Stabilization of Epidermal Growth Factor on Thermal and Proteolytic Degradation by Conjugating with Low Molecular Weight Chitosan

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ABSTRACT: Epidermal growth factor (EGF, 5900 Da) has been reported to have the high efficiency of wound repair. However, the half-life of EGF in the body is too short to exert the biological activity effectively when applied in free forms. Conjugation of the low molecular weight chitosan (LMC) to EGF was carried out to enhance its stability. EGF was conjugated with LMC activated by water-soluble carbodiimide. The formation of EGF–LMC was quantitatively measured by indirect enzyme-linked immunosorbent assay (ELISA). In a study of the thermal and the proteolytic stability of free EGF and EGF–LMC, EGF covalently attached to LMC was found to be more stable than free EGF in thermal and proteolytic stabilities. In animal experiments of which free EGF (control), EGF–LMC (test) and LMC (carrier) diluted in viscous carboxymethyl cellulose (CMC) solution (vesicle) were applied to the incisional wounds in rats, the EGF–LMC conjugates are considered to be potent wound healing agent with mitogenicity and wound-healing property. J Appl Polym Sci 102: 5072–5082, 2006

Key words: epidermal growth factor; low molecular weight chitosan; ELISA

INTRODUCTION

The carbohydrate moieties of natural glycoproteins function as the protectors of the polypeptide chain against proteolysis and the stabilizers of the tertiary structure of protein moieties.[1] Modification of properties of enzymes by attaching carbohydrate is one of the newest approaches in enzyme engineering.[2] It has been also known that the covalent attachment of low molecular weight sugars to enzymes greatly alters the properties of enzymes *in vitro* and their behaviors *in vivo*.[3]

On the other hand, wound repair is the result of complex interactions and well-coordinated biological processes.[4] Several polypeptide growth factors have been known to participate in the various phases of wound healing.[5] Among the growth factors, epidermal growth factor (EGF, 5900 Da) has been demonstrated particularly to stimulate *in vitro* cell proliferation and *in vivo* wound healing.[6] Because EGF induces the mitogenic response including the initiation of DNA synthesis and cell proliferation, or has the ability in the activation of RNA and protein synthesis, and the activation of the synthesis of extracellular macromolecules,[7] EGF has been applied on the wounding area as ointments or directly applied by injections *in vivo*.[8] However, EGF was rapidly internalized and degraded after binding to epidermal growth factor receptor (EGFR)[9,10] and quickly removed from the circulation in the liver and kidneys.[11,12] It was also reported that many proteases easily decompose EGF in the wounded or burned sites of skin as soon as it was applied.[13] Therefore, the protection of EGF against proteolysis by the conjugation of the low molecular weight sugars could be one possible approach to enhance its mitogenicity *in vivo*. Recently, EGH–dextran conjugates have been newly introduced as an example of EGF-low molecular weight sugar conjugates.[14] Chitosan, the copolymer of β-(1→4)-linked 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc) and 2-amino-2-deoxy-D-glucopyranose (GlcNAc), has been used in a wide variety of medical applications. It has been suggested that chitosan could be used for wound healing and tissue growth.[15] Chitosan oligomers affect on the mitogenic responses and the chemotactic activities of animal cells. They have also been used to form carbohydrate–protein conjugates with various biological activities.[16]

In the present study, we prepared the conjugation of LMC to EGF by using water-soluble carbodiimides

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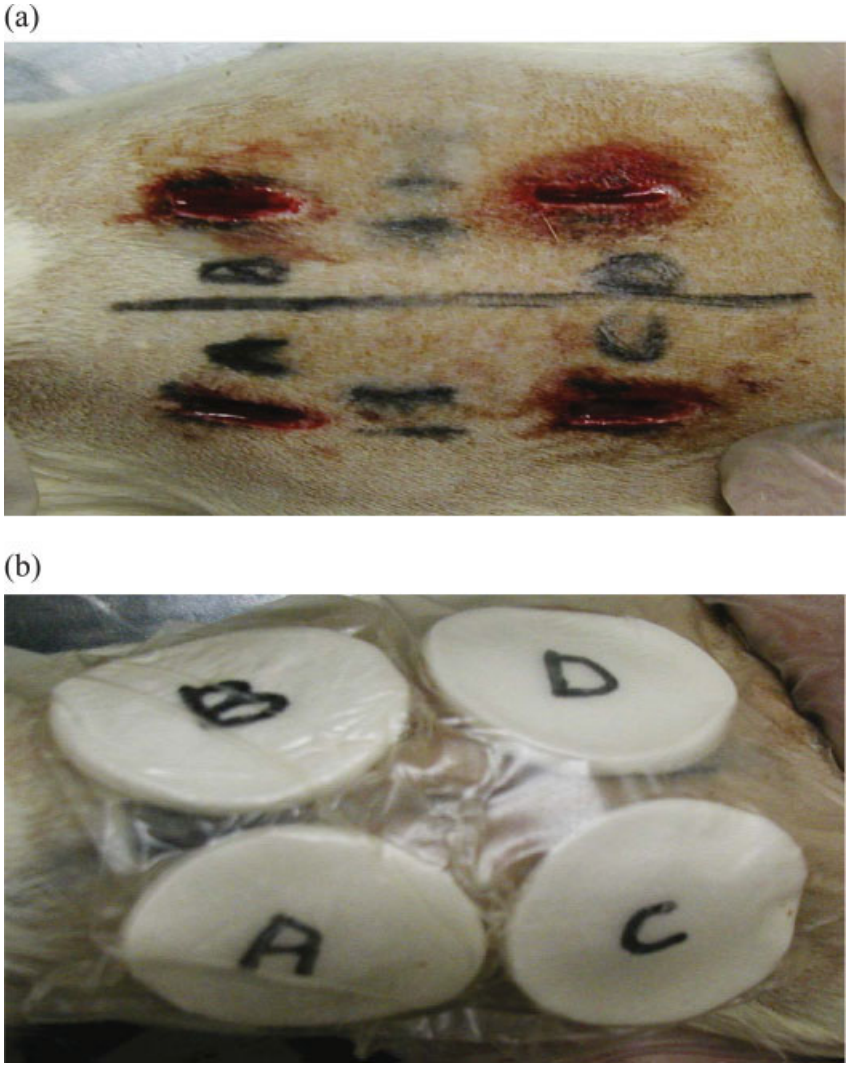

Figure 1 Anesthetized rat after wounding (a) and wounds covered with chitosan sponges (b). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

as a coupling agent and confirmed the formation of EGF–LMC through indirect enzyme-linked immunosorbent assay (ELISA). The thermal stability and the resistance of EGF–LMC against proteolytic digestion were also evaluated by comparing with free EGF (positive control). Three kinds of drugs (EGF, EGF–LMC, and LMC) in viscous carboxymethyl cellulose (CMC) solutions (100 g/L; vesicle control) were applied to each incisional wound, which was made on the back of rat. The effect of EGF–LMC on wound healing was also evaluated by the histological examination.

