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Production of a Biologically Active Epidermal Growth Factor Fusion Protein with High Collagen Affinity

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Collagen is generally incapable of capturing polypeptides such as growth factors in a specific manner. In this study, we established a collagen-binding growth factor (FNCBD-EGF) consisting of epidermal growth factor (EGF) and the fibronectin collagen–binding domain. A typical yield of FNCBD-EGF was approximately 200 μg/ml culture in an *Escherichia coli* expression system. This fusion protein bound to gelatin and fibrillar collagen sponges, and the bound protein was not effectively eluted even with 2 M NaCl. In addition, FNCBD-EGF bound to type I, II, III, or IV collagen–coated plates, and the specificity of binding was confirmed by competitive inhibition using fibronectin. FNCBD-EGF substantially stimulated cell growth after binding to collagen-coated culture plates, whereas EGF had no effect, indicating that this fusion protein acted as a collagen-associated growth factor. In an animal model of impaired wound healing, FNCBD-EGF, but not EGF, was retained with collagen sponges at wound sites 4 d after implantation, and repair of epidermis was observed underneath the sponges. These results suggested that our fusion protein with high collagen affinity would be useful for wound healing.

Key words: collagen-binding, epidermal growth factor, fibronectin, fusion protein, wound healing.

Efficient delivery systems of biologically active growth factors to lesions are essential in clinical management of damaged tissues, since most of these proteins have limited target specificity and short retention times in the tissues (*1*). Extracellular matrix (ECM) and growth factors are required for regeneration of tissues. Collagen is major and ubiquitous protein constituent of ECM that serves as a scaffold for cell growth. Collagen in damaged tissues may be a potential target of growth factors in inducing tissue repair. In addition, collagen materials may be useful as potential carriers for growth factors when applied into damaged tissues. Although a few studies indicated that collagen served as a specific ligand for some growth factors (*2–4*), this ECM protein is generally incapable of capturing various growth factors in a specific manner. One possible approach to overcome this issue is the modification of collagen as a carrier for growth factors. In recent studies, for example, acidic gelatin was prepared in order to make a complex with basic fibroblast growth factor (bFGF), and bFGF was released from this complex in a sustained manner (*5*, *6*).

Another approach may be the modification of growth factors to confer affinity to collagen by protein engineering (*1*). Tuan *et al.* (*7*) genetically engineered transforming growth factor-β (TGF-β1) fusion protein bearing a von Willebrand's factor–derived collagen-binding decapeptide (TGF-β1-vWF). Free TGF-β1-vWF exerted TGF-β1 activity to a certain extent, but it hardly stimulated cells when bound to collagen. Nishi *et al.* (*8*) constructed other fusion proteins (CBEGF/CBFGF) carrying epidermal growth factor (EGF) or bFGF with collagen-binding domain derived from bacterial collagenase. They reported that free CBFGF and CBEGF showed cell growth–promoting activity, but neither bound to any of the materials tested, including collagen gel, collagen film and collagen-coated tissue culture dishes. It was considered that employment of an appropriate collagen-binding fragment might be necessary in constructing bifunctional fusion proteins.

In this study, we established a collagen-binding fusion protein (FNCBD-EGF), two human polypeptides in a single chain, by fusing the gene encoding EGF to that of the fibronectin collagen-binding domain (FNCBD). Human EGF (hEGF) is a 53-residue single chain polypeptide that plays prominent roles in tissue regeneration, although its efficacy was not confirmed when applied to diabetic mice as an animal model of impaired wound healing (*9*). Fibronectin (FN) is a glycoprotein present in blood plasma, ECM, and also on the cell surface. FNCBD is one of the domains of FN and is responsible for stable binding to collagen. A polypeptide sequence of FN plasmin-chymotryptic 40-kDa fragment corresponds to FNCBD (*10*, *11*). This sequence was adopted as a fusion partner of hEGF. We report here that FNCBD-EGF showed high affinity to collagen and exhibited growth factor activity even after binding to collagen. The applicability of this fusion protein to skin wounds was also examined in an animal model.

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FN, fibronectin; FNCBD, fibronectin collagen–binding domain; HRP, horseradish peroxidase; PBS, phosphate buffered saline; TBS, Tris-buffered saline.

