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Construction of Chimeric Human Epidermal Growth Factor Containing Short Collagen-Binding Domain Moieties for Use as a Wound Tissue Healing Agent

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Among the various human growth factors, epidermal growth factor (hEGF, consisting of 53 amino acids) has various effects on cell regeneration, stimulation of proliferation, migration of keratinocytes, formation of granulation tissues, and stimulation of fibroblast motility, which are important for wound healing. Owing to their multiple activities, EGFs are used as pharmaceutical and cosmetic agents. However, their low productivity, limited target specificity, and short half-life inhibit their application as therapeutic agents. To overcome these obstacles, we fused the collagen-binding domain (CBD) of *Vibrio mimicus* metalloprotease to EGF protein. About 18 or 12 amino acids (aa) (of the 33 total amino acids), which were essential for collagen-binding activity, were combined with the N- and C-termini of EGF. We constructed, expressed, and purified EGF (53 aa)-CBD (18 aa), EGF (53 aa)-CBD (12 aa), CBD (18 aa)-EGF (53 aa), and CBD (12 aa)-EGF (53 aa). These purified recombinant proteins increased the numbers of cells in treated specimens compared with non-treated specimens and control hEGF samples. The collagen-binding activities were also evaluated. Furthermore, CBD-hybridized hEGF induced phosphorylation of the EGF receptor. These results suggested that these fusion proteins could be applicable as small therapeutic agents in wound tissue healing.

Keywords: Collagen-binding domain, fusion protein, human epidermal growth factor, wound healing agent

Introduction

Damage to tissue is commonly caused by acute or chronic diseases, accidental injuries, surgical procedures, congenital or acquired malformations, or cancer. Therefore, diverse techniques are required to repair this damage [21]. Tissue engineering technologies have been developed for the healing and regeneration of wound tissues, including synthetic materials for replacement, tactics for cell enclosure, delivery systems for various bioactive substances, artificial tissues and cells, bioactive scaffolds of complex tissue polymers, cells that can promote regeneration of various other cell types, and regulatory bioactive compounds, including growth factors for development, remodeling, and differentiation [19–21].

However, the replacement of tissue is restricted owing to an imbalance between patients and donors, infections during or after surgical procedures, and the rejection and deterioration of grafts [21]. Furthermore, artificial products are limited by a lack of technology for commercial scale-up and synthesis of highly complex biological systems [21]. Many studies have attempted to overcome these tissue engineering problems [21]. Therefore, many researchers focused on diverse bioactive materials and investigated

multifunctional and anti-rejection agents, including human growth factors [14].

Cell-to-cell communication using direct signal transduction or signaling molecules, including signal peptides and steroids, is important for cellular functions [19–21]. Among these signaling molecules, polypeptidyl substances are commonly involved in growth factors [19–21]. These growth factors are produced by various cell types, and play key roles in tissue production, regeneration, and differentiation during wound healing [11, 17, 18, 21, 23, 25]. Therefore, these molecules are important industrial compounds in tissue engineering [14, 21]. The effect of growth factors on wound healing is well understood, and human epidermal growth factor (hEGF, 6 kDa) is known to have various functions in diverse epidermal cell types [2, 9]. Furthermore, the physical, chemical, and biological properties of growth factors are well understood [2]. However, the low concentration of EGF at the target sites was the result of short half-lives and rapid cell diffusion [1, 4, 9]. Therefore, in this investigation, we explored the use of EGF as a wound healing agent and developed methods to address the above disadvantages with the target-binding system.

In this study, we concentrated on collagen, which is abundant protein in epidermal cells, as the target binding site of recombinant EGF proteins [14]. We fused hEGF to the bacterial collagen-binding domain (CBD) and expressed the resultant protein in *Escherichia coli* using the pET expression system to increase productivity. To evaluate bifunctional fusion proteins as epidermal cell healing agents, we also examined collagen-binding activities, and cell proliferation and phosphorylation signals.

Materials and Methods

Bacterial Strains, Plasmids and Cells

The cloned cDNA of the human mature type epidermal growth factor gene in a plasmid was used as the template for PCR amplification of the hEGF insert. Diverse lengths of collagen-binding domains were amplified from the VMCBD vector, and the pET-22b(+) vector (Novagen, USA) was selected as the expression partner [16]. *Escherichia coli* DH5α and BL21 (DE3) strains were used to construct and overexpress the recombinant plasmids and chimeric proteins, respectively. The A-431 human epidermoid carcinoma and HaCaT human adult skin keratinocyte lines were used for assessment of effects on proliferation.

