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Dermatopontin Promotes Epidermal Keratinocyte Adhesion via α3β1 Integrin and a Proteoglycan Receptor†

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ABSTRACT: Dermatopontin, an extracellular matrix component initially purified from bovine dermis, promoted cell adhesion of the human epidermal keratinocyte cell line (HaCaT cells). HaCaT cells spread on dermatopontin and formed actin fibers. Adhesion of HaCaT cells to dermatopontin was inhibited by both EDTA and heparin and was mediated in part by α3β1 integrin. A synthetic peptide (DP-4, PHGQVVVAVRS; bovine dermatopontin residues 33−43) specifically inhibited adhesion of cells to dermatopontin, and when the DP-4 peptide was coated on the well, it promoted cell adhesion in a dose-dependent manner. An active core sequence of the DP-4 peptide was localized to an eight-amino acid sequence (GQVVVAVR). These results indicate that dermatopontin is a novel epidermal cell adhesion molecule and suggest that the DP-4 sequence is critical for the cell adhesive activity of dermatopontin. Adhesion of cells to DP-4 was strongly inhibited by heparin. When HaCaT cells were treated with heparitinase I, the cells failed to adhere to DP-4 but chondroitinase ABC treatment did not influence the adhesion activity. DP-4 specifically interacted with biotinylated heparin, and this interaction was inhibited by unlabeled heparin. DP-4 peptide significantly promoted the adhesion of cells overexpressing syndecans, and syndecan bound to a DP-4 peptide affinity column. These results suggest that HaCaT cells adhere to dermatopontin through α3β1 integrin and a heparan sulfate proteoglycan-type receptor, which is likely a syndecan. We conclude that dermatopontin plays a role as a multifunctional adhesion molecule for epidermal cells.

Dermatopontin is a small 22 kDa extracellular matrix (ECM)[1] protein (*1*), which comprises ∼0.001% of the wet weight of the dermis (*2*, *3*). It was initially isolated from a bovine dermal extract during the purification of decorin (*1*). So far, more than 10 dermatopontin homologues have been identified in five different mammalian species (*1*, *4*−*7*) and in 12 different invertebrates (*8*−*14*). Mammalian dermatopontins are composed of 183 amino acids, containing five disulfide loop structures (*1*). Further, the molecule is tyrosine-rich, especially in the amino-terminal region (*1*, *5*).

Dermatopontin appears to have multiple and diverse functions. It accelerates collagen fibrillogenesis, makes the diameters of newly formed collagen microfibrils smaller (*15*), and forms a supramolecular complex with both decorin and TGF-β1 (*2*, *16*). Overexpression of dermatopontin in V16 cells reduced the extent of cell proliferation, and dermatopontin is expressed in quiescent cells (*6*). Another report indicated that dermatopontin enhanced human prostate cancer cell growth and that transgenic mice developed prostatic neoplasia (*17*). These findings suggest that dermatopontin regulates the cell cycle in a cell-type specific manner.

Dermatopontin promotes fibroblast and neurogenic cell adhesion (*18*). The adhesion requires high concentrations of dermatopontin, and the adhesion is inhibited by decorin, biglycan, or RGD-containing peptides (*18*). However, cell surface receptors for dermatopontin have not been identified. In invertebrates, dermatopontin from amoebocytes in a *Limulus polyphemus* crab shell induces amebocyte aggregation (*8*). Similarly, dermatopontin from demosponge *Suberites domuncula* was shown to have aggregation activity of the demosponge cells at low concentrations (*9*). These findings from invertebrates suggest that dermatopontin is an adhesive molecule for diverse cells.

Several reports indicate a change in the expression level of this protein in progressive systemic sclerosis (*19*), keloids, leiomyoma (*20*, *21*), and myocardial infarction (*7*), in which a rearrangement of collagen fibers is observed (*22*). Since the expression level of dermatopontin is increased in the myocardial infarct zone (*7*) and dermatopontin promotes fibroblast adhesion, we hypothesized that dermatopontin plays a role in wound healing. The process of wound healing is complex, and several interconnected steps occur, such as ECM deposition, cell proliferation, and cell migration.

Wound healing requires many different cell types, growth factors, and ECM proteins (*23*−*25*). Interaction between ECM proteins and cell surface receptors, such as integrins, syndecans, and growth factor receptors, is important for the healing

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[1]Abbreviations: ECM, extracellular matrix; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PVDF, polyvinylidene fluoride; SD, standard deviation.