MATERIALS AND METHODS

Materials

Crude chitosan [CS-0, degree of deacetylation (DAc): 81%] was purchased from Jakwang, Korea. The low molecular weight chitosan (LMC, 20,500 Da) was made from CS-0 via acid hydrolysis. The condensing agent, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl, was obtained from Nacalai Tesque., Japan. Major reagents including EGF, monoclonal anti-epidermal growth factor, rabbit anti-mouse IgG, SIGMA 104® phosphatase, and substrate (*p*-nitrophenyl phosphate, disodium) for indirect ELISA assay were purchased from Sigma (USA). The medium for cell culture was Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. Trypsin and lysozyme from Sigma were used for the enzymatic digestion of EGF and LMC, respectively. Askina Derm® (10×12 cm^2) obtained from B. Braun Hospicare (Irish) was used in wrapping the sponge on the wound. All were used without any further treatment or purification.

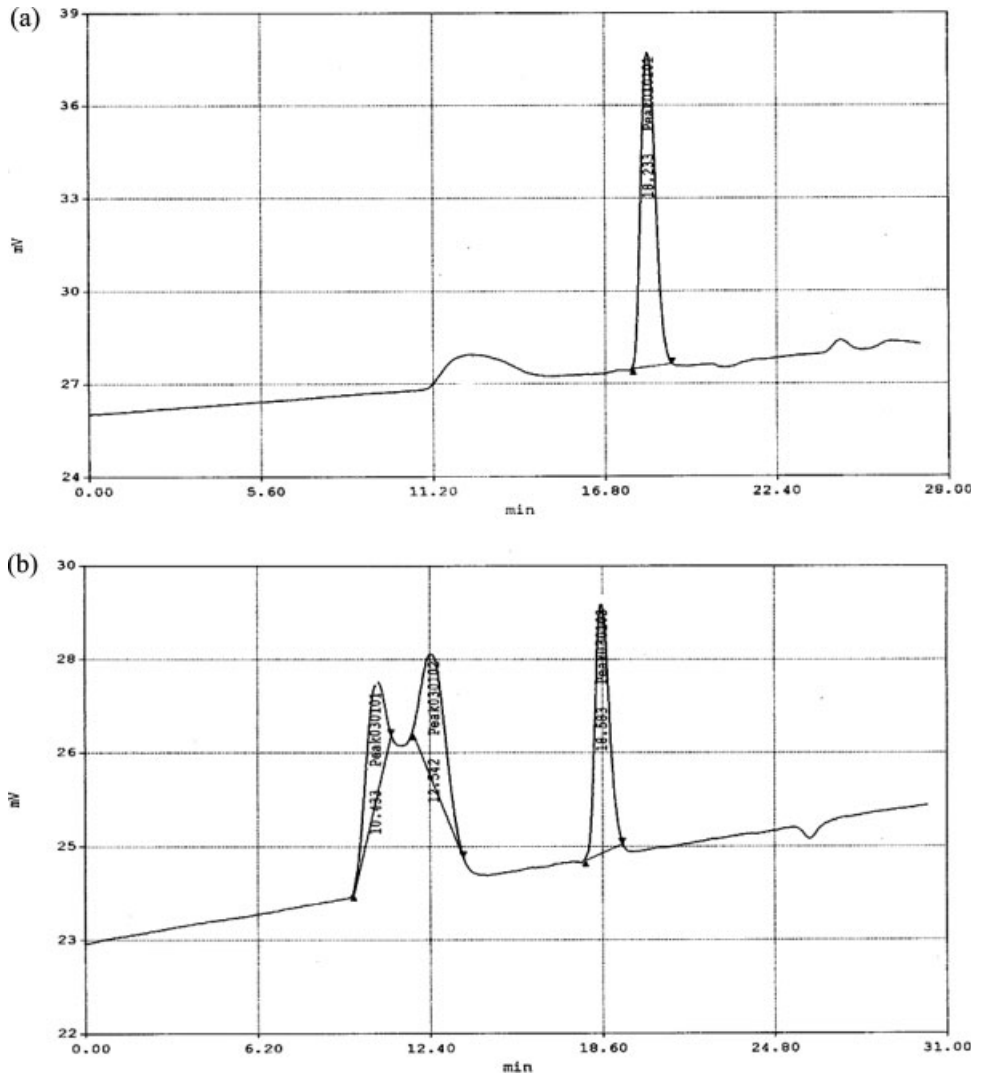

Figure 2 The HPLC elution profiles of the acid hydrolyzed chitosan sample (LMC). (a) The HPLC elution profiles of a mixture of standard compounds (P-10, P-20, and P-50). (b) Chromatographic conditions: column, Shodex Asahipak GS-320 HQ, 9 μm, 50 cm ×7.6); detection, RI detector.

Preparation of low molecular weight chitosan fractions

CS-0 was dissolved in 2% (v/v) acetic acid by using a mechanical stirrer. The dissolved sample was acid-hydrolyzed in 12.08*M* HCl (50°C) for 30 min. The reaction was stopped by adding with 1 volume of distilled water and the hydrolysate was held at −20°C for 2 days to precipitate the high molecular weight chitosan oligomers (HMC oligomers). The precipitates (HMC oligomers) were removed from the reactor to obtain the low molecular weight chitosan. HMC oligomers-free diluted hydrolysates were mixed with 1 volume of methanol to increase the precipitation yield. These precipitated oligomers were washed three times with ethanol/methanol/acetone and thoroughly dried under vacuum. Dried oligomers were dissolved in deionized water and ultrafiltered using YM3 (molecular weight cut-off 3000 Da) and YM10 (molecular weight cut-off 10,000 Da) membranes (Amicon, USA). The resultant was thoroughly evaporated and dried after washing by ether/acetone.

Characterization of LMC

The molecular weights of LMC were determined with a Waters liquid chromatograph (Waters Associates Milford, MA). The column (column, Shodex Asahipak GS-320, 7.6 × 30 cm^2) was packed with synthesized high molecular weight hard gel based on polyvinyl alcohol with a separation range of ca. < 40,000 Da (Pullulan standards). The eluent was 0.2*M* CH_3COOH/0.036*M* CH_3COONa. The sample concentration was 0.1% (w/w). The amount of injected sample was 0.2 mL. The flow rate was maintained at 0.5 mL/min. The temperature of column was maintained at 50°C. The eluent was monitored by a RI detector (model 410, Waters, USA).