Manipulation of Fusion Protein Expression Plasmids

We constructed four chimeric inserts, which consisted of human mature type EGF and 18 or 12 amino acids (aa) from the CBD; namely, EGF-CBD (18 aa), EGF-CBD (12 aa), CBD (18 aa)-EGF, and CBD (12 aa)-EGF. Each region of the collagen-binding domain and human epidermal growth factor were amplified by PCR using 11 unique primers (Table 1). Six forward and reverse primers with complementary sequences were created to connect the EGF and CBD fragments. We applied overlapping PCR to join the EGF and CBD moieties [14]. Other primers were designed to create the *Nde*I and *Xho*I restriction enzyme sites at the 5′ and 3′ ends, respectively, of the four inserts for ligation to pET-22b. During the second amplification step, 18 or 12 aa of CBD were connected to the N- or C-terminus of EGF genes without additional restriction enzyme sites, using PCR. The second PCR amplicons were analyzed using agarose gel electrophoresis, and named EC1 (EGF-18 aa CBD), EC2 (EGF-12 aa CBD), CE1 (18 aa CBD-EGF), and CE2 (12 aa CBD-EGF). The four inserts and the pET-22b vector were digested with the *Nde*I and *Xho*I enzymes, respectively. Purified inserts and vector were linked using the

Table 1. Primer sequences for amplification of hEGF and CBD fusion proteins.

Name and number of primer	Sequence (5′→3′)[a]
EC1-EGF-up (forward)-①	GGCC ***CATATG*** AATAGTGACTCTGAATGTCCC
EC1-EFG-rp (reverse)-②	TAAGTTTTTCAC GCGCAGTTCCCACCACTTC
EC1-CBD-up (forward)-③	TGGGAACTGCGC GTGAAAAACTTAGGTGAAC
EC1-CBD-rp (reverse)-④	GGCC ***CTCGAG*** TGTATCAAGCCAGACTGCAAAC
EC2-EGF-rp (reverse)-⑤	GGCGTTGTATTG GCGCAGTTCCCACCACTTC
EC2-CBD up (forward)-⑥	TGGGAACTGCGC CAATACAACGCCGAGTTTG
CE1-CBD-up (forward)-⑦	GGCC ***CATATG*** GTGAAAAACTTAGGTGAACAA
CE1-CBD-rp (reverse)-⑧	AGAGTCACTATT TGTATCAAGCCAGACTGCAA
CE1-EGF-up (forward)-⑨	TGGCTTGATACA AATAGTGACTCTGAATGTCC
CE1-EGF-rp (reverse)-⑩	GGCC ***CTCGAG*** GCGCAGTTCCCACCACTTCAG
CE2-CBD-up (forward)-⑪	GGCC ***CATATG*** CAATACAACGCCGAGTTTGCA

[a]The underlined letters in primer sequences mean complemented sequences for overlapping PCRs and bold italic letters indicate restriction endonuclease sites.

Quick Ligation kit (NEB, USA). After ligation, the four plasmids were transformed into *E. coli* DH5α to construct expression plasmids, and then isolated and retransformed into *E. coli* BL21 for overexpression.

Isolation of Pure Chimera Proteins

A total of four *E. coli* transformants carrying the EC1, EC2, CE1, and CE2 plasmids were incubated in Luria-Bertani broth with 50 μg/ml ampicillin at 37°C to an OD_{600} of 0.4. The expression of target proteins was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 1 mM, followed by incubation for an additional 4 h at 37°C. Cell pellets were obtained from the culture media by centrifugation, dissolved in 50 mM Tris-HCl (pH 8.0), and lysed by sonication. Insoluble inclusion bodies from the four sonicates were obtained by centrifugation and incubated in 6 M urea containing 20 mM Tris-HCl (pH 8.0) at 4°C for 16 h. The four denatured chimeric proteins were dialyzed in 20 mM Tris-HCl (pH 8.0) at 4°C for 16 h with constant stirring. After dialysis, fusion proteins were purified by Ni-NTA his-binding resin affinity column chromatography (Novagen, USA) and the purities of target proteins were analyzed using Tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (Tricine SDS-PAGE) and western blotting.

Affinity Test with Collagen

To estimate the collagen-binding activities of the four recombinant proteins, we used the methods of Kim *et al*. [14] and Lee *et al*. [16], with minor modifications. Briefly, we chose insoluble type I collagen from bovine Achilles tendon (Sigma, USA) as binding substrates and conducted a pre-swelling step, as Tris-HCl follows: 10 mg of collagen was swollen with 50 mM Tris-HCl buffer (pH 8.0) containing 5 mM $CaCl_2$ for 1 h at room temperature. After a buffer elimination step using filtered centrifugation (Corning, USA), 0.1 mg of purified target proteins and moist collagen fibers were reacted in the same buffer system for 6 h at room temperature. Reactants were then treated with 1% SDS for 1 h at room temperature and filtered by centrifugation. The binding activities of filtrates were analyzed by Tricine-SDS-PAGE.