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process (*26*−*28*). These receptors are ubiquitously expressed in adult tissues, suggesting that ECM components, such as collagen, laminin, and proteoglycans, bind to various types of cells through the receptors.

Although dermatopontin is mainly expressed in the dermis, its biological activity may not be limited to the dermis since dermal components can regulate the behavior of epidermal cells during skin repair (*29*−*31*). Here, we examined the biological role of dermatopontin with keratinocytes. We found that dermatopontin promoted keratinocyte adhesion, and active sequences in dermatopontin for keratinocyte adhesion were identified using synthetic peptides. We also characterized the cellular receptors for dermatopontin. Together with its role in the dermis, these new findings suggest that dermatopontin plays a role in skin repair.

MATERIALS AND METHODS

Materials. Dermatopontin was purified from newborn calf dermis as reported previously (*2*). Biotin-conjugated heparin (molecular mass of approximately 15 kDa) was purchased from Sigma (St. Louis, MO). Heparitinase I and chondroitinase ABC were purchased from Seikagaku Kogyo (Tokyo, Japan). Porcine intestinal heparin and fluorescamine were obtained from Wako (Osaka, Japan). Functional blocking anti-integrin subunit antibodies (FB12 for α1, P1E6 for α2, ASC-1 for α3, P1D6 for α5, NKI-GoH3 for α6, and 6S6 for β1) were obtained from Chemicon (Temecula, CA). Antibodies used for Western blotting (I-19 for α3, H-104 for α5, H-87 for α6, and N-20 for β1) and anti-syndecan-1 antibody (DL-101) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). An affinity support, Affi-gel 10, was obtained from Bio-Rad (Hercules, CA). CNBr-activated Sepharose 4B and enzyme chemiluminescent (ECL) reagent were obtained from GE Healthcare (Buckinghamshire, U.K.). Horseradish peroxidase (HRP)-conjugated streptavidin was obtained from Pierce (Rockford, IL). ARH-77 human lymphoid cell lines transfected with syndecan constructs were provided by Y. Yamada from the National Institutes of Health (Bethesda, MD).

Cell Culture. The human epidermal keratinocyte cell line (HaCaT cells) was cultured as a monolayer in 75 cm^2 bottles at 37 °C in a humidified 5% CO_2/95% air atmosphere. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum (FCS). Two cell lines were used, namely, ARH-77- Synd-1 and ARH-77- Cont which express recombinant syndecan-1 and an empty vector, respectively (*32*, *33*). These cells are derivatives of an ARH-77 human lymphoid cell line that expresses small amounts of heparan sulfate proteoglycan (HSPG) on the cell surface but does not express syndecan (*34*, *35*). These cell lines were maintained in a suspension culture in RPMI 1640 medium supplemented with 5% FCS and antibiotics (penicillin at 100 U/mL and streptomycin at 100 μg/mL).

Cell Adhesion Assay. Dermatopontin and synthetic peptides were coated on a 96-well plate in 0.14 M NaCl and 30 mM phosphate buffer (pH 7.3) overnight at room temperature. After immobilization, the wells were rinsed once with PBS and then blocked with 1% BSA in PBS for 1 h, followed by three washes with PBS. A monolayer of cultured cells was detached with 5 mM EDTA in PBS, and the cells were suspended in DMEM. The ARH-77 cell lines were collected by centrifugation and then suspended in RPMI 1640. The cells were inoculated in the wells at a density of 30000 cells/well and were incubated at 37 °C in an incubator. After incubation, the wells were rinsed once with warm PBS, and the attached cells were fixed with 1% glutaraldehyde for 30 min and then stained with 0.1% crystal violet for 1 h. After staining had been completed, the wells were rinsed with running water and dried. Finally, the dye was eluted with 0.1% Triton X-100 for 30 min, and the absorbance at 595 nm was measured with an ELX808 UV spectrometer (BioTek, Winooski, VE). In inhibition experiments, dermatopontin was coated at a concentration of 5 μg/mL and EDTA, heparin, antibodies, and peptides were included during the cell adhesion assay.

For the enzyme treatment of the cells, HaCaT cells were first treated with cycloheximide (CHx) at 2 μg/mL at 37 °C for 2 h, and then the cells were detached with EDTA. The same concentration of CHx was included in all the following steps. The cell suspension was treated with either 10 mIU/mL heparitinase I or 50 mU/mL chondroitinase ABC at 37 °C for 30 min. For control, PBS was added to the cell suspension. After the enzyme treatment, cell adhesion was assessed for 1 h.