EXPERIMENTAL PROCEDURES

Construction of Expression Plasmids—FNCBD and EGF gene fragments were amplified from total RNA of human mesangial cells and human kidney by reverse transcription–polymerase chain reaction (RT-PCR), respectively. RNA LA PCR kit Ver. 1.1 (Takara Shuzo) was used with the following RT-PCR primers: FW-FNCBD (5′-GAGGTACCATGGTACATATGGCAGCTGTTTACCAACCGCAGCCTCACCC-3′), RV-FNCBD (5′-CGGGATCCTTACTCGAGCCACTGGATGGGGTGGGAGTTGGGCTGAC-3′), FW-EGF (5′-GTGTCGACGACGATGATAAGAATAGTGACTCTGAATGTCCCCTG-3′), and RV-EGF (5′-GAATTCTTAGCGCAGTTCCCACCACTTCAG-3′). Amplified gene fragment encoding human FNCBD or hEGF was introduced into the *Kpn*I–*Bam*HI or *Sal*I–*Eco*RI sites of the cloning plasmid pBluescript SK (Stratagene), respectively, and the introduction was confirmed by DNA sequencing analysis. The 5′-terminus of the EGF gene fragment was tagged with a sequence encoding Asp-Asp-Asp-Asp-Lys derived from the RT-PCR primer FW-EGF. The resulting plasmids were named pBS [FNCBD] and pBS [EGF], respectively. The FNCBD gene fragment was introduced into the *Kpn*I–*Sal*I site of pBS [EGF] and the resulting plasmid was named pBS [FNCBD-EGF]. The FNCBD gene fragment and the FNCBD-EGF fusion gene fragment were introduced into the *Nde*I–*Not*I and *Nde*I–*Eco*RI sites of the expression plasmid pTYB1 (New England Biolabs), respectively. The resulting plasmids were named pTY [FNCBD] and pTY [FNCBD-EGF], respectively.

Preparation of FNCBD and FNCBD-EGF—*Escherichia coli* strain ER2566 carrying plasmid pTY[FNCBD] or pTY-[FNCBD-EGF] was grown to express recombinant proteins. Cells were harvested by centrifugation at 4°C and immediately subjected to sonication in cold sonication buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA). After washing, the insoluble fractions were solubilized in 8 M urea (8 M urea, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA) and dialyzed against serial twofold dilutions of urea and finally against sonication buffer. Aliquots of quantified samples were used to assay gelatin-binding activity. The remaining samples were purified with gelatin-Sepharose 4B, then dialyzed against 10 mM phosphate buffer (pH 7.4) containing 50 mM NaCl. Purified FNCBD-EGF was quantified with an enzyme-linked immunosorbent assay (ELISA) kit for hEGF (R&D Systems). The concentration of purified FNCBD was determined by the method of Bradford (*12*) using FNCBD-EGF as a standard.

Immunoblotting Analysis—FNCBD and FNCBD-EGF were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were incubated with monoclonal antibodies (mAb) against FNCBD (FNC-4-4; Takara Shuzo) or hEGF (clone 10825.1; R&D Systems), then treated with horseradish peroxidase (HRP)–conjugated secondary antibodies (Dako). Peroxidase activity was detected with a Konica immunostaining kit HRP-1000.

Gelatin Binding Assay—Protein samples and commercial hEGF (Pepro Tech) were incubated with 500 μl of gelatin-Sepharose 4B (Amersham Pharmacia Biotech) in microtubes on a rotator at 4°C for 1 h. The gelatin was washed, twice per solution (500 μl), with sonication buffer, Tris-buffered saline (TBS ; 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA), and 1 M NaCl and 2 M NaCl in 50 mM Tris-HCl, pH 8.0 at 4°C for 30 min. The bound proteins were then eluted, twice per solution (500 μl), with 1, 2, 4, and 8 M urea in 50 mM Tris-HCl, pH 8.0 at 4°C for 30 min, and SDS-sample buffer (*13*) at 90°C for 10 min. Proteins in 1/100 volume of each supernatant after gelatin binding, washing, and elution were analyzed by Coomassie blue staining of 10–20% gradient gels after SDS-PAGE.

Collagen Binding Assay—Ninety-six-well plates (Falcon 3072) were coated overnight at 4°C with 0.2 ml/well of pepsin-digested bovine type I, II, III, or IV collagen at 3 mg/ml in hydrochloride solution, pH 3.0 (Koken), BSA at 3 mg/ml in PBS, or blocking reagent (BlockAce; Dai-Nippon Pharmaceuticals). The plates were washed six times with PBS containing 0.05% Tween 20 (PBST) and once with PBS. The plates were then incubated with 0.1 ml/well of serial dilutions of FNCBD-EGF or EGF in Dulbecco's modified Eagle's medium (DMEM) at 37°C for 2 h. After washing six times with PBST, the plates were reacted with anti-hEGF mAb (R&D Systems). Bound antibodies were detected by ELISA with HRP-conjugated secondary antibodies with H_2O_2 and *o*-phenylenediamine. Collagen-binding activity was determined by subtracting the absorbance at 660 nm from that at 490 nm.