Effects on Cell Proliferation

To examine the effects of the epidermal growth factor fusion proteins on cell proliferation, we used the A-431 and HaCaT human epidermal cell lines, as described previously [14]. A-431 and HaCaT cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) containing 10% (v/v) fetal bovine serum (FBS, Gibco, USA) at 37°C in a 5% carbon dioxide atmosphere. The two cell lines were inoculated onto 6-well plates and incubated overnight, and then rinsed with phosphate-buffered saline (PBS) twice and added to serum-free DMEM medium. Purified fusion recombinants and hEGF (Sigma, USA) were added at 100 pg to 100 ng per well. Samples were washed with PBS after 2 days, and incubated for 2 more days with fresh Rosewell Park Memorial Institute's (RPMI) (-) medium. Cell proliferation was determined based on the increase in the number of cells using the MTS assay kit (Promega, USA). Furthermore, we coated type I collagen to the bottoms of well plates to perform the collagen-bound cell mitogenetic activity assay as described above [17].

Western Blotting for the Phosphorylation of EGF Receptors

We used western blotting to evaluate the level of phosphorylation of the EGF receptor stimulated by the four fusion proteins. Cell culture and preparation of phosphorylated samples were performed as described by Sahasrabuddhe *et al*. [22], with minor modifications [22]. Briefly, A-431 cells were incubated and washed as described above. A total of 5×10^5 cells were seeded in each well of 6-well plates, and cells were washed with PBS and treated with 100 ng of the four mitogens. After 5 min, the cells were harvested by centrifugation and analyzed by SDS-PAGE. Target proteins were transferred to nitrocellulose membranes and investigated by western blotting using an anti-phosphotyrosine antibody (BioSource Europe, Belgium).

Results

Construction of the Chimera Protein Expression Plasmid

For fusion protein expression plasmids, the sequences encoding hEGF, 18 aa of CBD, and 12 aa of CBD were prepared using PCR and purified for use in the secondary PCR step (Fig. 1A). After PCR, we identified approximately 180 bp of the hEGF gene, 80 bp of the CBD gene (18 aa), and 60 bp of the CBD gene (12 aa) products on the agarose gel (Fig. 1B). EGF genes were amplified efficiently, while CBD genes showed lower amplification efficiencies (Fig. 1B). For the second process, amplified EGF and CBD genes were linked and amplified by overlapping PCR, and subsequently analyzed by agarose gel electrophoresis (Fig. 1B). After the second reaction, we confirmed the sizes of EC1, EC2, CE1, and CE2 products (Fig. 1B). The four chimeric inserts and pET-22b(+) vectors were digested, connected, and transformed into *E. coli* DH5α to construct recombinant plasmids. The introduced plasmids were isolated from *E. coli* DH5α and re-transformed into *E. coli* BL21 for overexpression of fusion proteins.

Purification of Target Proteins

EC1, EC2, CE1, and CE2 proteins were overexpressed using IPTG, and *E. coli* BL21 cells were harvested and disrupted. Cell lysates were separated and purified by his-tag affinity chromatography. All steps were analyzed by PAGE and purified single bands of EC1, EC2, CE1, and CE2 were observed at ~7–8 kDa on the gel by Coomassie brilliant blue staining (Fig. 2A). The concentrations of the four purified chimeric proteins were determined using the

