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Recombinant tetra-cell adhesion motifs supports adhesion, migration and proliferation of keratinocytes/fibroblasts, and promotes wound healing

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Abbreviations: ECM, extracellular matrix; EPDIM, Glu-Pro-Asp-Ile-Met; FGF, fibroblast growth factor, FN, fibronectin; PHSRN, Pro-His-Ser-Arg-Asn; RGD, Arg-Gly-Asp; VN, vitronectin

Abstract

An extracellular matrix protein plays an important role in skin wound healing. In the present study, we engineered a recombinant protein encompassing the 9th and 10th type III domains of fibronectin, and 4th FAS1 domain of βig-h3. This recombinant protein, in total, harbors four known-cell adhesion motifs for integrins: Pro-His-Ser-Arg-Asn (PHSRN) and Arg-Gly-Asp (RGD) in 9th and 10th type III domains of fibronectin, respectively, and Glu-Pro-Asp-Ile-Met (EPDIM) and Try-His (YH) in 4th FAS1 domain of βig-h3, were designated to tetra-cell adhesion motifs (T-CAM). *In vitro* studies showed T-CAM supporting adhesion, migration and proliferation of different cell types including keratinocytes and fibroblasts. In an animal model of full-thickness skin wound, T-CAM exhibited excellent wound healing effects, superior to both 4th FAS1 domain of βig-h3 or 9th and 10th type III domains of fibronectin. Based on these results, T-CAM can be applied where enhancement of cell adhesion, migration and proliferation are desired, and it could be developed into novel wound healing drug.

Keywords: βIG-H3 protein; cell adhesion; cell movement; cell proliferation; fibronectin; wound healing

Introduction

Wound healing is essential process for organisms to regenerate the injured tissues. The cutaneous wound healing that follows injury to the skin and other soft tissues represents a dynamic and well-ordered biological process (Martin, 1997). This requires integration of complex biological and molecular events of homeostasis, inflammation, epithelization, fibroplasia, extracellular matrix (ECM) deposition, angiogenesis, and tissue remodeling (Falanga, 2005). The fundamental processes like adhesion, migration and proliferation of keratinocytes/fibroblasts at wound play a key role for synthesis and reconstitution of injured cutaneous tissues. The deposition of provisional matrix comprising ECM proteins like collagens, fibronectin (FN), vitronectin (VN) and laminin-5 at wound bed and its remodeling are essential aspects of wound healing. These ECM proteins serve as substratum for migrating and proliferating keratinocytes and fibroblasts. In addition, various growth factors and cytokines (TGF-β, EGF, PDGF, FGF, ILs, INF) secreted and/or present in wound sites influence wound healing by promoting and/or regulating the activities of migrating/proliferating keratinocytes and fibroblasts (Singer and Clark, 1999; Werner and Grose, 2003).

FN, an adhesive glycoprotein, is one of the components of initial plasma clot and provisional matrix at cutaneous injury sites and is involved in multi-step process in wound healing and scar formation (Singer and Clark, 1999). It is a disulfide-linked dimer of identical 230-250 kDa subunits, each subunit consists of three repeating domains: type I, type II, and type III domains (Pankov and Yamada, 2002). It is present in soluble dimeric form in plasma whereas in multimeric insoluble form in ECM. Several domains in FN are associated with various functions including cell adhesion, migration, proliferation, differentiation, chemotaxis, tissue-remodeling and wound healing (Grinnell, 1984; Pankov and Yamada, 2002). The specific sequences identified in FN, Pro-His-Ser-Arg-Asn (PHSRN) in 9th and Arg-Gly-Asp (RGD) in 10th type

III repeats are recognized to interact with bewildering arrays of integrins including $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha v\beta 1$, $\alpha v\beta 3$ and $\alpha_{IIb}\beta 3$ and mediate cell binding to FN (Pierschbacher and Ruoslahti, 1984; Grant *et al.,* 1997; Li *et al.,* 2003). These two sequences act synergistically to mediate cell adhesion.

βig-h3 is an FAS1 domain-containing matrix molecule, originally cloned by Skonier *et al.* (1994) from adenocarcinoma cells treated with TGF-β. It consists of EMI domain followed by tandem repeats of four FAS1 domains (each consisting of about 140 amino acids) which are similar to its prototype protein *Drosophila* fasciclin-I (Bastiani *et al.,* 1987). The cell adhesion represents one of the functions of FAS1 domain-containing proteins like fasciclin I, periostin and βig-h3. The precise function of βig-h3 is not known yet but a number of studies suggest βig-h3 playing role in cell adhesion, migration, proliferation, differentiation, osteogenesis and wound healing (Kim *et al.,* 2002; Park *et al.,* 2004; Lee *et al.,* 2006). At present, NKDIL in second, Glu-Pro-Asp-Ile-Met (EPDIM) in fourth FAS1 domain and Try-His (YH) in each FAS1 domain are the known-cell adhesion motifs so far identified in βig-h3 and interact with various integrins mediating adhesion of cells to βig-h3 (Kim *et al.,* 2000, 2002). The previous two integrin-binding sites are known to interact with $\alpha 3\beta 1$ integrin and mediate the adhesion of keratinocytes to βig-h3 (Bae *et al.,* 2002) whereas YH motif interacts with $\alpha v\beta 3/5$ integrins supporting the adhesion of various cell types including fibroblasts, endothelial cells and osteoblasts (Kim *et al.,* 2002; Thapa *et al.,* 2005). Although, role of βig-h3 in wound healing is obscure, its expression is observed in endothelium and stroma-derived cells in healing corneal wound (Rawe *et al.,* 1997) and reactive astrocytes at stab wound sites in rat cerebral cortex (Yun *et al.,* 2002). It is present in papillary dermis (LeBaron *et al.,* 1995) and secreted by keratinocytes (Katz and Taichman, 1999). *In vitro* study shows βig-h3 supporting adhesion and migration of dermal fibroblasts and keratinocytes (LeBaron *et al.,* 1995; Bae *et al.,* 2002).