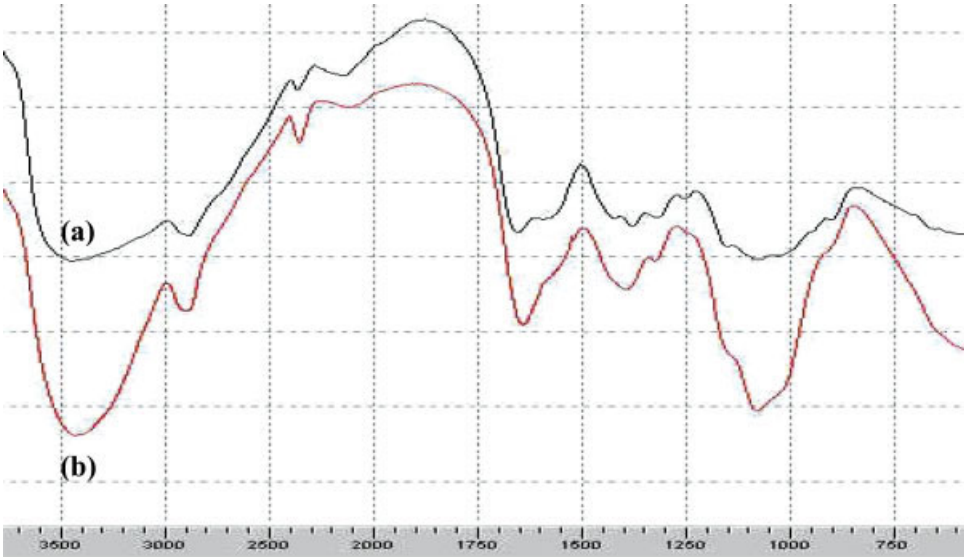

Figure 3 The FTIR spectra of CS-0 (a), and LMC (b). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The standards used to calibrate the column were Pullulan Shodex Standard P-82 (Showa Denko K. K., Japan). The degree of deacetylation values of LMC were obtained by ^{1}H-NMR spectroscopy as described previously by Varum et al.[17] ^{1}H-NMR spectra were recorded at 400 MHz in a JEOL ECP 400 spectrometer. The water-soluble sample (LMC) was dissolved in D_2O and the water-insoluble sample (CS-0) was dissolved in 4% CD_3COOD in D_2O. The chemical shifts were referenced to internal tetramethylsilane-d_4 (ACROS, Japan). The main macromolecular structure of LMC was compared with that of CS-0 by using Fourier-transform infrared (FTIR) analysis. IR spectra were obtained in IR-400 JASCO FTIR spectrometer using KBr pellets.

Preparation of EGF–LMC conjugates

The conjugation of EGF with LMC was performed in a manner similar to the method reported by Andersson et al.[14] Briefly, EGF (1 μg) in 500 μL of phosphate-buffered saline (PBS), pH 6.5, was mixed with 1.61 μg (5-fold molar excess over the EGF) of 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide HCl (EDC) and shaked gently for 2 min at 4°C. The modified EGF was mixed with LMC (20,500 Da, 250-fold molar excess over the EGF) in PBS (pH 8.5). The coupling of LMC with EDC-activated EGF was allowed to proceed during 4 h at 25°C. After this period, glycine (0.81 mg) in 100 μL of PBS, pH 8.5, was added to block residual active groups. Incubation continued for 90 min at 25°C. The reaction mixture was then dialyzed for 24 h at 4°C against PBS in pH 7.4 (membrane cut-off 22,000 Da, Sigma). The EGF–LMC conjugate was finally sterilized by the passage of the solution through a 0.22 μm filtration membrane (Millipore, USA).

Indirect ELISA for EGF–LMC conjugates

The ELISA for EGF was carried out to measure the amount of EGF in EGF–LMC conjugate. Briefly, 90 μL of each sample (EGF–LMC) or standard (EGF) diluted in PBS-T [PBS solution containing 0.05% (v/v) of Tween-20] was coated to 96-well flat microtiter plates (TPP, USA) by incubating relevant wells (triplicate) at 37°C for 90 min. After washing each well twice with 200 μL PBS-T, 100 μL of primary antibody (monoclonal anti-EGF, Sigma) solutions diluted 1/1000 in PBS-T were added to plates. The solutions were incubated for 90 min at 37°C. Wells were washed twice with PBS-T. 90 μL of the secondary antibody [rabbit anti-mouse IgG conjugated with alkaline phosphatase (AP), Sigma] solutions diluted 1/1000 in PBS-T were added to each well and incubated for 90 min at 37°C. The enzyme reaction was initiated by adding 90 μL of fresh substrate solution containing *p*-nitrophenyl phosphate disodium (Sigma 104®) after washing and emptying the plate five times repeatedly. After incubation for 45 min at room temperature, the absorbance was measured at 405 nm and 650 nm in a microplate reader (Biorad, USA). The concentrations of EGF in EGF–LMC were estimated by comparing the absorbances measured at 405 nm from unknown samples (EGF–LMC) with those from the corresponding EGF samples (10, 1000, and 2000 ng).

Relative thermal and proteolytic stability of EGF–LMC

Each of 0.2 mL EGF (2 μg/mL) or EGF–LMC solutions (maximally 2 μg/mL) was incubated at different times (30, 90, and 180 min) at 37°C in PBS (pH 7.4). After each incubation, 90-μL aliquots were withdrawn in duplicate to determine the relative thermal stability of both EGF and EGF–LMC by indirect ELISA. As controls,