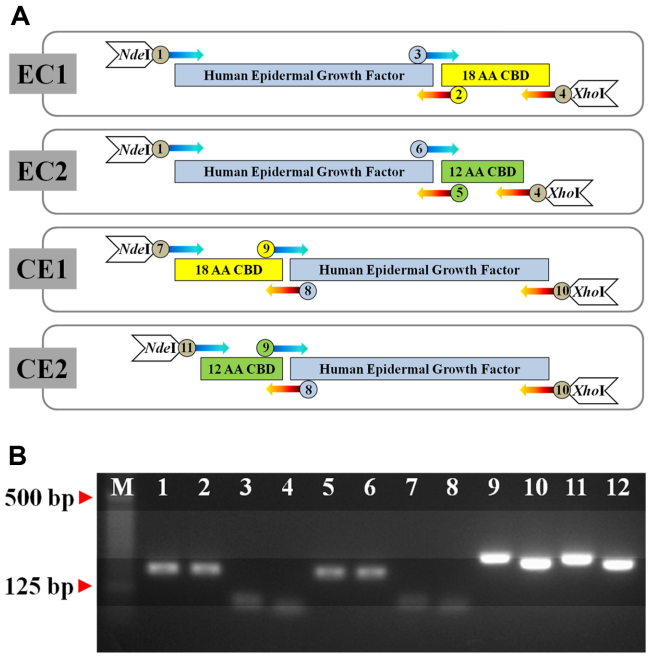

Fig. 1. (**A**) Schematic illustration of overlapping PCR for EGF and the CBD fusion plasmid and (**B**) conventional PCR and overlapping PCR amplification for EGF-CBD.
Lane M, DNA ladder; lanes 1 and 2, amplified human mature EGFs for C-terminus fusion; lanes 3 and 7, CBD of 18 amino acids (aa); lane 4 and 8, CBD composed of 12 aa; lanes 5 and 6, PCR products of hEGF for N-terminal fusion; lane 9, EGF-CBD (18 aa) amplicon; lane 10, EGF-CBD (12 aa); lane 11, CBD (18 aa)-EGF; and lane 12, CBD (12 aa)-EGF.

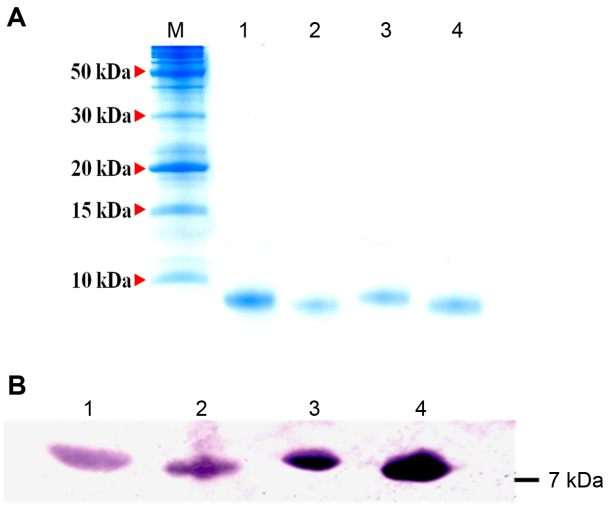

Fig. 2. SDS-PAGE and western blotting of the four purified fusion proteins.
(**A**) Tricine-SDS-PAGE analysis of purified fusion proteins. Purified EC1, EC2, CE1, and CE2 proteins were loaded on Tricine-SDS-PAGE after dialysis. M, protein marker; lanes 1–4, purified EC1, EC2, CE1, and CE2 proteins, respectively. (**B**) Western blotting of purified EC1, EC2, CE1, and CE2 proteins. Purified EC1, EC2, CE1, and CE2 proteins were analyzed by western blotting with anti-C-terminus 6-histidine antibody. Lanes 1–4, purified EC1, EC2, CE1, and CE2, respectively.

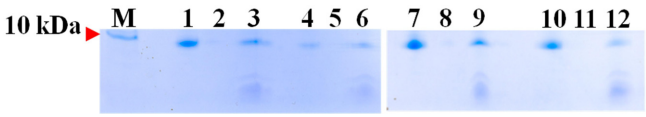

Fig. 3. Collagen-binding activities of EGF-CBD fusion proteins.
The 18- and 12-amino-acid CBD moieties fused with EGF recombinant proteins were reacted with/without type I bovine collagen. Filtrated protein specimens were analyzed using Tricine-SDS-PAGE. M, protein marker; lanes 1, 4, 7, and 10 show EC1, EC2, CE1, and CE2 without type I collagen, respectively; lanes 2, 5, 8, and 11 show EC1, EC2, CE1, and CE2 with type I collagen, respectively; lanes 3, 6, 9, and 12 show eluted EC1, EC2, CE1, and CE2 proteins, respectively.

Bradford method and loaded onto Tricine PAGE gels for western blotting. After gel electrophoresis, target proteins were transferred to a nitrocellulose membrane (GE Healthcare, USA). We performed western blotting using the Western Breeze kit (Invitrogen, USA). An anti-C terminus histidine antibody was used as the primary antibody (Invitrogen, USA). We observed the expression and purification of the target proteins, as evidenced by clear single bands in the 7–8 kDa region (Fig. 2B).