Competitive Inhibition of Collagen Binding—Ninety-six-well plates were coated with type I or IV collagen and incubated with 20 nM FNCBD-EGF at 37°C for 90 min, followed by washing. The plates were then incubated with serial dilutions of human plasma FN (Life Technologies) or BSA at 37°C for 90 min. The plates were reacted with anti-hEGF mAb, followed by ELISA as described above.

Growth Factor Activity—NRK49F cells (*14*) were seeded in 24-well plates at 1×10^4 cells/well in 2% fetal bovine serum (FBS)-DMEM, and 7 h later protein sample was added to culture. Cell growth-promoting activity was examined by WST-1 assay (Dojindo) after a 4-day culture (*15*). The activity was evaluated at 450 nm by subtracting the background at 660 nm.

Growth Factor Activity after Collagen Binding—Twenty-four-well plates (Falcon 3047) were coated with type I collagen, then incubated with serial dilutions of FNCBD-EGF at 37°C for 2 h. The plates were washed twice with PBST and four times with PBS, then incubated with DMEM at 37°C for 7 d. Collagen-coated wells without bound FNCBD-EGF were incubated with serial dilutions of EGF at 37°C for 2 h. Both EGF-added wells and FNCBD-EGF–bound wells were washed thoroughly. NRK49F cells were seeded at 1×10^4 cells/well in 2% FBS-DMEM, and cell culture was continued for 4 d. Cell growth of NRK49F was observed at the center of each well. Cell growth–promoting activity was examined by WST-1 assay.

In Vivo Studies—Genetically diabetic mice (C57BL/KsJ-db/db Jcl) (*16*), 9–11 weeks old, were anesthetized with pentobarbital, and shaved. Two full-thickness skin wounds 8 mm in diameter were prepared on the back of each mouse with a punch biopsy instrument. Wounds were covered with a transparent occlusive dressing (Bioclusive; Johnson and Johnson) fixed in place with adhesive strips. Three days after wounding, collagen sponges 9 mm in diameter and 3 mm in thickness (Terudermis; Terumo) (*17*) were soaked with 50 μl of FNCBD-EGF or 50 μl of mixture of EGF and FNCBD at 0.8 μM (0.25 μg as hEGF), and were implanted into the wounds in each mouse. A single

mouse received the same sample in both wounds. Mice were euthanized 4 d (day 7) after implantation of the sponges.

Immunohistochemical Analyses—Implanted sponges and their surrounding tissues were excised, fixed in 10% neutral-buffered formalin and embedded in paraffin. Five-micrometer sections of the specimens were treated with M.O.M. immunodetection kit (Vector Laboratories) to prevent staining of endogenous Ig. The sections were examined by staining with anti-hEGF mAb (R&D Systems) or normal mouse IgG as a negative control, followed by reaction with a biotinylated anti–mouse IgG. The sections were then treated with avidin–biotin peroxidase complex and 3,3′-diaminobenzidine tetrahydrochloride solution. The stained sections were counterstained with hematoxylin. The other sections of the same tissues were subjected to Azan staining.

RESULTS

High Affinity Binding of FNCBD-EGF to Gelatin—A typical yield of FNCBD (40 kDa) and FNCBD-EGF (46 kDa) was approximately 200 μg/ml culture, and their identities were confirmed by immunoblotting (data not shown). Figure 1 shows Coomassie blue staining of proteins after SDS-PAGE via gelatin-binding assay. After incubation with gelatin-Sepharose in microtubes, supernatants of the protein samples contained small amounts of recombinant and *E. coli* proteins. In contrast, EGF had no affinity to gelatin. FNCBD-EGF was not effectively eluted with sonication buffer, TBS, 1 M NaCl, 2 M NaCl, or 1 M urea, whereas EGF was washed out with sonication buffer or TBS. Gelatin-bound FNCBD-EGF was slightly dissociated from gela-