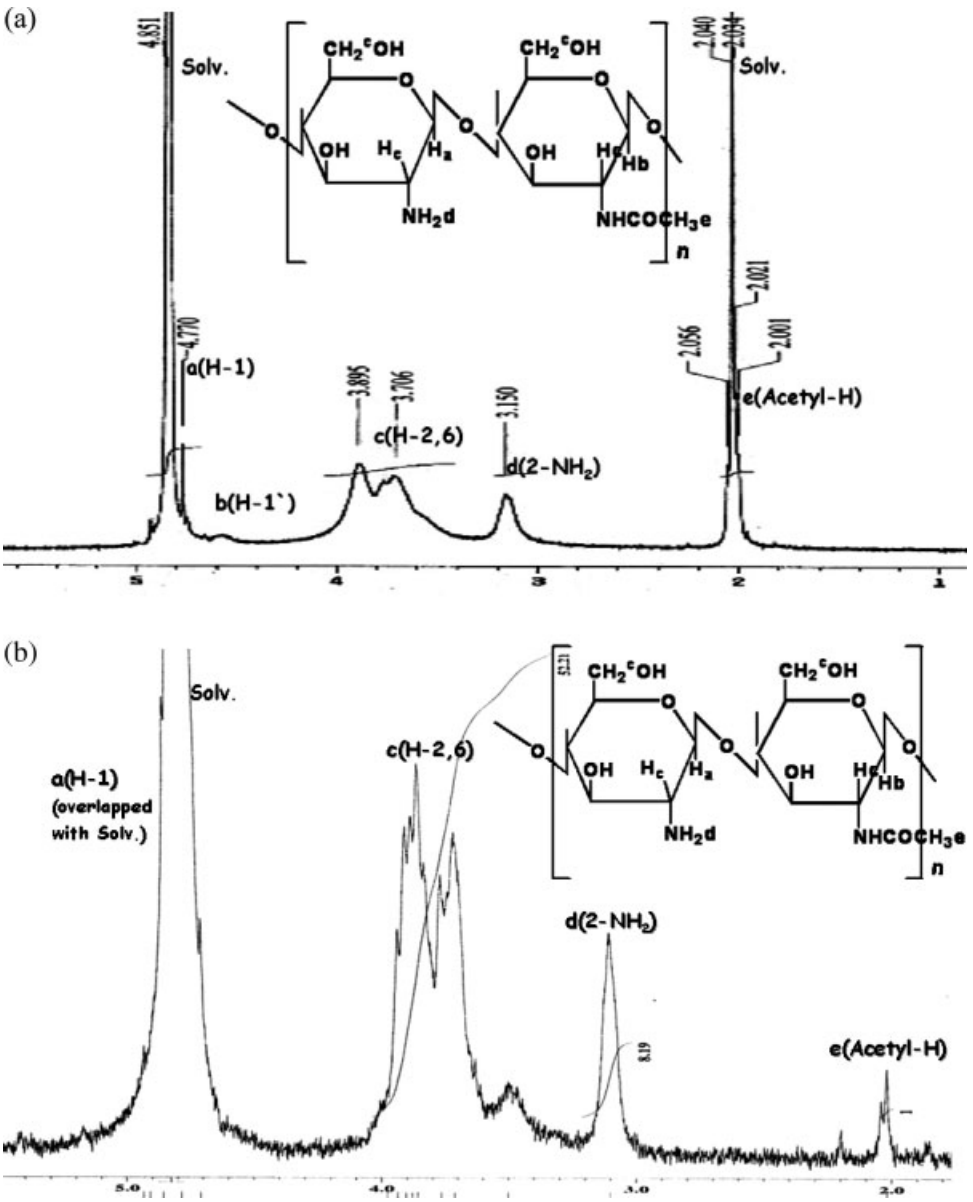

Figure 4 The 400 MHz ^{1}H-NMR spectra of CS-0 in 4% CD_3COOD in D_2O (a), 400 MHz ^{1}H-NMR spectra of LMC in D_2O (b).

samples in duplicate were immediately withdrawn at 0 time point and then determined. Each of 0.2 mL EGF (2 μg/mL) and EGF–LMC solutions (maximally 2 μg/mL) was incubated at 37°C with 192 ng lysozyme (L-7651, Sigma) or 900 ng trypsin (T-6763, Sigma) added, respectively. At different times (30, 90, and 180 min), 90 μL aliquots were withdrawn in duplicate and the relative proteolytic stability of EGF and EGF–LMC was measured by indirect ELISA. As controls, each sample was incubated at 37°C for 30, 90, and 180 min without lysozyme or trypsin addition.

Animal model

Adult, male, Sprague-Dawley rats (200–250 g) were used in this study. They were individually housed in stainless-steel mesh cages at constant temperature and relative humidity, and fed with a standard diet. A viscous chitosan sponge (28 mm in diameter, 4 mm in thickness) was employed as an inductive matrix for repair tissue.

Production of incisional wounds

The rats were anesthetized with ether and ketamine. The dorsal hair was clipped and the skin was sterilized

TABLE I
Chemical Shifts (δ) of Proton Resonances for Chitosan in D_2O at 90°C (pD 3)

	Proton			
Residue	H-1	H-2	H-2/6	Acetyl-H
GlcNAc (A)	4.55–4.65 (=b)		3.5–4.0	2.04 (=*e*)
GlcN (D)	4.85 (=*a*)	3.15 (=*d*)	3.5–4.0 (=*c*)	

with 70% (v/v) ethanol. Four full-thickness dorsal linear incisions (each area = 0.48 cm^2), each 1.5 cm long, were made with a scalpel [Fig. 1(a)]. On each animal, 1 mL of viscous carboxymethyl cellulose (CMC) solution (100 g/L) was applied to the first wound area as vesicle control. The second wound was treated with 0.445 mg of LMC (carrier control) diluted in 1 mL of CMC solution. The third wound was treated with 200 ng of EGF (positive control) diluted in 1 mL of CMC solution. The fourth wound was treated with EGF–LMC (test) containing maximally 100 ng of EGF diluted in 1 mL of CMC solution. Then, each wound area was covered with the chitosan sponge (6.15 cm^2) and the commercially available adhesive polyurethane film (Askina Derm®) [Fig. 1(b)]. These wounds were treated on days 0 after wounding. Animals were killed by ether over-dosage on days 3, 7, and 14 after wounding. After sacrificing animals, specimens encompassing the whole area were removed.

Histological examination

Specimens were fixed in 10% neutralized formalin. They were dehydrated in a graded series of ethanol and embedded in paraffin. 5 μm thin sections were prepared and stained with hematoxylin and eosin for histologic evaluations.

RESULTS AND DISCUSSION

Characterization of LMC

All chitosan fractions were obtained from acid hydrolysis and ultrafiltration. Of some chitosan fractions, only one chitosan fraction (LMC, about 10,000 Da) and main functional groups of chitosan itself were used to prepare EGF-low molecular weight sugar conjugates. The weight–average molecular weight (M_w) of LMC was determined by gel-permeation chromatography (GPC). The GPC profiles of LMC [Fig. 2(a)] and the mixture [Fig. 2(b)] of the standards of Pullulan (P-10, 11,800 Da; P-20, 22,800 Da; P-50, 47,300 Da) are shown in Figure 2. These standards showed the corresponding signals at the retention time of 10.43, 12.54, and 18.58 min, respectively, and the standard curve was made for calculation of molecular weight of LMC obtained from hydrolysis of crude chitosan. As shown in Figure 2, single GPC pattern peak at 12.11 min was the major product (LMC), whose M_w was 20,500 Da. LMC as a low molecular weight sugar is considered sufficiently proper for the preparation of EGF-low molecular weight sugar conjugates. It has been reported that the molecular weight of dextran is ~ 20,000 Da in the case of EGF–dextran conjugates.[14]

IR analysis was performed to know whether there are still structural similarities between CS-0 (control) and LMC (test) even after acid hydrolysis. The FTIR spectra of CS-0 [Fig. 3(a)] and LMC [Fig. 3(b)] are shown in Figure 3. The typical IR bands of CS-0 as a crude chitosan appeared at 3387, 2925, 1634, and 1374 cm^{-1} due to an —OH group, a $—CH_3$ group, a $—CH_3—C=O$ group and a C—O stretching of a primary alcoholic group, respectively. As expected, the spectral profile of LMC is similar to that of CS-0, indicating that the main macromolecular structure of LMC still remains unmodified[18] after acid hydrolysis in concentrated HCl. LMC is particularly expected to exert its own biological activities when applied *in vitro* and *in vivo* because of its structural similarity with CS-0.[19] Therefore, LMC was considered as an appropriate material for the preparation of EGF-low molecular weight sugar conjugates.