The defective wound healing is associated with various pathophysiologies including diabetes and uses of topical therapeutic agents have been proven beneficial. The soaking of wound dressing with FN, preparing growth factors- and ECM-containing topical formulations are reported to accelerate wound healing (Livant *et al.,* 2002; Lariviere *et al.,* 2003; Qiu *et al.,* 2007). Moreover, the use of ECM proteins and their derivatives is common practice in tissue engineering (Badylak, 2002). With the aim of designing new recombinant protein endowed with multiple integrin-binding properties, we made hybrid molecule, T-CAM encompassing 9th and 10th type III domains of fibronectin and the 4th FAS1 domain of βig-h3. The ability of T-CAM to support cell adhesion, migration and proliferation of cells was examined. Topical application of T-CAM and its wound healing effect were tested in full-thickness cutaneous wound model in rabbit.

Materials and Methods

Generation of recombinant proteins

Expression plasmid for wild type βig-h3 protein (βigh3-WT) is described previously (Kim *et al.,* 2000). The fragment for fourth FAS1 domain (encoding amino acids 498-637) was obtained by PCR using βig-h3 cDNA, and cloned into the *Eco*RV site of pET-29b (designated to βigh3 D-IV). The PCR fragment for 9th and 10th type III fibronectin domain was cloned into the *Eco*RV site of pET-29b (designated to FN115). To generate T-CAM, above *Eco*RV fragment of FN was inserted into the *Eco*RV and *Xho*I sites of βigh3 D-IV in pET-29b. All the constructs were confirmed by DNA sequencing. Recombinant proteins for all of these constructs were induced and purified as described previously (Kim *et al.,* 2000).

Western blot analysis

Purified proteins βigh3 D-IV, FN115 and T-CAM were separated through 12% SDS-PAGE gel (50 ng each) and transferred to polyvinylidine difluoride membrane. The membrane was blocked with 5% skim milk in TBS (50 mM Tris-HCl, pH 7.4; 150 mM NaCl) for one h at room temperature and then incubated with His-probe HRP mouse monoclonal IgG (Santa Cruz Biotechnology, Inc) at the dilution of 1:4000 in TBS-T (TBS containing 0.05% Tween 20) for 1 h at room temperature. The membrane was extensively washed and the proteins were visualized with an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech., Piscataway, NJ).

Cell culture

HaCaT and NIH3T3 cell lines were cultured in DMEM (Gibco-BRL) with 4.5 g/L glucose, 10% FBS and antibiotics at 37^{o}C in 5% CO_2. The normal human fibroblast cells were grown in DMEM medium supplemented with 4.5 g/L glucose, 10% fetal calf serum and antibiotics. Normal human keratinocytes (NHEK) were grown in keratinocyte

medium kit (KMK, Sigma). CHO cells were grown in Minimum Essential Medium-Alpha (α-MEM) and MRC5 cells were grown in RPMI-1640 with 4.5 g/L glucose, 10% FBS and antibiotics at 37°C in 5% CO_2.

Cell adhesion and spreading assay

The cell adhesion assay was performed as described previously (Kim *et al.,* 2000). Briefly, 96-well micro-plates (Falcon, Becton-Dickinson, Mountain View, CA) were coated with protein by incubating at 37°C for 1 h and then blocked with PBS-containing 0.2% BSA for 1 h at 37°C. The different proteins used for coating were as follows: βigh3-WT, βigh3 D-IV, purified human plasma fibronectin (pFN), FN115, T-CAM, and heat-inactivated BSA (Sigma). Cells were trypsinized and suspended in the culture media at a density of 2.5 $\times$ 10^4 cells/ml and 0.1 ml of the cell suspension was then added to each well of the plates. Cell attachment was analyzed as follows. After incubation for 1 h at 37°C, unattached cells were removed by rinsing twice with PBS. Attached cells were incubated for 1 h at 37°C in 50 mM citrate buffer, pH 5.0, containing 3.75 mM *p*-nitrophenyl-N-acetyl β-D-glucosaminide (hexosaminidase substrate) and 0.25% Triton X-100. Enzyme activity was blocked by addition of 50 mM glycine buffer, pH 10.4, containing 5 mM EDTA, and the absorbance was measured at 405 nm in a Multiscan MCC/340 microplate reader (Titertek Instruments, Inc., Huntsville, AL).