Collagen-Binding Assay

To examine their collagen-binding ability, the four purified recombinant proteins were incubated with or without pre-swelled collagen. After the binding reaction, unbound proteins were released from the filter tubes by centrifugation and the filtrates were examined by Tricine-SDS-PAGE (Fig. 3). We identified protein bands in lanes 1, 4, 7, and 10, which were incubated without collagen (Fig. 3). However, no protein bands were identified for samples incubated with type I collagen (Fig. 3). This was due to binding of EGF-fused CBDs to collagen, resulting in their becoming completely insoluble and so could not be eluted from the filter tube. To confirm our results, collagen-bound fusion proteins were mixed with 1% SDS solution to extract bound fusion proteins, and then isolated by filtration and resolved by Tricine-SDS-PAGE (Fig. 3). Chimeric proteins were detected in lanes 3, 6, 9, and 12 of the stained gel (Fig. 3). Recovered unbound proteins were identified at the same molecular masses as the four purified chimeric proteins (about 7 kDa), and the four eluted proteins

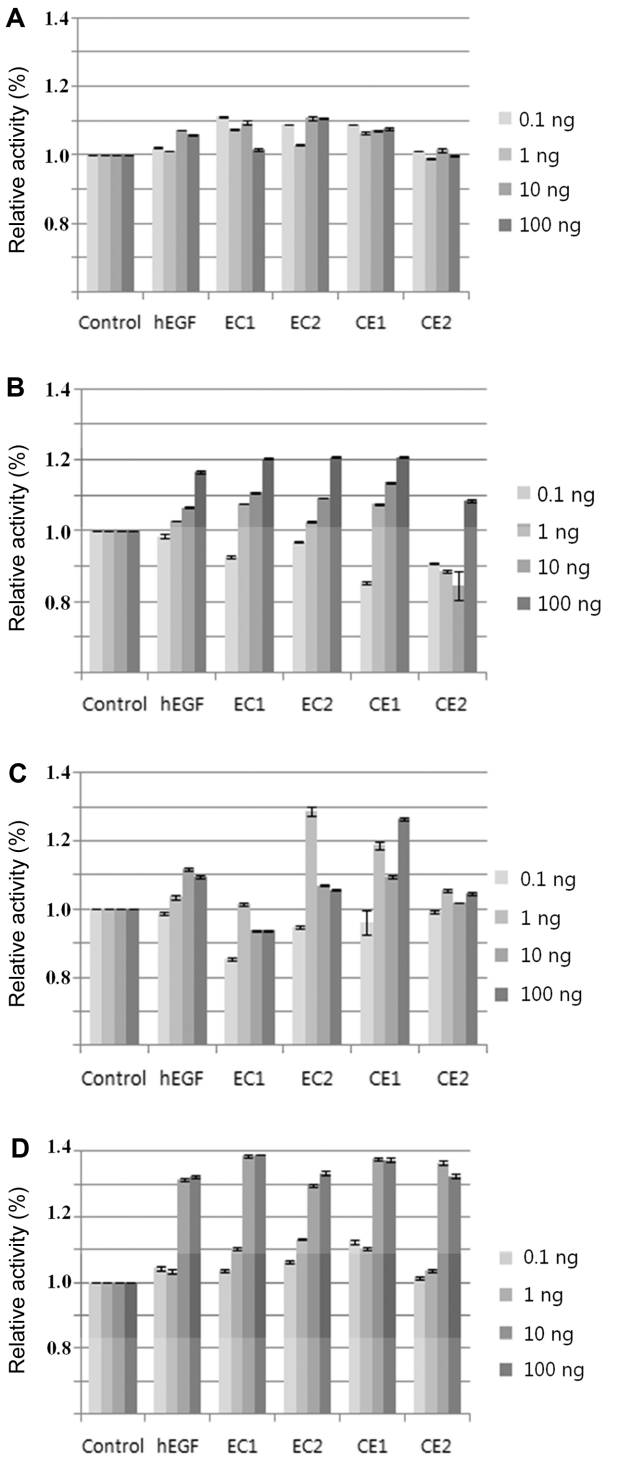

Fig. 4. Effects of the four fusion proteins on the growth of epidermal cells.

(**A**) Effects of fusion proteins on the proliferation of the A-431 human cell line. (**B**) Mitogenic effects of fusion proteins on the HaCaT human cell line. (**C**) Growth of A-431 cell line on collagen-coated plates with fusion proteins. (**D**) HaCaT cell mitogenic activities of fusion proteins on collagen-coated culture plates.

were present at slightly lower concentrations than those obtained in the absence of collagen (Fig. 3). Therefore, the EGF moieties did not affect the collagen binding domain.