The degree of deacetylation of LMC was determined by 1H-NMR spectroscopy. The ^{1}H-NMR spec-

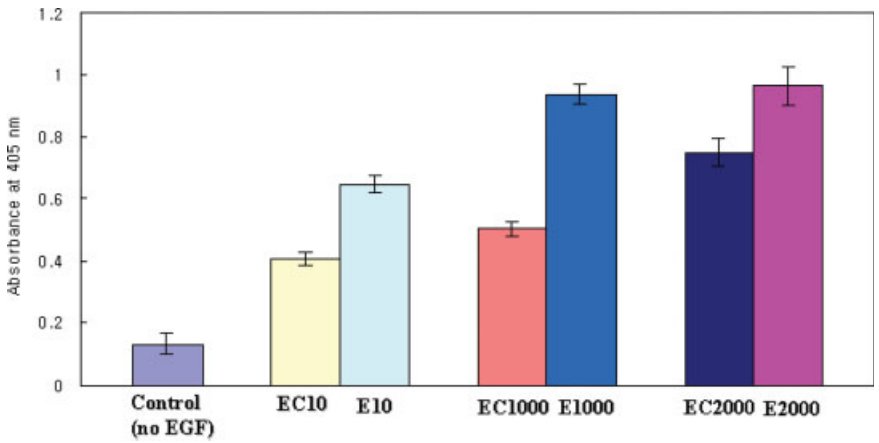

Figure 5 Absorbance of EGF–LMC samples at 405 nm. EC and E denote the conjugates obtained from the condensation reaction with some amounts of EGF (10, 1000, and 2000 ng) and free EGF (10, 1000, and 2000 ng), respectively. Data ($n = 3 \pm$ SEM) are presented as absorbance at 405 nm. There is no EGF–LMC sample in the control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

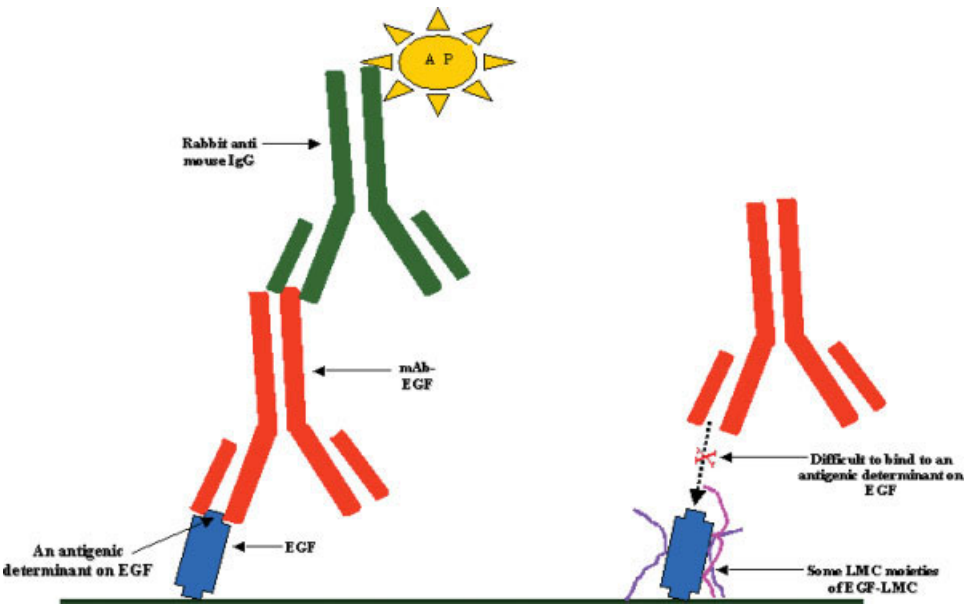

Figure 6 The ELISA assay for the detection of EGF–LMC conjugates. In the case of EGF–LMC conjugates (b), mAb-EGF is more difficult to bind with the antigenic determinant of EGF than free EGF due to the steric hindrances caused by LMC moieties (pink) around the conjugates. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

tra of CS-0 and LMC are shown in Figures 4(a) and 4(b), respectively. In the 400 MHz ^{1}H-NMR spectra, five resonances (a–e) for chitosan were identified by Varum et al. (Table I).[17] It was reported that the resonance at 2.04 ppm was due to the CH_3 residue on the acetamide group and the resonance of H2–H6 occurred in the range 3.1–4.0 ppm. From the identification of resonances [(a–e) in Fig. 4], it was possible to determine the degree of deacetylation of CS-0 and LMC. The degree of deacetylation[17] may be expressed as

$$\text{Degree of deacetylation } (\%) = [1 - \{7(I_b + I_e)/(4(I_a + I_c + I_d) + I_b + I_e)\}] \times 100.$$

The degree of deacetylation values of CS-0 and LMC were 80.7% and 97%, respectively. The *N*-acetyl content and molecular weight of LMC probably decreased under the drastic hydrolysis conditions with concentrated HCl because of the acid-catalyzed hydrolysis of the *N*-acetyl linkages (S_N2 reaction) and the glycosidic linkages (S_N1 reaction).[20] Therefore, LMC with high

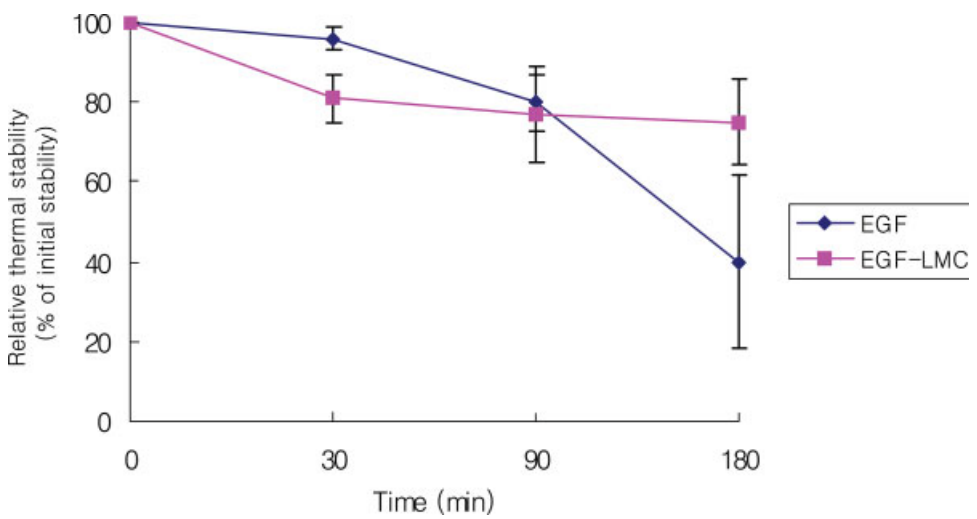

Figure 7 Thermal inactivation of EGF and EGF–LMC conjugate. The relative thermal inactivation determined as the percentage of initial stability. Experimental points represent the average of triplicate determinations. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