For spreading assay, cells were applied into 96-well culture plates-coated with proteins as described above. The attached cells were fixed with 8% glutaraldehyde (Sigma) and then stained with 0.25% crystal violet (Sigma) in 20% methanol (w/v). Cell area was measured using Image-Pro plus software (Media Cybernetics, Silver Spring, MD). Experiments were repeated in triplicate with 200 or 300 measurements per site for each experiment. Data are reported as the mean area at specific time point $\pm$ S.E.

Migration assay

Cell migration assay was performed in trans-well plates (8 μm pore size, Costar, Cambridge, MA). The undersurface of the transwell-membrane were coated by incubating in protein-containing PBS at 4°C for overnight, and blocked with 2% BSA. The fibroblast (1 $\times$ 10^4 cells) and HaCaT (1 $\times$ 10^5 cells) per well in 200 μl medium were seeded in the upper compartment of filters. After 2 h and 24 h of migration, cells in the upper chamber of the filter were removed and nonmigrating cells on the top of the filters were removed with a cotton swab. Cells on the lower side of the filter were fixed with 8% glutaraldehyde and then stained with 0.25% crystal violet in 20% methanol (w/v). Each experiment was repeated in duplicate and within each well, cell counting was done in nine randomly selected microscopic high power fields (HPF $\times$ 200).

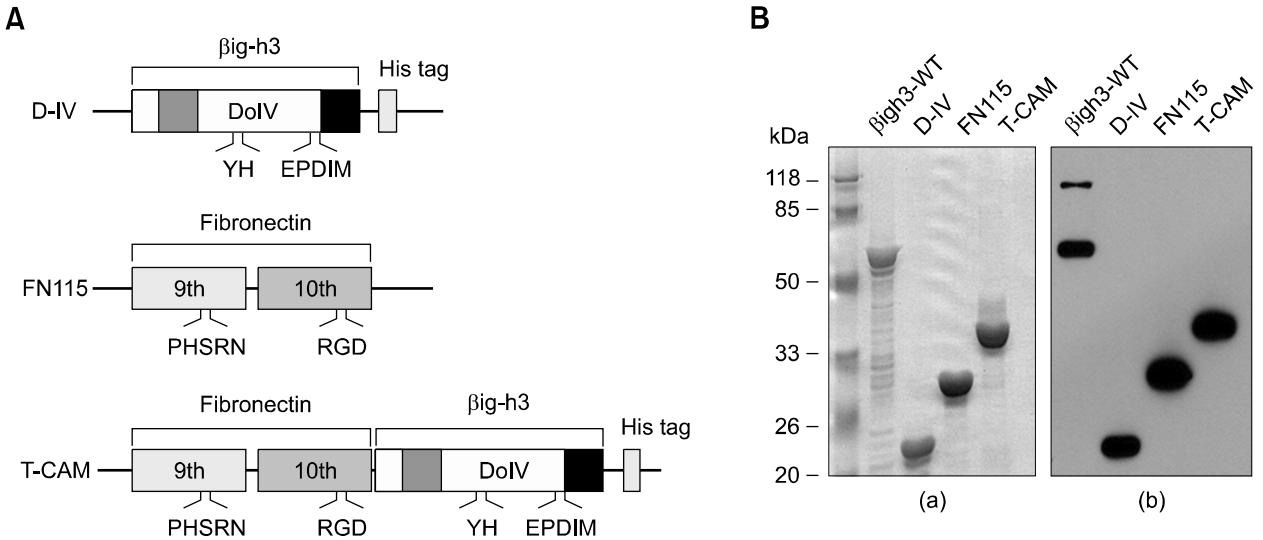

Figure 1. Schematic diagram of βigh3 D-IV, FN115 and T-CAM. A, Positions of YH and EPDIM motifs in βigh3 D-IV are shown. The 9th and 10th FN type III domain (FN115) contain PHSRH and RGD motif, respectively. The T-CAM consists of N-terminus FN115 and C-terminus FAS1 domain. B, The purity and integrity of protein used are shown by SDS-PAGE (a) and western blotting using anti-his antibody (b).

Proliferation assay

Twenty-four-well culture plates were coated with the indicated proteins diluted in PBS, for overnight at 4°C. The plates were rinsed three times with PBS and uncoated surfaces were blocked with 2% BSA in PBS for 1 h at 37°C. Then, plates were rinsed and keratinocytes prepared by 0.25% trypsin-EDTA treatment, were added to each well in 1 ml culture medium. Although the initial cell adhesion efficiency was different depending on the substrate used, most of the cells became adherent within a few hours, thus, giving the same cell numbers. The cell proliferation was assessed by manual cell counting in hematocytometer at the interval of 24 h for total 96 h. Cell numbers at 0 h indicate numbers at 24 h after initial cell seeding, showing that there were no differences in the initial cell numbers at 0 h point among different conditions.

