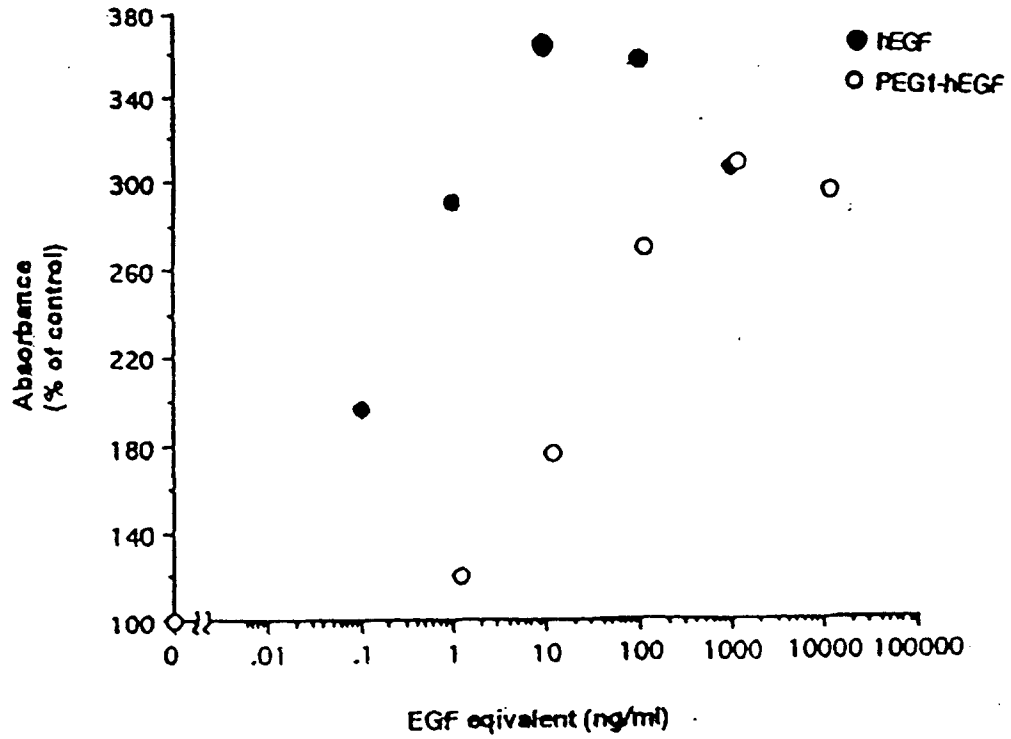
Purified
fraction
b
PEG 1-hEGF
PEG 5-hEGF
PEG 10-hEGF


Absorbance
0.6
0.5
0.4
0.3
0.2
1
10
100
1000
hEGF equivalent (ng/ml)
hEGF
PEG1-hEGF
PEG5-hEGF
PEG10-hEGF
mean ± S.D.


380
340
300
260
220
180
140
100
Absorbance
(% of control)
0
.01
.1
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10000
100000
EGF eqivalent (ng/ml)
hEGF
PEG1-hEGF