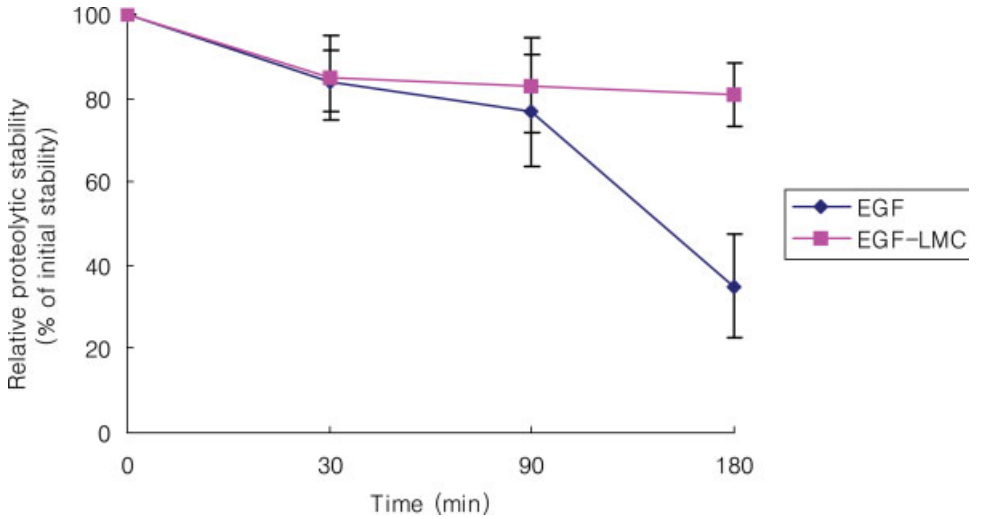

Figure 8 Proteolytic degradation of EGF and EGF–LMC conjugate by trypsin. As controls, EGF and EGF–LMC conjugates were incubated at different times and at 37°C without addition of trypsin. The relative proteolytic stability was determined as the percentage of controls at different times by indirect ELISA. The experimental points represent the average of triplicate determinations. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

degree of deacetylation value (96.7%) would react easily with EDC and the higher yield of EGF–LMC conjugates would be obtained. In addition, degree of deacetylation level of LMC was a key factor in the mitogenic activity on human dermal fibroblasts[21,22] though the primary amine groups of LMC were be attached with the carboxyl groups of EGF to form new amides.

Quantitative analysis of EGF–LMC

The ELISA assay for the EGF isolated from human serum (hEGF) was used to measure the amount of hEGF in EGF–LMC samples. The monoclonal anti-EGF (primary antibody) was chosen as capture antibody. The rabbit anti-mouse IgG (secondary antibody) was used as the detector. A recombinant human EGF or EGF–LMC conjugate was added to form conjugate with its ligand (monoclonal anti-EGF). As shown in Figure 5, the absorbance values obtained from the various concentrations of EGF–LMC samples (EC10, EC1000, and EC2000) were 0.408, 0.506, and 0.75, respectively. This result indicates that ~50–60% of applied EGFs in the coupling step were bound to the LMC since EGF–LMC (EC) series contained more than half amount of

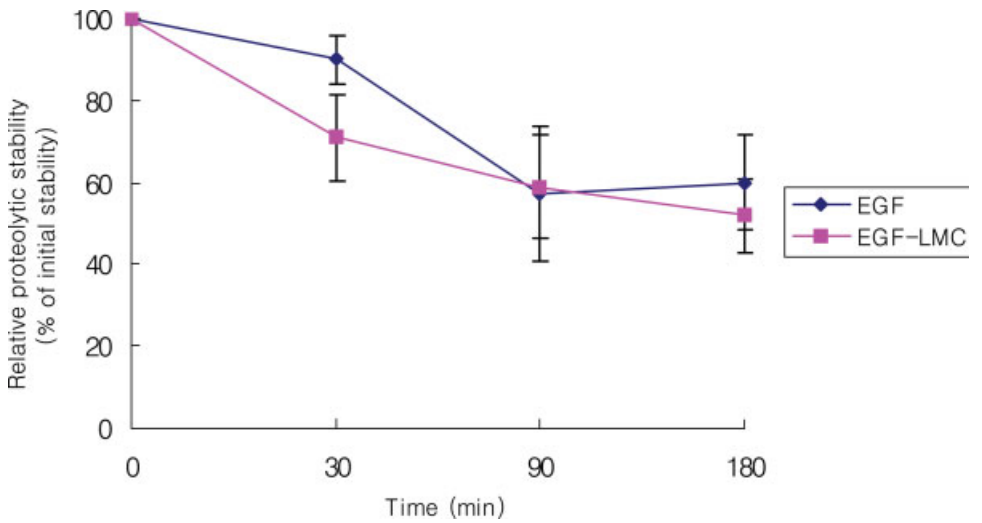

Figure 9 Schematic representation of a EGF–LMC conjugates showing the principle of EGF stabilization by the covalent attachment of LMC (bold line) at multipoints of EGF. The formation of new hydrogen bonds between LMC and EGF molecules allows LMC to stabilize the tertiary structure of native EGF against ''poisons'' such as heating and protease. A macromolecular aggregate may also be formed by intermolecular cross-linking based on hydrogen bonding (dotted line) between LMC and EGF molecules. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

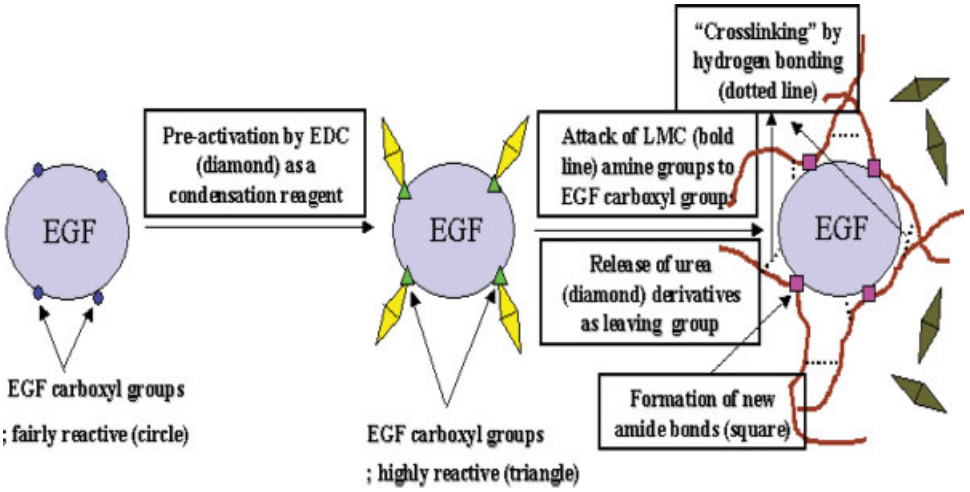

Figure 10 Inactivation of EGF and EGF–LMC conjugate at different times and 37°C after treatment with lysozyme as an enzyme that is able to degrade LMC molecules. As controls, EGF and EGF–LMC conjugates were incubated at different times and at 37°C without addition of lysozyme. The relative proteolytic stability was determined as the percentage of controls at different times by indirect ELISA. Experimental points represent the average of triplicate determinations. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