Full-thickness skin wound preparation and treatment

The dorsal skin of the New Zealand white rabbits were shaved and cleaned with iodine solution, and a full-thickness skin wound (approximately 30 mm in diameter) were created (Saaristo *et al.,* 2006). Then, wounds were treated with the cream con-

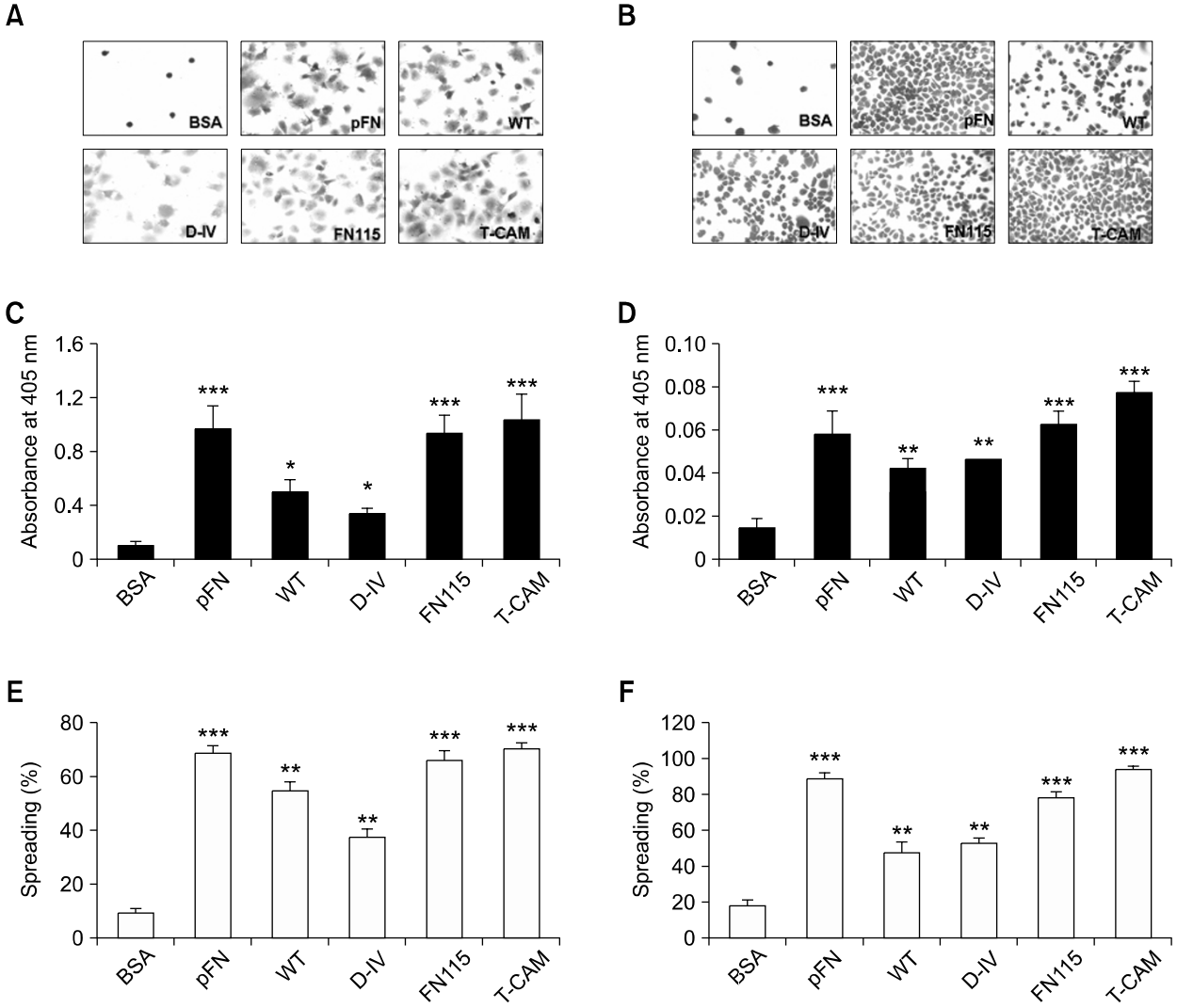

Figure 2. T-CAM supports adhesion and spreading of fibroblast and HaCaT cells. A, Low power microscopic picture of adhering fibroblasts (A) and HaCaT cells (B) to various proteins are shown. The cell adhesion assay was carried out in 96-well plate pre-coated with various proteins and number of cells adhering was quantified by enzymatic method as described in "Materials and Methods". C, fibroblasts, D, HaCaT. BSA was used as negative control and other proteins used were pFN, βigh3-WT, FN115, and T-CAM. Spreading of cells to above proteins and their measurement are shown in E (fibroblasts) and F (HaCaT cells). Data represent mean ± SD of three separate experiments. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$

taining various recombination proteins, βigh3-WT, βigh3 D-IV, FN115, and T-CAM (500 μg each) and covered with a permeable adhesive dressing. The dressing was removed on day 3 after wounding to enable visual analysis. Digital photographs of the dorsal wounds were taken every 3 days, and the wound areas were calculated as a percentage of the original wound. Sixty rabbit with paired wounds were analyzed for each time point.