Affinity Chromatography and Western Blotting. HaCaT cells were extracted in 50 mM Tris-HCl and 0.15 M NaCl (pH 7.40) containing 100 mM *n*-octyl β-D-glucoside (n-OG), 1 mM $CaCl_2$, and $MgCl_2$ for 1 h on ice. Dermatopontin was immobilized on Affi-gel 10 in 0.1 M MOPS buffer containing 80 mM $CaCl_2$ and 6 M urea (pH 7.5) at a protein (μg):bead slurry (μL) ratio of 1:1, according to the manufacturer's instructions. BSA was immobilized in 0.1 M MOPS buffer in the same manner. The beads were added to the cell extract and incubated at 4 °C for 2 h. The beads were placed in a column, and they were rinsed with buffer A [50 mM Tris-HCl (pH 7.40) containing 25 mM n-OG, 1 mM $CaCl_2$, and 1 mM $MgCl_2$]. The column was eluted with buffer A containing 10 mM EDTA. Aliquots of each fraction from the affinity columns were subjected to 7.5% SDS−PAGE under reducing conditions and blotted onto a PVDF membrane. Integrins were identified with anti-integrin antibodies, followed by the HRP-conjugated anti-mouse IgG antibody. Images were visualized using an ECL reagent. Under nonreducing conditions, the integrin α3 subunit was detected at a position near 150 kDa both in the cell extract and in the gel-bound fraction. Little reactivity with antibodies against α5 and β1 subunits was found under the nonreducing conditions. Therefore, the detection of integrin subunits was conducted under reducing conditions. For peptide affinity chromatography, DP-4 peptide with additional glycine at the N-terminus (GPHGQVVVAVRS) was immobilized on CNBr-activated Sepharose 4B. The n-OG extract of HaCaT cells was added and the mixture was incubated overnight at 4 °C. Then, the beads were treated with heparitinase I (0.1 mIU/mL) and with chondroitinase ABC (0.5 mU/mL) for 18 h at room temperature in 40 μL of buffer containing 40 mM Tris-HCl (pH 8.0), 40 mM NaOAc, 5 mM $Ca(OAc)_2$, 0.01% BSA, and complete protease inhibitor cocktail, and the supernatant fraction of each sample was collected. Eluates were subjected to 7.5% SDS−PAGE under reducing conditions, and syndecan was detected as described above by Western blotting.

Cytoskeletal Staining. Dermatopontin (10 μg/mL) was coated on a silan-coated glass plate (Dako Japan, Kyoto, Japan), and cell adhesion for 1 h was performed as described above. The adherent cells were fixed with 70% methanol for 10 min and then blocked with 10% skim milk in PBS for 30 min. Actin filaments were stained with fluorescein isothiocyanate-conjugated phalloidin (Sigma-Aldrich, St. Louis, MO), with 3 × 5 min intervals of rinsing with PBS. In some experiments, synthetic peptides were

coated on the dish instead of dermatopontin and the dishes were processed identically as described above.

Synthetic Peptides of Dermatopontin. Peptides were generally were 12 amino acid residues in length and overlapped with neighboring peptides by four amino acids as described previously (*36*). If the N-terminal amino acid was either glutamine or glutamic acid, one amino acid was extended to avoid pyroglutamine formation. Cysteine residues were omitted to avoid the influence of disulfide bonds. Peptides were synthesized manually by the Fmoc-based solid-phase method with a C-terminal amide form (*36*). *N*,*N*-Dimethylformamide (DMF) was used as a solvent. Diisopropylcarbodiimide/*N*-hydroxybenzotriazole and 20% piperidine in DMF were used for condensation and deprotection of Fmoc groups, respectively. Respective amino acids were condensed using 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy or NovaSyn TGR resin in a stepwise fashion. Side chains of aspartic acid, glutamic acid, serine, threonine, and tyrosine were protected by *tert*-butyl. Asparagine, glutamine, and histidine were protected by trityl. Lysine was protected by *tert*-butoxycarbonyl. Arginine was protected by 2,2,5,7,8-pentamethyldihydrobenzofuran-5-sulfonyl. The protected peptide resins were cleaved from the resin and deprotected via incubation in a trifluoroacetic acid/thioanisole/*m*-cresol/ethanedithiol/H_2O mixture (80:5:5:5:5) for 3 h at room temperature. Purification was performed using HPLC on a Mightysil RP-18 (5 mm × 250 mm, Kanto Chemical, Tokyo, Japan) column with a water/acetonitrile gradient containing 0.1% trifluoroacetic acid. The purity and identity of the peptides were confirmed with an analytical HPLC and an electrospray ionization mass spectrometer at the Central Analysis Center, Tokyo University of Pharmacy and Life Sciences.