free EGF (E). All negative control groups (PBS-T, LMC/EDC mixture, EGF/LMC mixture) purified with dialysis tubing represented very low absorbance values (lower than 0.11 at 405 nm, data not shown). A typical standard curve obtained with the ELISA was produced (data not shown). The minimum detection limit estimated by serial dilution was 10 pg/mL recombinant human EGF. However, the correlation coefficient (R^2) of the standard curve was 0.89, suggesting that the LMC moieties of EGF–LMC possibly

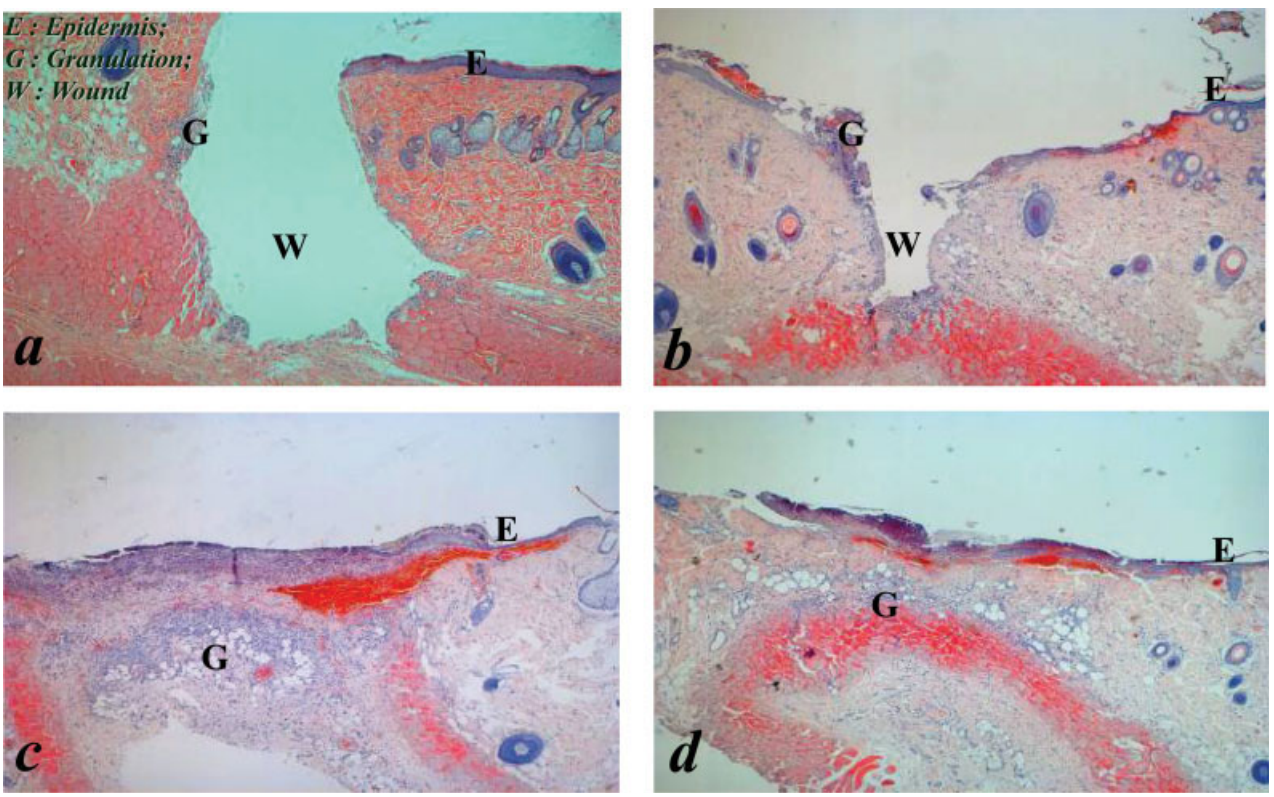

Figure 11 Histological findings of the wounded skins treated with different wound-healing agents at 3 days after initial wounding by hematoxylin and eosin: (a) CMC solution; (b) CMC solution + LMC; (c) CMC solution + EGF; (d) CMC solution + EGF–LMC. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