Effects on the Growth of Epidermal Cells

The effects of the recombinant fusion proteins on cell proliferation were examined using the MTS assay. The results of the A-431 and HaCaT cell lines were compared with control groups (non-treated specimens) as a standard (Fig. 4). The A-431 cell line showed similar or greater cell numbers than the hEGF-treated and fusion-protein-treated groups (Fig. 4A). However, CE2-treated samples showed lower cell numbers than the hEGF-treated and control groups (Fig. 4A), but the response of the HaCaT cell line was greater than that of A-431 cells and exhibited dose-dependence (Fig. 4B). Similar to the effect of CE2 on A-431 cells, CE2 protein exhibited little effect on the HaCaT cell line (Fig. 4B). Furthermore, we used collagen-coated well plates to evaluate the effects on collagen binding and cell growth. By MTS assay, the numbers of A-431 and HaCaT cells with bound EGF fusion proteins were similar or greater than in untreated well plates (Figs. 4C and 4D). EC2 and CE1 showed greater effects on A-431 cells, but the effect of EC1 was reduced by collagen addition (Fig. 4C). CE2 had a similar effect on the number of cells (Fig. 4C). In contrast, all recombinant proteins exhibited dose-dependent effects on HaCaT cells (Fig. 4D). The greatest stimulation of the growth of both cell lines was >20% (Figs. 4C and 4D).

Interaction between Chimeric Proteins and EGF Receptors

Cell proliferation and mitogenesis are the result of signal transduction, which is stimulated by a combination of growth factors and receptors, and evaluated by immuno-detection of phosphorylation. A-431 cells were incubated with the various factors and subjected to western blotting. Phosphorylation levels were measured based on the intensities of bands detected using an anti-phosphotyrosine antibody (Fig. 5). The concentration that resulted in the greatest difference of A-431 cell number between the EGF and fusion proteins was 100 ng [14]. Band intensities were

Fig. 5. Effects of EGF fusion proteins on phosphorylation of the EGF receptor.

Lane 1, control A-431; lane 2, hEGF (Sigma) treated A-431; lane 3, EC1-treated A-431; lane 4, EC2-treated A-431; lane 5, CE1-treated A-431; and lane 6, CE2-treated A-431.

in the order of control < EC1 < hEGF, CE2 < EC2, CE1 (Fig. 5). These results were similar to those of the MTS assay (Fig. 4A).

Discussion

Various factors must be considered for application of EGF as a therapeutic agent, including side effects, target specificity, stability, appropriate cell type in question, and suitable strategies for delivery to the target site [14, 21]. The major obstacle for application of EGF as a healing agent is the low concentration in target areas [1, 4, 9, 21]. Therefore, high concentrations in the target region of EGF are essential for its use as a therapeutic agent, because side effects and decreased susceptibility of target cells cause elevated EGF concentrations [10]. Some studies have reported effective production of anchoring partner-fused growth factors to increase target specificity, and have applied microbial expression systems to improve productivity and purification [3, 6, 7, 12–15, 21, 24]. EGF fusion proteins exhibit usefulness as therapeutic agents for injury, and these binding domains have been used as anchoring partners of growth factors [6–8, 12–15, 21, 24]. Nishi *et al*. [20] produced and characterized collagen-binding growth factors by generating fusion proteins consisting of the 5.4 kDa rat epidermal growth factor (rEGF) or 17.4 kDa human basic fibroblast growth factor (bFGF) and 23.9 kDa of the collagen-binding domain from the collagenase moiety of *Clostridium histolyticum*. Their recombinants showed high collagen-binding activities and similar or low proliferation promoting abilities [20]. Ishikawa *et al*. [12, 13] produced 40 kDa of a human fibronectin collagen-binding domain (FNCBD) fused to human EGF. Approximately 46 kDa of the fusion protein exhibited binding and mitogenic bifunctional activities [12, 13]. Elloumi *et al*. [7, 8] fused ~47 kDa and ~61 kDa of human fibronectin-derived CBD (fnCBD) and/or artificial cell adhesive peptide, respectively, to the human EGF protein. Both biomimetic materials showed comparable binding and cell-promoting abilities [7, 8]. Kitajima *et al*. [15] created a novel chimeric protein fused with the human fibronectin fibrin-binding domain (FBD) and EGF. The 17 kDa chimeric EGF induced a dose-dependent increase in binding affinity and improved cell growth [15]. Kim *et al*. [14] constructed fusion proteins composed of human EGF (6 kDa) and a 33 aa CBD peptide from metalloprotease of *V. mimicus*. They described the binding and cell proliferation properties of the fusion proteins and proposed its use as a healing agent [14]. Yang *et al*. [24] constructed a 10.5 kDa collagen-binding human epidermal growth factor, which was a combination of human EGF and a 7 aa moiety of mammalian collagenase short CBD. This construct exhibited binding ability and promoted cellularization of scaffolds [24]. Egawa *et al*. [6] examined an engineered EGF that included the CBD of von Willebrand factor. In their investigation, structural folding, binding competence, and enhanced mitogenetic functions were observed [6].