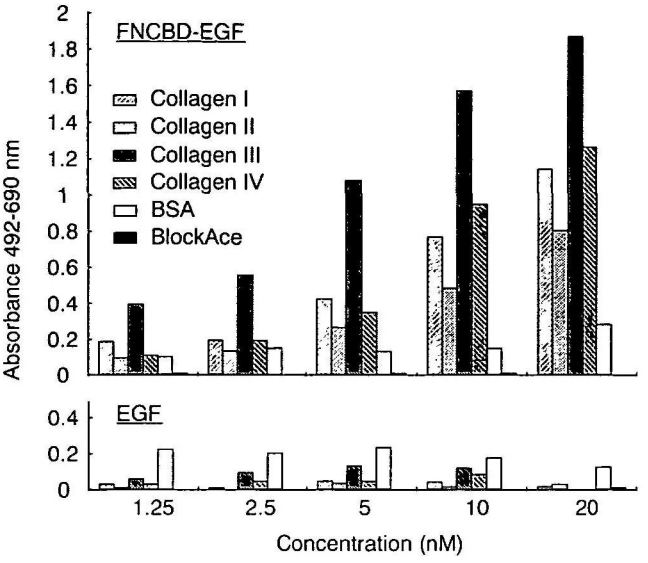

Fig. 2. **Collagen-binding activity of FNCBD-EGF.** The amount of FNCBD-EGF or EGF bound to wells was measured by ELISA using anti-hEGF mAb. Absorbance values for wells without added samples were adjusted to 0.00. The experiment was repeated three times.

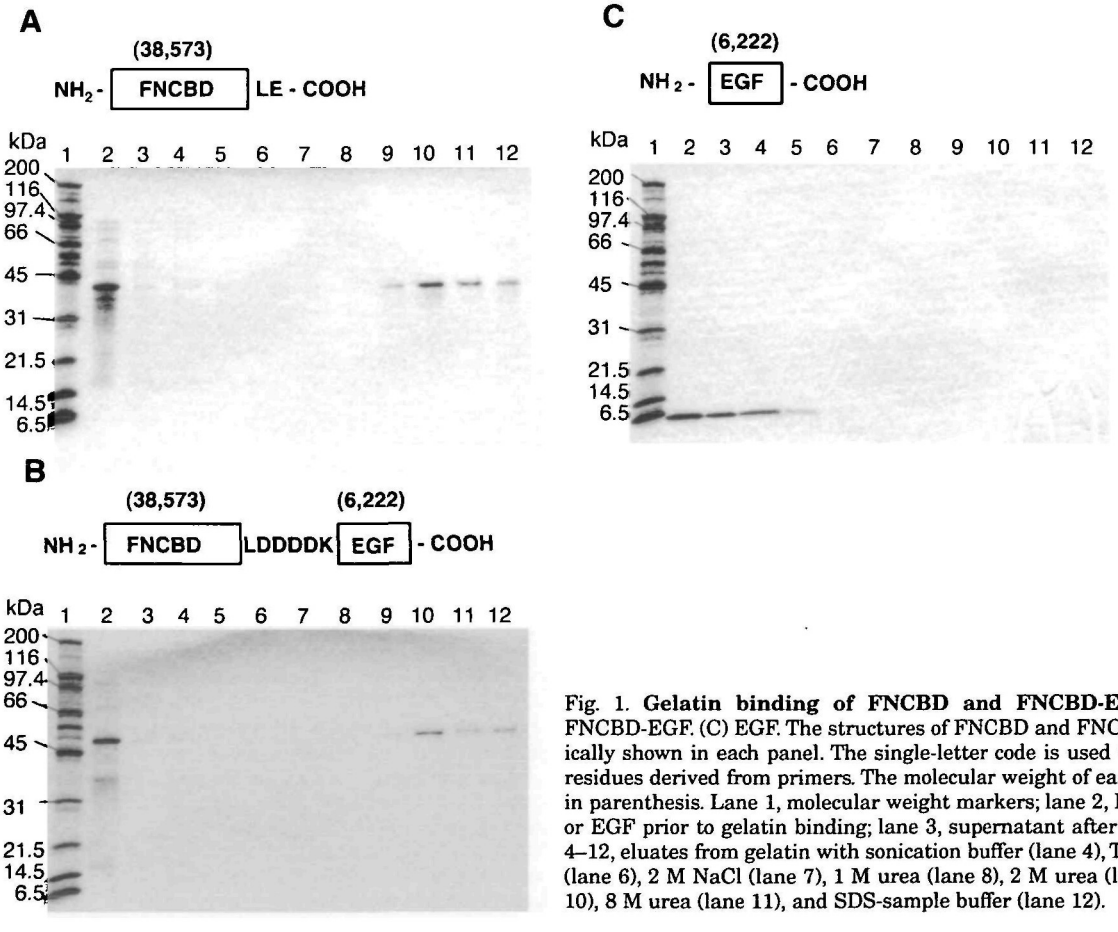

Fig. 1. **Gelatin binding of FNCBD and FNCBD-EGF.** (A) FNCBD. (B) FNCBD-EGF. (C) EGF. The structures of FNCBD and FNCBD-EGF are schematically shown in each panel. The single-letter code is used to indicate amino acid residues derived from primers. The molecular weight of each domain is indicated in parenthesis. Lane 1, molecular weight markers; lane 2, FNCBD, FNCBD-EGF, or EGF prior to gelatin binding; lane 3, supernatant after gelatin binding; lanes 4–12, eluates from gelatin with sonication buffer (lane 4), TBS (lane 5), 1 M NaCl (lane 6), 2 M NaCl (lane 7), 1 M urea (lane 8), 2 M urea (lane 9), 4 M urea (lane 10), 8 M urea (lane 11), and SDS-sample buffer (lane 12).