Interaction between the Peptides and Heparin. The interaction between the peptides and heparin was analyzed as previously reported (*37*). Synthetic peptides were coated on the wells at 10 μg/mL and blocked by BSA as described in the section on cell adhesion. Biotinylated heparin was dissolved in PBS containing 0.1% BSA at 10 μg/mL and added to the wells, and the wells were incubated at room temperature for 1 h. The wells were rinsed with PBS containing 0.1% BSA three times, and then the bound biotinylated heparin was detected by HRP-conjugated streptavidin according to the manufacturer's instructions. Finally, the color was allowed to develop via addition of 0.057 M citric acid and 0.086 M disodium hydrogen phosphate containing 10 mM 2,2′-azidobis(3-ethylbenzothiazoline-6-sulfonic acid) and 0.03% hydrogen peroxide in the dark. The color reaction was terminated by adding aliquots of 0.1 M citric acid containing 1.5 mM sodium azide, after which the absorbance at 405 nm was determined. For inhibition experiments, heparin was included during the assay.

Calculation of Immobilization Efficiency. After immobilization of peptides on the plate and rinsing as described above, 150 μL of PBS containing 3% Triton X-100 was added to each well, and the wells were incubated for 15 min. Then aliquots of the same buffer containing 20 μL of fluorescamine in acetone (1 mg/mL) were added. Fluorescence emission at 465 nm was determined using a Spectra Fluor Plus microplate fluorometer (Tecan, Salzburg, Austria) with excitation at 405 nm. Coating efficiencies (percent) were determined relative to corresponding peptide standards made in PBS containing 3% Triton X-100.

Statistical Analysis. Statistical analysis was conducted using the Student's *t* test, and a *P* value of < 0.05 was considered to be statistically significant.

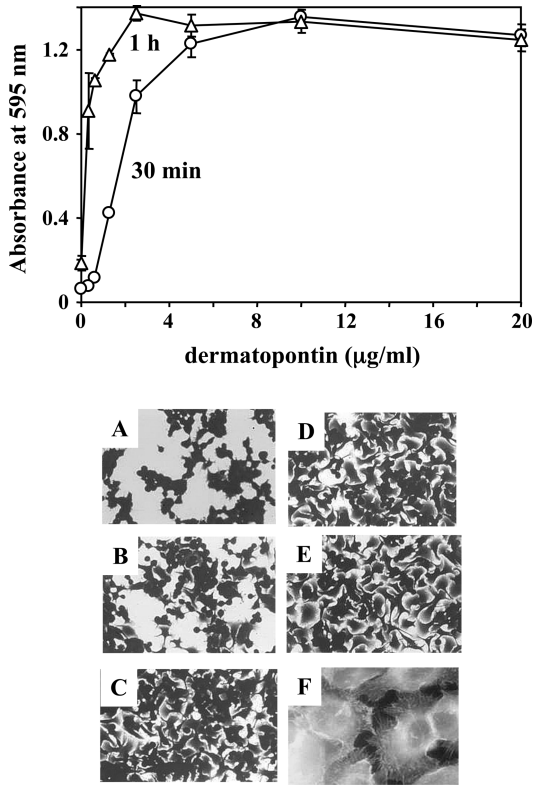

FIGURE 1: Adhesion of HaCaT cells to dermatopontin. In the top panel, dermatopontin was coated with various concentrations and HaCaT cells were incubated for 30 min (○) or 1 h (△). Data are expressed as means ± SD of triplicate results. (A−E) Stains of adhered HaCaT cells after a 1 h incubation on dermatopontin. Dermatopontin concentrations were (A) 0.625, (B) 1.25, (C) 2.5, (D) 5, and (E) 10 μg/mL. The magnification was 200×. (F) Staining of phalloidin. The magnification was 400×. Experiments were performed three times and gave similar results.