In a previous study, we reported that the 33 aa region of the C-terminus of the collagen-binding domain from the metalloprotease of *V. mimicus* contained two FAXWXXT motifs, which were essential for collagen-binding activity [16]. Owing to the abundance of collagen in the extracellular matrix (ECM) of human epidermal cells, we used collagen as the binding target for recombinant EGF [14]. Moreover, the ECM and collagen in epidermal tissues are important for the storage and interaction of growth factors that promote cell growth [14, 19, 20, 21]. Among the repeated FAXWXXT portion, the carboxyl terminus was more important for binding [16]. Therefore, the indispensable FAXWXXT region that included 18 and 12 aa of the C-terminus was selected for the anchoring partner. We also combined EGF and CBD in various orders for a bifunctional effect (Fig. 1A).

Additional amino acids have been included between growth factors and binding domains to improve the flexibility of the functional and binding sites of growth factors [6, 13, 24]. However, insertion of additional amino acids in recombinant proteins caused misfolding and interfered with growth and binding activities [14]. Furthermore, recombinant proteins larger than 10 kDa are easily recognized as endotoxins or antigens by the human immune system [5]. For the above reasons, we constructed small fusion proteins of less than 10 kDa in molecular mass without insertional amino acids.

In the present investigation, we designated the smallest chimeric EGF proteins as wound repair agents and addressed issues with EGF as a therapeutic agent. We also compared the optimal EGF and CBD composition. Interestingly, after incubation with collagen, bound samples were eluted and confirmed their adhesion (Fig. 3). The binding abilities of fusion proteins were also compared using type I collagen and the staining intensities of eluted protein bands (data not shown). Recombinant proteins exhibited high adhesive affinities to insoluble type I collagen and similar binding levels to the combination order (N-terminus or C-terminus of EGF), but differed according to the length of the CBD motif (18 aa of CBD recorded more higher affinities than 12 aa; data not shown).

With the exception of CE2, whole fusion proteins demonstrated enhanced or similar proliferation of A-431 and HaCaT cells than mature-type EGF (Fig. 4). Furthermore, tests with the collagen-coated well plate and HaCaT cell line showed better growth, higher susceptibilities, and dose-dependent results than the non-treated well plate and A-431 cell line (Fig. 4D). However, CE2 recombinant protein showed a low proliferation affinity with the cell lines (Fig. 4). The marked effects of EC2 and CE1 on mitogenesis were demonstrated by western blotting to detect phosphorylation (Fig. 5). Therefore, CE1 was a suitable fusion protein as a wound healing agent in terms of collagen-binding affinity, cell proliferation, and signal transduction. Although CE1 contained 18 aa, more than the 12 aa CBD moiety used in this study, it was smaller than other reported chimeric EGF proteins. Based on its collagen adhesion and enhancement of cell growth, CE1 is suitable as a therapeutic agent, and the 18 aa collagen-binding domain could be applied in various pharmaceutical and cosmetic industries. However, further *in vivo* studies are required to determine the clinical efficacy of this fusion protein.