Statistical analysis

All data were expressed as means ± SEM. Comparisons among group were performed using one or two-way ANOVA. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ were considered to be statistically significant.

Results

T-CAM supports adhesion and spreading of cells

The schematic diagram of βigh3 D-IV, FN115 and T-CAM are shown in Figure 1A. The purity and integrity of each protein used were assessed by SDS-PAGE/Coomassie staining (Figure 1B-a) and western blotting using his-tag antibody (Figure 1B-b). The expected sizes of proteins are: 68 kDa (βig-h3), 21 kDa (βigh3 D-IV), 27 kDa (FN115) and 45 kDa (T-CAM). The microscopic picture of fibroblast and HaCaT cells adhering to these proteins are shown in Figure 2A and 2B, respectively. The cell adhesion assay showed remarkably high cell adhesion activity of T-CAM for these cells when compared with βig-h3 and its FAS1 domain (Figure 2C and 2D). However, its cell adhesion activity was comparable with that of FN or FN115, and no significant increase in its cell adhesion activity was observed even with inclusion FAS1 domain.

The cell adhesion and spreading are intimately associated processes and involves the complex rearrangements of the actin cytoskeleton in spreading cells. The number and surface areas of fibroblast and HaCaT cells that adhered and spread to T-CAM were clearly greater than those adhered and spread to BSA, βigh3-WT or βigh3 D-IV (Figure 2E and 2F). Like cell adhesion, cell spreading of these cells to T-CAM was comparable to FN or FN115 and no further enhancement in spreading activity of T-CAM was observed.

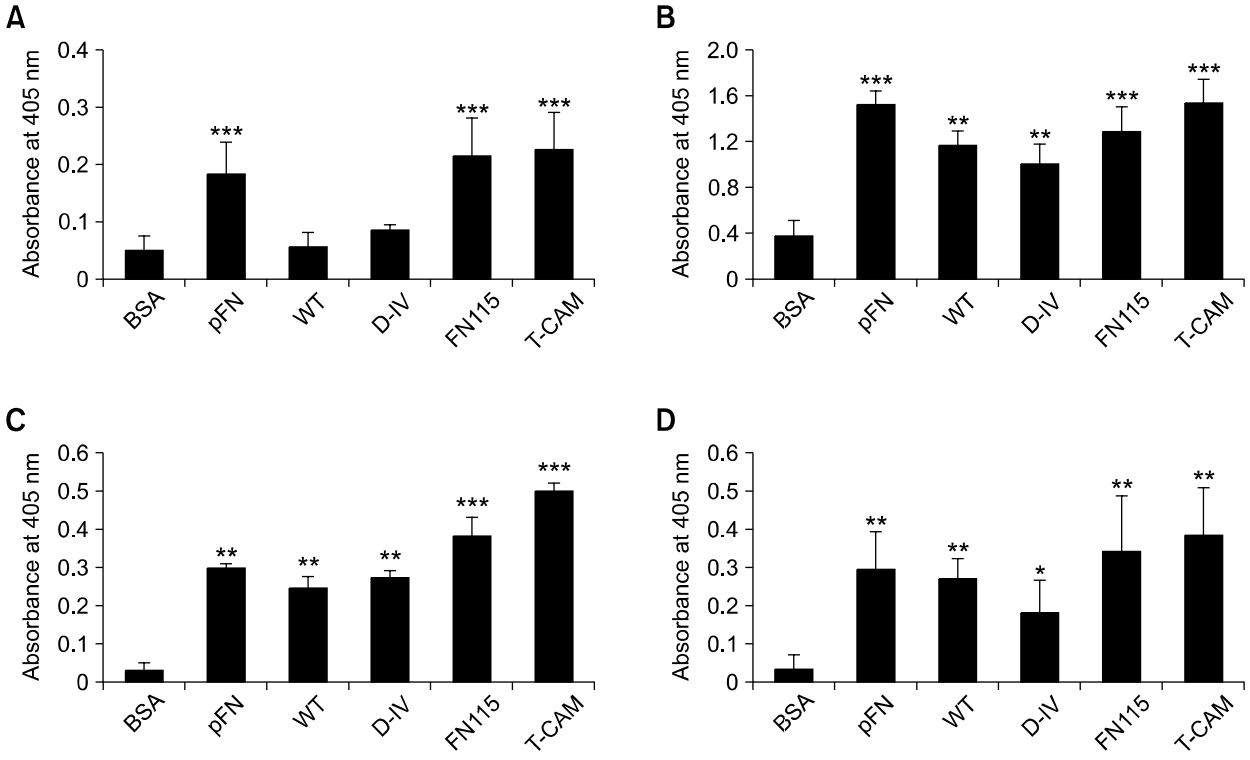

Figure 3. T-CAM supports adhesion of various cell types. Cell adhesion assay was performed as described above and various cell types used are A (CHO cells), B (MRC5 cells), C (NHEK cells), and D (NIH3T3 cell). Data represent mean ± SD of three separate experiments. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$

The cell adhesion activity of T-CAM was tested for a number of other cell lines including Chineses hamster ovary (CHO), lung fibroblast (MRC5), normal human keratinocyte (NHEK) and mouse fibroblast (NIH3T3). As shown in Figure 3, T-CAM is equally active in supporting the adhesion of these cell lines. It was expected because four known-cell adhesion motifs present in T-CAM are supposed to interact with a large numbers of integrins and can mediate adhesion of these cell types.