tin in 2 M urea. The majority of FNCBD-EGF was eluted with 4 M urea, 8 M urea, and SDS-sample buffer. The elution pattern of FNCBD-EGF was indistinguishable from that of unfused FNCBD. Likewise, we obtained an analogous pattern in an experiment using fibrillar collagen (honeycombed sponge; Koken) according to the same procedure except performed at 37°C (data not shown). These results indicated that almost all of the FNCBD-EGF prepared had high collagen affinity comparable to that of unfused FNCBD. It was also indicated that most of the contaminating proteins were eliminated from these recombinant proteins by affinity purification. Protein samples, which were eluted with 8 M urea after washing, were dialyzed without any precipitation. Purified protein samples were used for collagen-binding assays, cell growth assays, and *in vivo* studies as follows.

FNCBD-EGF Binding to Major Collagen Types—An assay was performed to evaluate whether FNCBD-EGF has affinity to pepsin-digested type I, II, III, and IV collagens. Gelatin-purified FNCBD-EGF and EGF were incu-

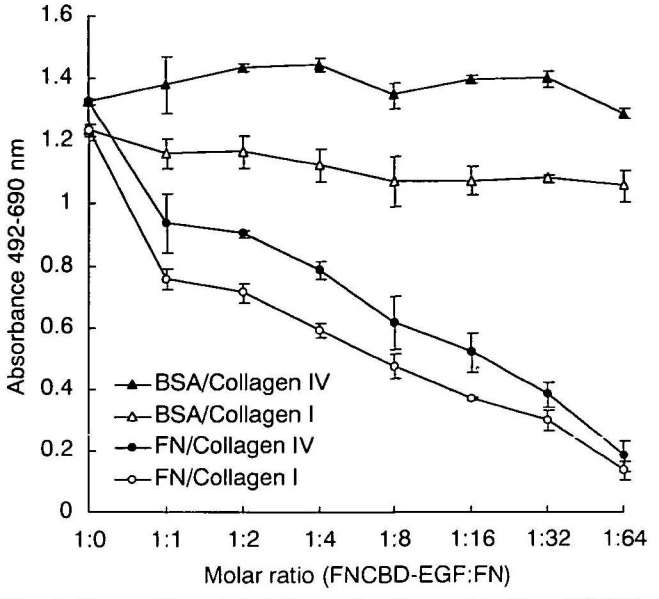

Fig. 3. **Competitive inhibition of collagen binding.** FNCBD-EGF (20 nM) was bound to collagen-coated wells, and amount of FNCBD-EGF remaining in the presence of fibronectin (FN) or BSA was measured by ELISA using anti-hEGF mAb. The concentration of FN is shown as the molar ratio to FNCBD-EGF. The concentration of BSA corresponds to that of FN in weight per volume. Absorbance values for collagen-coated wells without added of FNCBD-EGF were adjusted to 0.00. Each point represents the mean ± SD of duplicate wells. The experiments were repeated twice.

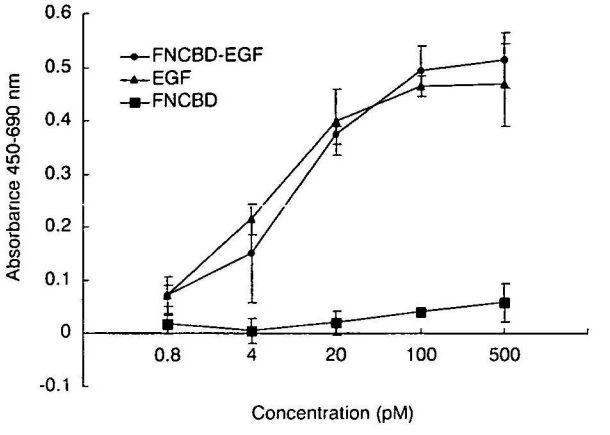

Fig. 4. **Growth-factor activities of FNCBD-EGF and EGF.** Protein sample was added to NRK49F cell culture and growth-promoting activity was examined by WST-1 assay. Absorbance of control wells containing buffer solution was adjusted to 0.00. Each point represents the mean ± SD of triplicate wells. The experiment was repeated three times.