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References

1. Carpenter G, Cohen S. 1976. ^{125}I-labeled human epidermal growth factor. Binding internalization, and degradation in human fibroblast. *J. Cell Biol.* **71:** 159-171.
2. Carpenter G, Cohen S. 1979. Epidermal growth factor. *Ann. Rev. Biochem.* **48:** 193-216.
3. Chen X, Cen P, Chen J. 2005. Enhanced production of human epidermal growth factor by a recombinant *Escherichia coli* integrated with *in situ* exchange of acetic acid by macroporous ion-exchange resin. *J. Biosci. Bioeng.* **5:** 579-581.
4. Chung KH, Park SH, Kim MK, Park HD, Son TI. 2006. Stabilization of epidermal growth factor on thermal and proteolytic degradation by conjugation with low molecular weight chitosan. *J. Appl. Polym. Sci.* **102:** 5072-5082.
5. Dagmar P, Anspach FB. 2000. Endotoxin removal from protein solution. *J. Biotechnol.* **76:** 97-119.
6. Egawa EY, Kato K, Hiraoka M, Nakaji-Hirabayashi T, Iwata H. 2011. Enhanced proliferation of neural stem cells in a collagen hydrogel incorporating engineered epidermal growth factor. *Biomaterials* **32:** 4737-4743.
7. Elloumi I, Kobayashi R, Funabashi H, Mie M, Kobatake E. 2006. Construction of epidermal growth factor fusion protein with cell adhesive activity. *Biomaterials* **27:** 3451-3458.
8. Elloumi HI, Nakamura M, Mie M, Kobatake E. 2009. Construction of multifunctional proteins for tissue engineering: epidermal growth factor with collagen binding and cell adhesive activities. *J. Biotechnol.* **139:** 19-25.
9. Hardwicke J, Schmaljohann D, Boyce D, Thomas D. 2008. Epidermal growth factor therapy and wound healing – past, present and future perspective. *Surgeon* **6:** 172-177.
10. Hayashi M, Tomita M, Yoshizato K. 2001. Production of EGF-collagen chimeric protein which shows the mitogenic activity. *Biochem. Biophys. Acta* **1528:** 187-195.
11. Imanishi J, Kamiyama K, Iguchi I, Kita M, Sotozono C, Kinoshita S. 2000. Growth factors: importance in wound healing and maintenance of transparence of the cornea. *Prog. Retin. Eye Res.* **19:** 113-129.
12. Ishikawa T, Terai H, Kitajima T. 2001. Production of a biologically active epidermal growth factor fusion protein with high collagen affinity. *J. Biochem.* **129:** 627-633.
13. Ishikawa T, Terai H, Yamamoto T, Harada K, Kitajima T. 2003. Delivery of a growth factor fusion protein having collagen-binding activity to wound tissues. *Artif. Organs* **27:** 147-154.
14. Kim DG, Min MK, Ahn SC, Kim JK, Kong IS. 2009. Expression of a fusion protein containing human epidermal growth factor and the collagen-binding domain of *Vibrio mimicus*. *Biotechnol. Lett.* **31:** 259-264.
15. Kitajima T, Makoto S, Hirokazu H, Takeshi O, Yoshihiro I. 2009. A chimeric epidermal growth factor with fibrin affinity promotes repair of injured keratinocyte sheets. *Acta Biomater.* **5:** 2623-2632.
16. Lee JH, Ahn SH, Lee EM, Jeong SH, Kim YO, Lee SJ, Kong IS. 2005. The FAXWXXT motif in the carboxyl terminus of *Vibrio mimicus* metalloprotease is involved in binding to collagen. *FEBS Lett.* **579:** 2507-2513.
17. Liu YL, Huang LM, Lin WP, Tsai CC, Lin TS, Hu YH, *et al.* 2006. Secretion of biologically active human epidermal growth factor from *Escherichia coli* using *Yersinia pestis* Caf1 signal peptide. *J. Microbiol. Immunol. Infect.* **39:** 366-371.
18. Nakagawa S, Yoshida S, Hirao Y, Kasuga S, Fuwa T. 1985. Biological effects of biosynthetic human EGF on the growth of mammalian cell *in vitro*. *Differentiation* **29:** 284-288.
19. Nimni ME. 1997. Polypeptide growth factors: targeted delivery systems. *Biomaterials* **18:** 1201-1225.
20. Nishi N, Matsushita O, Yuube K, Miyanaka H, Okabe A, Wada F. 1998. Collagen-binding growth factors: production and characterization of functional fusion protein having a collagen-binding domain. *Proc. Natl. Acad. Sci. USA* **95:** 7018-7023.
21. Rustad KC, Sorkin M, Levi B, Longaker MT, Gurtner GC. 2010. Strategies for organ level tissue engineering. *Organogenesis* **6:** 151-157.

22. Sahasrabuddhe AA, Ahmed N, Krishnasastry MV. 2006. Stress-induced phosphorylation of caveolin-1 and p38, and down-regulation of EGFr and ERK by the dietary lectin jacalin in two human carcinoma cell lines. *Cell Stress Chaperones* **11:** 135-147.
23. Schlessinger J, Schreiber AB, Levi A, Lax I, Libermann T, Yarden Y. 1983. Regulation of cell proliferation by epidermal growth factor. *CRC Crit. Rev. Biochem.* **14:** 93-111.
24. Yang Y, Zhao Y, Chen B, Han Q, Sun W, Xio Z, Dai J. 2009. Collagen-binding human epidermal growth factor promotes cellularization of collagen scaffolds. *Tissue Eng. A* **15:** 3589-3596.
25. Yin J, Xu K, Zhang J, Kumar A, Yu FX. 2007. Wound-induced ATP release and EGF receptor activation in epidermal cells. *J. Cell Sci.* **120:** 815-825.