T-CAM support proliferation of fibroblast and HaCaT cells

The various ECM proteins in concert with growth factors show mitogenic property on adhering cells by providing the necessary intracellular signaling. The fibroblast and HaCaT cells synchronized by serum starvation were seeded onto plates coated with T-CAM or other proteins and allowed to grow. The cell proliferation assessed by manual cell counting showed that growth rate of these cells seeded onto cultured plates coated with T-CAM and other proteins were higher than the growth rate of the cells seeded onto culture plates coated with BSA (Figure 4). However, growth promoting activities for T-CAM, FN, FN115, βigh3-WT and D-IV were more or less similar. Previous studies have also demonstrated growth promoting activities of pFN, βig-h3 and its FAS1 domain (Bae *et al.,* 2002).

T-CAM enhances fibroblast and HaCaT cells migration

Migration promoting activity of T-CAM was desirable as migration of keratinocytes and fibroblasts from the vicinity of wound is crucially required during wound healing. To test whether T-CAM could mediate fibroblast and HaCaT cells migration, we employed transwell plate migration assay. Cells were seeded onto transwells coated with T-CAM or other proteins. The cells started to migrate through the T-CAM-coated filter within 6 h of cell seeding. The microscopic pictures of migrating cells are shown in Figure 5A and B. The quantitaion of migrating cells showed profoundly greater migration-promoting activity of T-CAM compared with other proteins used for both types of cells (Figure 5C and D).

T-CAM accelerates wound closure

After testing for cell adhesion, proliferation and migration of cells to T-CAM, *in vivo* test was conducted to test its effect on cutaneous wound healing in rabbit. The paired full-thickness skin wounds in the back skin of the rabbit were covered with cream-containing T-CAM or other proteins. As shown in Figure 6, T-CAM-treated wounds showed significantly accelerated repair when compared with βigh3-WT, βigh3 D-IV or FN115 treated wounds. T-CAM treated wounds showed 93% reduction in wound size by day 12 whereas βigh3 D-IV and FN115-treated wounds had 87% and 82% reduction in wound size by day 12, respectively.

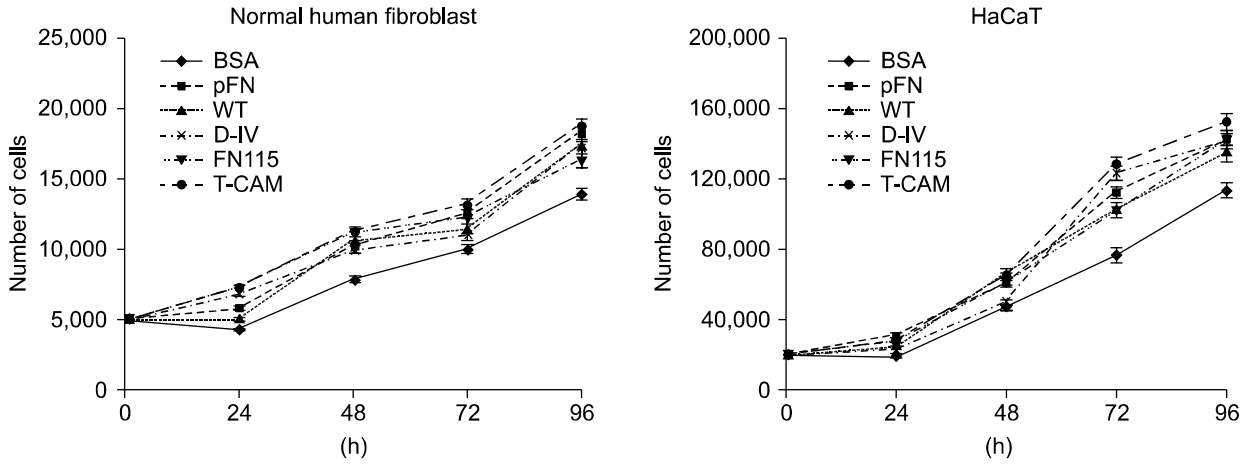

Figure 4. T-CAM supports proliferation of normal human fibroblast and HaCaT cells. The 24-well cultured plates were coated with either of proteins: BSA, pFN, βigh3-WT, FN115 or T-CAM. The normal human fibroblasts (5×10^3 cells) and HaCaT cells (1×10^4 cells) were seeded in serum-containing medium and cell numbers were counted at 24, 48, 72 and 96 h after the cell seeding by manual cell counting methods. The results were derived from three independent experiments, with duplicated in each experiment.