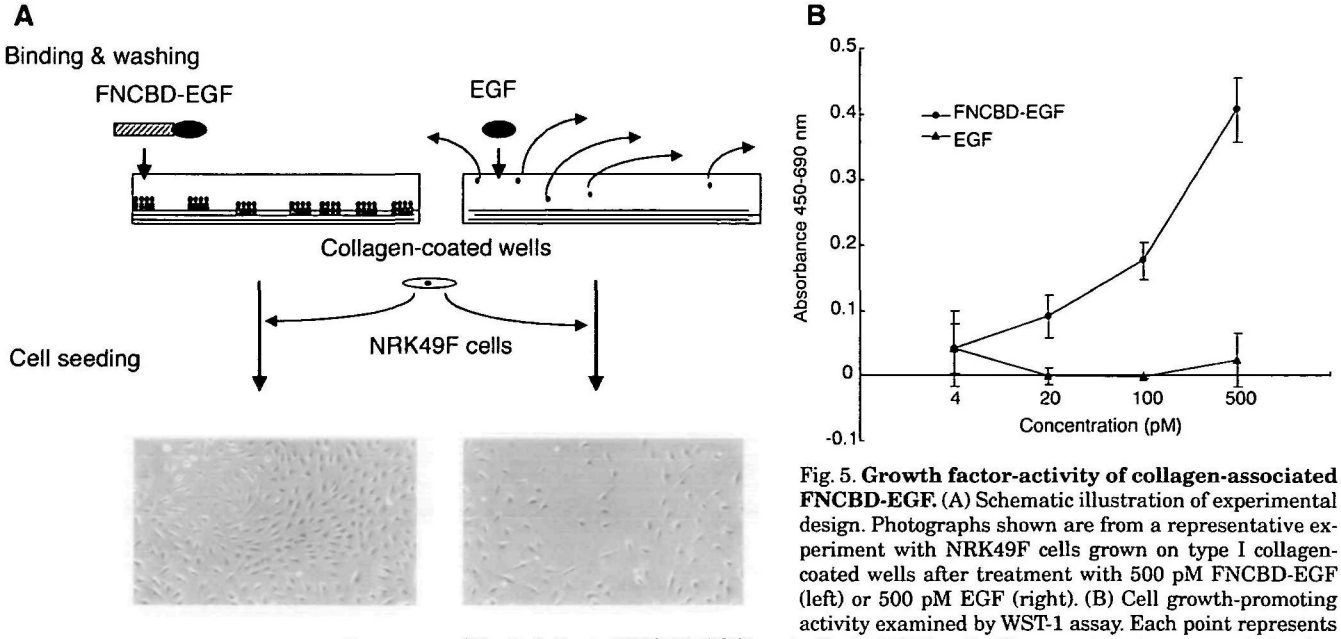

Fig. 5. **Growth factor-activity of collagen-associated FNCBD-EGF.** (A) Schematic illustration of experimental design. Photographs shown are from a representative experiment with NRK49F cells grown on type I collagen-coated wells after treatment with 500 pM FNCBD-EGF (left) or 500 pM EGF (right). (B) Cell growth-promoting activity examined by WST-1 assay. Each point represents the mean ± SD of triplicate (FNCBD-EGF) or duplicate (EGF) wells. The experiment was repeated twice.

bated in collagen-coated multiwell plates, then thoroughly washed. Collagen-bound FNCBD-EGF was detected and measured by ELISA using anti-hEGF mAb. As shown in Fig. 2, the amount of FNCBD-EGF bound to collagens increased in a dose-dependent manner, while interaction of FNCBD-EGF with BlockAce or BSA was considered as non-specific binding. FNCBD-EGF showed the highest affinity to type III collagen, medium affinity to type I and IV collagens, and the lowest to type II collagen. No binding of EGF was observed to any type of collagen.

Specific Binding of FNCBD-EGF to Collagen—Competitive inhibition experiments were performed to determine whether the FNCBD moiety was responsible for specific binding of FNCBD-EGF to collagen. Representative major collagen types, type I and IV collagens, were employed as ligands for competition experiments. FNCBD-EGF at 20 nM bound to collagen-coated multiwell plates, and FN efficiently reduced the binding of FNCBD-EGF in a molar ratio-dependent manner (Fig. 3). FN at 1,280 nM (the ratio of FNCBD-EGF to FN, 1:64) blocked the binding of FNCBD-EGF to type I and IV collagen by nearly 80%, whereas BSA did not act as a competitor. The competitive effect of FN against FNCBD-EGF was similar in the binding to type I and IV collagen.