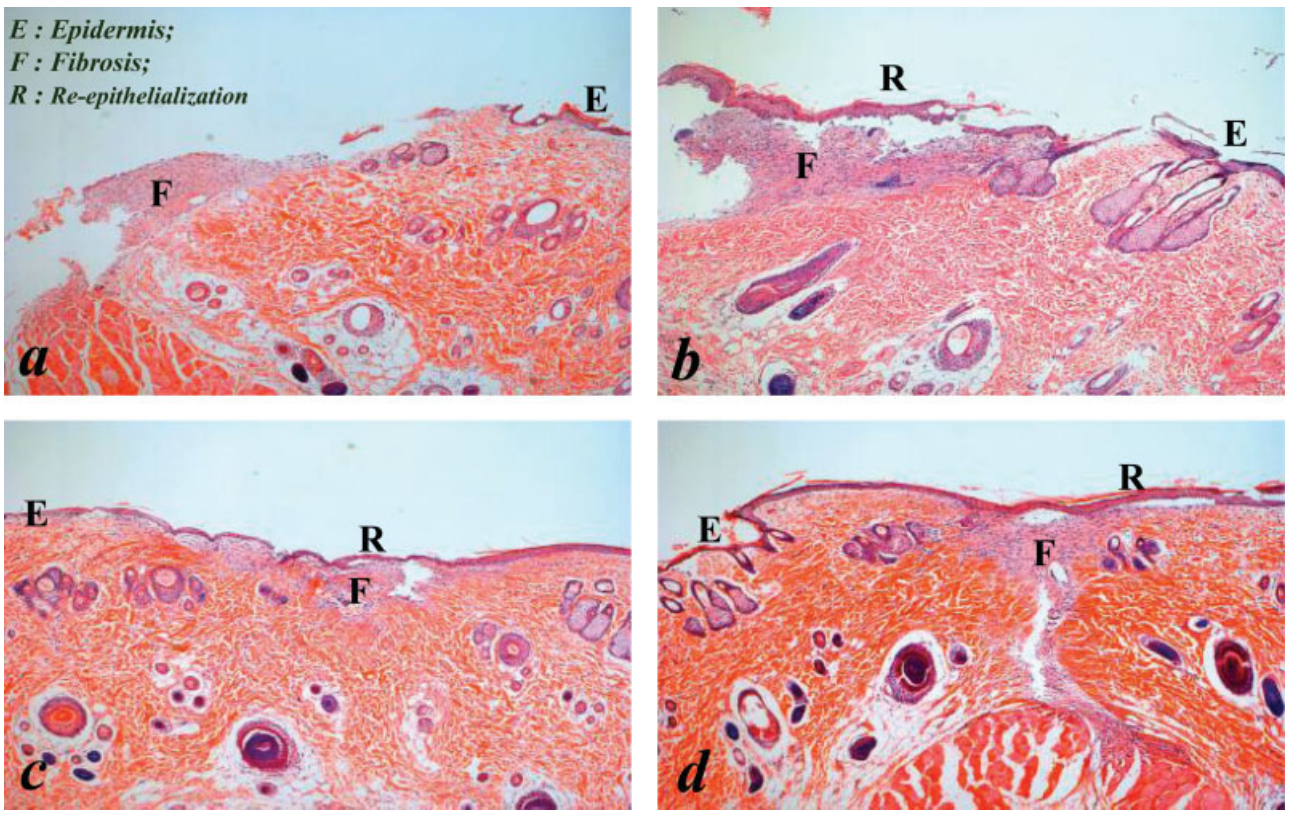

Figure 12 Histological findings of the wounded skins treated with different wound-healing agents at 7 days after initial wounding by hematoxylin and eosin: (a) CMC solution; (b) CMC solution + LMC; (c) CMC solution + EGF; (d) CMC solution + EGF–LMC. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

interfere with the binding of monoclonal anti-EGF to the antigenic determinant on EGF (Fig. 6)[23] due to the high sensitivity of ELISA using two antibodies (primary and secondary antibodies). It is considered that the EGF–LMC conjugates consist of a heterogeneous mixture of conjugates with different conformations.[24] EGF–LMC conjugates are formed via the random reaction between the free carboxyl groups of EGF and the free amine groups of LMC. The heterogeneity of EGF–LMC was indirectly characterized by the relative stability of EGF–LMC with high standard deviations against thermal, trypsin, and lysozyme.

Comparison of the relative thermal and proteolytic stability of EGF–LMC and EGF

The experiment for the stability of EGF–LMC conjugates was carried out to confirm the possibility regarding the enhancement of EGF stability due to covalently bound LMC. The relative thermal stability of EGF and its conjugates is depicted in Figure 7. After 90-min incubation at 37°C, EGF retained 80% of initial stability that is approximately equivalent to EGF–LMC (77%). However, EGF lost 60% of initial stability as compared with 25% of initial stability reduction for EGF–LMC after 180-min incubation at 37°C. It was observed that EGF–LMC was more heat-stable than EGF. This result is well consistent with the thermal stability of β-galactosidase–dextran conjugates[25] and trypsin–dextran conjugates.[23] After 180-min trypsin digestion, EGF–LMC showed higher relative stability (81%) than EGF (35%). As shown in Figure 8, EGF–LMC has good resistance to proteolysis by trypsin. This is similar to the experimental fact that trypsin–dextran conjugates show resistance to inhibition by naturally-occurring protease inhibitors,[2] suggesting that LMC bound on EGF may be a part of the carbohydrate-induced stabilization. As shown in Figure 9, it is possible that the higher stability of EGF–LMC than that of EGF can be explained by the enhanced conformational stability caused by hydrogen-bonding between LMC and EGF. In addition, the effect may be due to steric shielding a specific domain against protease by the covalently attached carbohydrate.[26] As shown in Figure 10, the treatment of EGF–LMC with lysozyme destabilizes EGF–LMC. As expected, after 180-min incubation with lysozyme, both EGF and EGF–LMC showed approximately the equivalent stability. It is indicated that lysozyme treatment causes the complete breakdown of LMC part in EGF–LMC after the enzymatic reaction between lysozyme and EGF–LMC for 180 min at 37°C. It is considered that LMC attached at the multipoints of EGF contributes to the stabilizing the tertiary structure of EGF molecule.

It is likely that the EGF–LMC possibly exert its biological activities effectively *in vivo* due to the LMC-induced EGF stabilization.

Histological examination

The histological findings of the wounded skins at 3 and 7 days after initial wounding, stained with hematoxylin and eosin, are shown in Figures 11 and 12, respectively. At 3 days after the wounding (Fig. 11), the wound of the vesicle control and carrier control showed similar findings, which were focally replaced by granulation tissues. However, the EGF-treated and the EGF–LMC treated wound were completely replaced by granulation tissues with cluster formation. There was no significant histological difference between EGF and EGF–LMC treated groups. At 7 days after the wounding (Fig. 12), the wound of vesicle control showed focal replacement by fibrous tissues and re-epithelialization was not observed. The wound treated with only LMC (carrier control), however, was focally replaced with tissue fibrosis and re-epithelialization partially. It is suggested that chitosan itself may stimulate cell proliferation and hence wound healing indirectly.[27] The wound treated with EGF–LMC similar to EGF showed the total replacement by fibrous tissues and complete re-epithelialization even though the amount of EGF in effect on the EGF–LMC treated wound was less than the half of the amount of EGF (416 ng/cm^2 of wound) effected on EGF treated wound. It is probably that EGF and LMC act synergistically to improve several wound-healing characteristics in wounded rat.[28,29] LMC-conjugated EGF exhibits the enhanced resistance against the proteolysis of many proteases, which is activated in the injured tissue.[13] At 14 days after the wounding, each group showed basically similar histology, which represents the complete wound healing with dense fibrous scar formation (data not shown). Based on the epidermal growth rate (E) and re-epithelialization (R) (Fig. 12), the rate of wound healing increased in the following order: control < LMC < EGF, EGF–LMC. It is likely that EGF in EGF–LMC, in comparison to free EGF, efficiently affects the proliferation of fibroblasts and wound healing in a manner similar to the controlled-release system by the action of lysozyme on LMC.[21] The study about the burn wound healing of EGF–LMC will be reported in the near future.

CONCLUSIONS

The LMC (10,535 Da, Dac: 97%) was successfully obtained by acid hydrolysis of CS-0 and then was coupled with EGF via the carbodiimide condensation reaction using EDC as a condensating agent. EGF–LMC was sufficiently noncytotoxic on fibroblast cells for the animal experiments. The healing efficiency of EGF–LMC is twice as much as that of EGF on wound healing. It was found that LMC attached at multipoints of EGF stabilized the structure of EGF against proteolysis of many proteases in the wounded tissue of rats. Therefore, EGF–LMC would be considered as a potent wound healing agent due to the mitogenicity and high efficient wound-healing property obtained from EGF since EGF itself is largely known to have high mitogenicity.

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