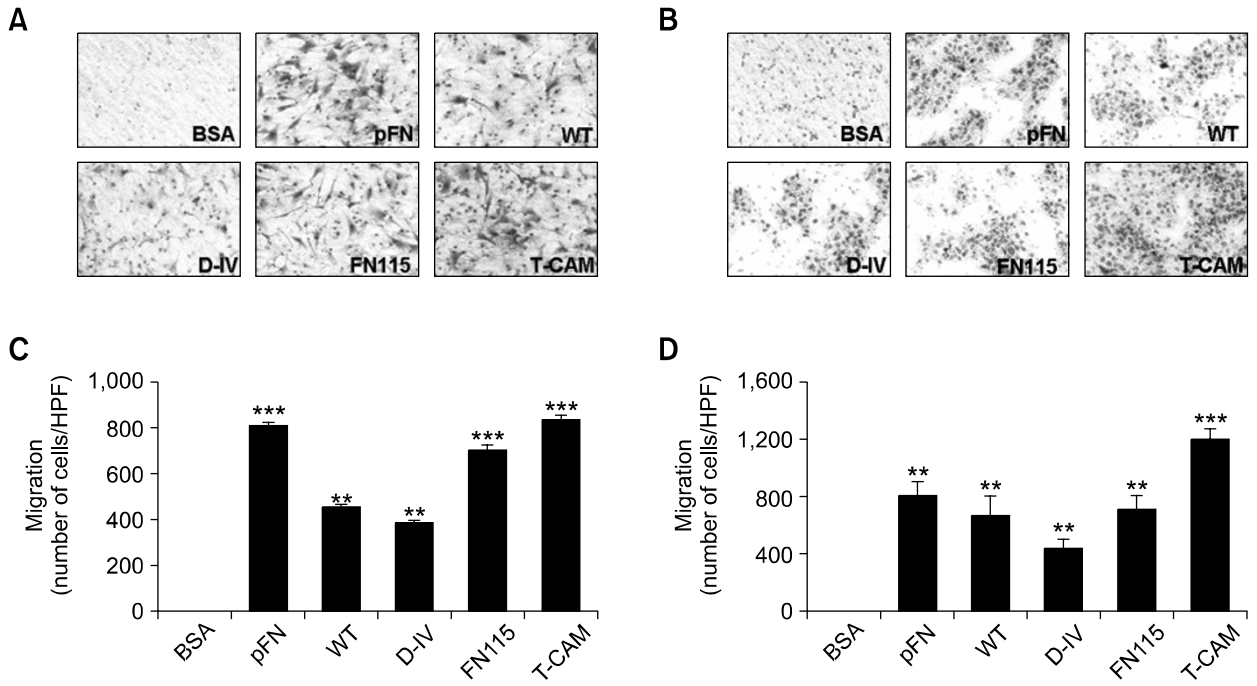

Figure 5. Migration-promoting effect of T-CAM. The transwell membranes were pre-coated with various proteins as described above. Fibroblast and keratinocyte cells were seeded and allowed to migrate for 2 h and 24 h through transwell plates and migrating cells were quantified as described in "Materials and Methods". Data represent mean ± SD of three separate experiments. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$

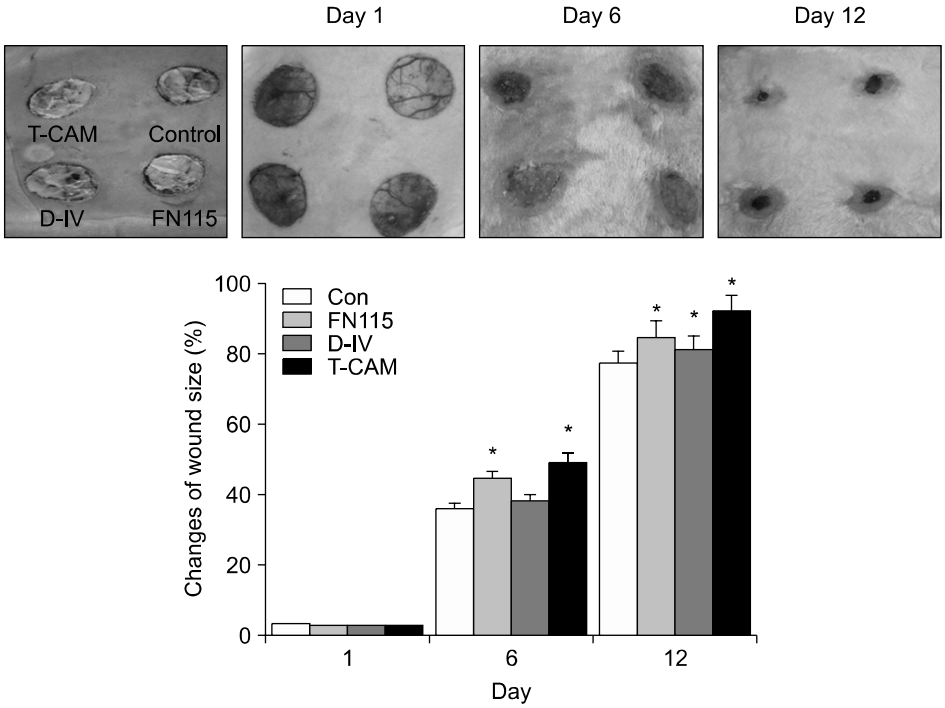

Figure 6. T-CAM promotes the wound healing. Wound was made on dorsal area of a rabbit. T-CAM, βigh3 D-IV and FN115 were applied to the wounds at final concentration of 500 μg/ml. Wound closure was measured on days 1, 6, and 12 after wounding. *$P < 0.05$ was considered to be statistically significant.