Intact Growth Factor Activity of FNCBD-EGF—Cell growth-promoting activity of FNCBD-EGF was compared with that of EGF in a WST-1 colorimetric assay (Fig. 4). The dose-response curve of FNCBD-EGF was similar to that of EGF, indicating that FNCBD-EGF retained the active EGF form. In contrast, FNCBD had no EGF activity. Taken together, these findings demonstrate that FNCBD-EGF has both collagen-binding and cell growth-promoting activities comparable to those of the corresponding unfused proteins.

FNCBD-EGF as a Collagen-Associated Growth Factor—An assay was performed to examine whether biologically active FNCBD-EGF remains bound to collagen. Collagen-coated multiwell plates were incubated with FNCBD-EGF and washed. The plates were incubated with DMEM for 7 d before cell culture. Photographs indicating NRK49F cell growth are shown with a schematic illustration of the experimental set-up in Fig. 5A. Collagen-associated FNCBD-EGF stimulated NRK49F cell growth 7 d after collagen binding in a dose-dependent manner, whereas EGF had no effect (Fig. 5B).

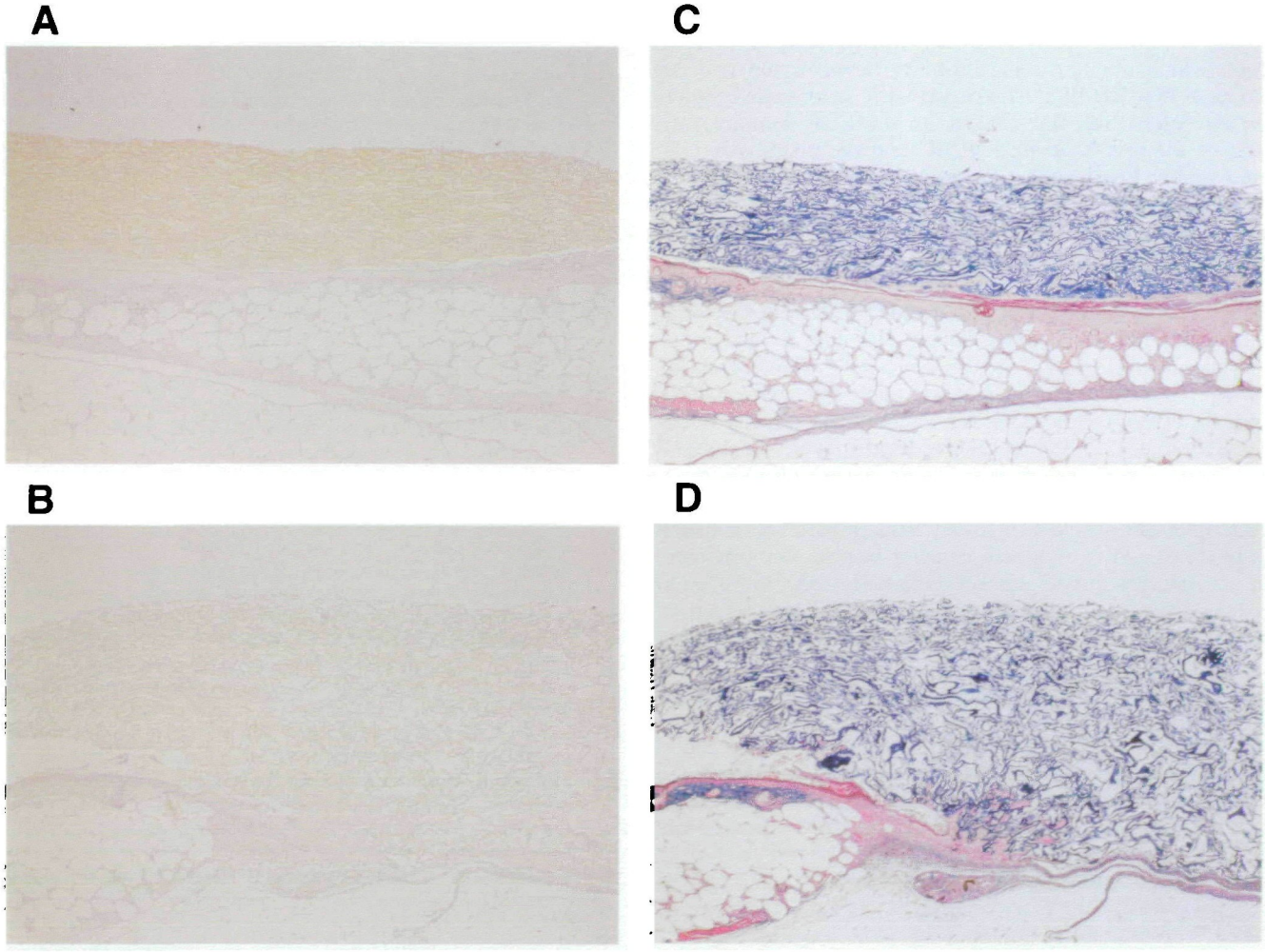

Fig. 6. **Immunohistochemical and Azan stainings of wound tissues.** Collagen sponges soaked with FNCBD-EGF (A and C) or EGF plus FNCBD (B and D) were implanted into diabetic mice 3 d after wounding The collagen sponges and their surrounding tissues were examined 4 d after implantation (day 7). Anti-hEGF mAb (A and B). Azan staining (C and D). Original magnification, 10 ×.

FNCBD-EGF Function in Injured Tissue—The effectiveness of FNCBD-EGF was evaluated in diabetic mice as an animal model of impaired wound healing. FNCBD-EGF was applied to wounds in combination with the collagen sponges. FNCBD-EGF was detectable with the sponge 4 d after administration (Fig. 6A) and epidermal tissue was observed underneath the sponge (Fig. 6C). On the contrary, the collagen sponge treated with EGF plus FNCBD showed negligible staining (Fig. 6B), indicating that the EGF level was reduced by diffusion, and it had no effect on the wound (Fig. 6D). These results indicated that the retention of FNCBD-EGF promoted regeneration of epidermis on intractable wounds.

DISCUSSION

We designed a biologically active fusion protein that could stably bind to collagen materials and exerted its growth-factor activity after collagen binding. The sequence corresponding to FN plasmin-chymotryptic 40-kDa fragment was adopted as a fusion partner of EGF to make the molecule without any functional impairment. The inclusion of the sequence corresponding to the proteolytic FNCBD appears to be critical for generation of the bifunctional fusion protein, since this FNCBD retains its function with an active conformation. In addition, the FNCBD employed had a long C-terminal peptide sequence with no cysteine residues, which might confer flexibility between the two domains of FNCBD-EGF. The region with an extended conformation might link the FNCBD to the EGF domain similarly to the link between FNCBD and the neighboring domain of FN. Furthermore, we added no purification tags to the termini of the fusion protein. The results of the present study confirmed that our molecular design was optimized to construct the collagen-binding fusion protein.

EGF is synthesized as a transmembrane precursor and released from cells as a mature form (*18*). This precursor is a biologically active growth factor, although it anchors to cell membrane (*19*). This mode of action is termed the juxtacrine mechanism (*20*, *21*), and many members of EGF gene family and some cytokines exhibit their activity in the same manner (*22*). It was also reported that EGF exhibited mitogenic activity when it was chemically cross-linked to synthetic polymers (*23*, *24*). As shown in this study, FNCBD-EGF was not released from gelatin or collagen unless exposed to reagents causing protein denaturation. In addition, the fusion protein remained bound to collagen-coated dishes even after long incubation followed by extensive washing, and it stimulated cells that were seeded into the dishes. These results suggested that collagen-associated FNCBD-EGF might act as a growth factor in a nondiffusible fashion similar to the juxtacrine mechanism.

In our preliminary observation, we unexpectedly found that little or no collagen was exposed upon wounding in the animal model when FNCBD-EGF alone was administered into the injured tissue. Although EGF immunostaining was occasionally observed locally with fibrous substance on the surface of the wounds, the localization of the staining was not necessarily identical to that of collagen revealed by Azan staining (data not shown). This implied that the application of collagen-binding EGF alone might have a minimal effect on severe defects such as full-thickness skin wounds. As a result of these observations, we employed collagen sponges combined with FNCBD-EGF and observed the epidermal repair underneath the sponge retaining this fusion protein. Our results suggested the application of the fusion protein in combination with collagen materials might be more effective than that of this protein alone.

Although it is generally accepted that EGF is essential for epithelialization in the wound-healing process, its efficacy was not significant in clinical studies (*25*) or in diabetic mice as observed in the present study. This may be due to inefficient retention of EGF in a wound (*26*). FNCBD-EGF might be a useful alternative to EGF as a therapeutic agent, since its retention at wound sites would minimize the amount and number of applications necessary. Further studies on animal models may confirm the effectiveness of our fusion protein on intractable wounds such as diabetic ulcers.

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