Discussion

The specific purpose of this study was to examine the beneficial aspect of topical application of protein endowed with multiple integrin-binding sites in cutaneous wound healing process. For this purpose, T-CAM was designed by combining the 9th and 10th type III domain of FN and 4th FAS1 domain of βig-h3. This new fusion protein, T-CAM harbors, in total, four well known-integrin binding sites: PHSRN in 9th and RGD in 10th type III fibronectin domain, and EPDIM and YH in 4th FAS1 domain of βig-h3. The PHSRN and RGD motifs in FN are known to act synergistically in mediating cell adhesion (Grant *et al.,* 1997). The EPDIM in 4th FAS1 domain is known to mediate α3β1 integrin-mediated adhesion of human corneal epithelial cells and keratinocytes to βig-h3 (Kim *et al.,* 2000; Bae *et al.,* 2002). The YH motif which is present in all four FAS1 domains of βig-h3 is active in mediating adhesion of fibroblasts (Kim *et al.,* 2002). We hypothesized that inclusion of these integrin-binding sites will enable T-CAM to interact with diverse repertoire of integrins, specially, those expressed in migrating and transmigrating cells at wound healing sites. Migrating keratinocytes/ fibroblasts at the vicinity of wounds are known to show enhanced expression of already existing or new integrins that will enable them to interact with various ECM proteins present at wound sites (Santoro and Gaudino, 2005). The reason for selecting integrin-binding domains of FN is supported by the fact that FN is a major constituent of provisional matrix proteins at wound healing sites and its role in wound healing is supported by a numbers of studies (Lariviere *et al.,* 2003). Although, βig-h3 is reported to be expressed in healing corneal wound (Rawe *et al.,* 1997) and reactive astrocytes at stab wound sites in rat cerebral cortex, its precise role in wound healing process is yet to be known.

As a new fusion protein from two well-known ECM proteins, FN and βig-h3, the ability of T-CAM to support the adhesion, migration and proliferation of cells was examined. The T-CAM showed remarkable cell adhesion activity supporting the adhesion of different cell types including keratinocytes and fibroblasts. As expected, cell adhesion activity was higher than that of pFN, FN115, βigh3-WT and βigh3 D-IV. The ability of T-CAM to support the adhesion of different cell types was conceivable because T-CAM constitute four well-known integrin binding sites that can interact with diverse family of integrins. However, we did not examine functional contribution of specific integrin mediating the adhesion of neither of these cells to T-CAM. We do not know whether T-CAM retains the integrin-specificity of its parent molecules and if there is change in integrin specificity and affinity because of fusion of two distinct domains.

Keratinocytes migration and epithelization over provisional matrix composed of fibrin and FN is an essential process of wound healing (Guo *et al.,* 1990; Zhang and Kramer, 1996; Putnins *et al.,* 1999; Yamada, 2000). We tested migration-promoting activity of T-CAM for keratinocytes and fibroblasts because migration of these cells is required for wound healing. The T-CAM support migration of fibroblast and HaCaT cells and quantitation of migrating cells to T-CAM was clearly higher than to pFN, FN115, βigh3 and D-IV.

In the context of wound healing, the synergy between integrin, ECM and growth factors is one of the key factors in the regulation of tissue regeneration. The ligation of ECM by integrins is known to regulate/amplify the intracellular signaling of growth factors that are intimately associated with wound healing processes. As a cell adhesion substrate, T-CAM support proliferation of both keratinocytes and fibroblasts in a serum-free condition. The mitogenic and proliferative activity is generally expected for many ECM proteins.

In vivo study was performed to test the wound healing effect of the T-CAM in a full-thickness cutaneous wound model in rabbit. This represents the most common and simplest model of wound repair mimicking that of human (Greenhalgh, 2005). In previous studies, the topical application of FN and short peptide PHSRN derived from FN has been reported to promote the wound healing in the genetically obese diabetes model (Livant *et al.,* 2002; Qiu *et al.,* 2007). The various peptides derived from ECM proteins such as collagen type IV and laminin and with increased adhesion, motility and chemotaxis effects are sought to develop into wound healing topical formulations (Rosso *et al.,* 2005). We found more effective wound healing in T-CAM-treated groups than FN115 and βigh3 D-IV-treated groups. It is likely that adhesion, migration and proliferation-promoting effect of T-CAM might be the underlying molecular clues that account for the wound healing effect of T-CAM.

In conclusion, T-CAM may represent a novel fusion protein from two prevalent and distinct ECM proteins, FN and βig-h3, that could be developed into wound healing drug.

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