RESULTS

Dermatopontin Is a Potent Adhesion Molecule for HaCaT Cells. Cell adhesive activity of dermatopontin was examined using the human epidermal keratinocyte cell line (HaCaT). Dermatopontin induced strong HaCaT cell adhesion in a dose-dependent manner (Figure 1, top panel). At 30 min, an adhesion plateau was observed at 5 μg/mL dermatopontin, and at 1 h, an adhesion plateau was reached at 2.5 μg/mL. A large fraction of the adhered HaCaT cells showed spreading at 1 h on coated dermatopontin at concentrations of > 2.5 μg/mL (Figure 1C−E). On the basis of the results, cell adhesion was conducted with a 1 h incubation in the following experiments unless otherwise mentioned. Upon adhesion to dermatopontin, actin filaments were organized (Figure 1F), but the level of organization was lower than the levels of those formed in the cells which adhered to type I collagen (data not shown). Fibroblasts also adhered to dermatopontin in a dose-dependent manner; however, > 20 μg/mL dermatopontin was needed to reach a plateau, and the final level of adhesion (0.35) was lower than that of HaCaT cells (1.4) (data not shown). These results suggested that dermatopontin promotes cell adhesion in a cell-type specific manner.

Next, we examined the effect of EDTA and heparin on adhesion of cells to dermatopontin (Figure 2). Adhesion of HaCaT cells to dermatopontin was completely inhibited by EDTA at concentrations of > 2 mM (Figure 2A). In contrast, adhesion was partially inhibited by heparin (Figure 2B). These results suggest that the adhesion of HaCaT cells to

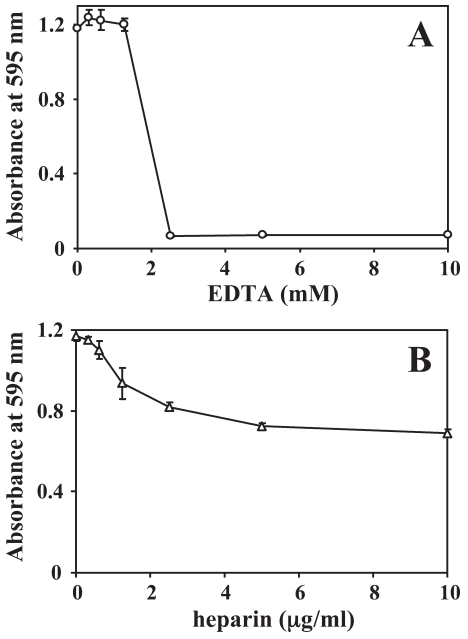

FIGURE 2: Effect of EDTA and heparin on adhesion of cells to dermatopontin. EDTA (A) and heparin (B) solutions in DMEM were applied to the dermatopontin-coated wells and were incubated for 5 min at room temperature. HaCaT cells were added to the wells and incubated for 1 h. The horizontal lines represent the final concentrations of EDTA (A) and heparin (B). Data are expressed as mean ± SD of triplicate results. Experiments were performed three times and gave similar results.

dermatopontin is cation- and heparin-dependent, and that dermatopontin interacts with multiple receptors.

Dermatopontin Binds to α3β1 Integrin. Since the inhibition of cell adhesion by EDTA may indicate the involvement of integrins as a receptor, a panel of functional blocking antibodies against integrin subunits was tested for blocking adhesion of cells to coated dermatopontin. Both anti-integrin α3 and β1 subunit antibodies strongly inhibited adhesion of cells to dermatopontin, whereas other anti-integrin subunit antibodies were inactive (Figure 3A). We also confirmed the receptor interaction using affinity chromatography. Chromatography of a cell extract using a dermatopontin-conjugated gel revealed that both α3 and β1 integrin subunits were eluted by 10 mM EDTA (Figure 3B), while trace amounts of these subunits were seen in the eluted fractions from a BSA-conjugated gel (Figure 3B, lane 5). In addition, the α5 integrin subunit, which is also expressed on HaCaT cells, did not bind to the dermatopontin-conjugated gel (Figure 3B). Taken together, these results suggest that binding of α3β1 integrin is involved in adhesion of HaCat cells to dermatopontin.

Peptide Screening of Cell Binding Sequences in Dermatopontin. To identify the cell-binding sites on dermatopontin, 16 synthetic peptides covering most of dermatopontin sequence were generated (Table 1). The 16 peptides were screened for their ability to block adhesion of cells to dermatopontin. As shown in Figure 4A, the DP-4 peptide significantly inhibited adhesion of cells to dermatopontin to the background level, whereas the rest of the 15 peptides were inactive (Figure 4A). Next, these synthetic peptides were coated on wells, and their cell adhesion activity was examined (Figure 4B). DP-4 peptide induced strong cell adhesion. We also found that DP-8 and DP-13 peptides had cell adhesion activity, but these activities were lower than that of the DP-4 peptide (Figure 4B). The remainder of the peptides were

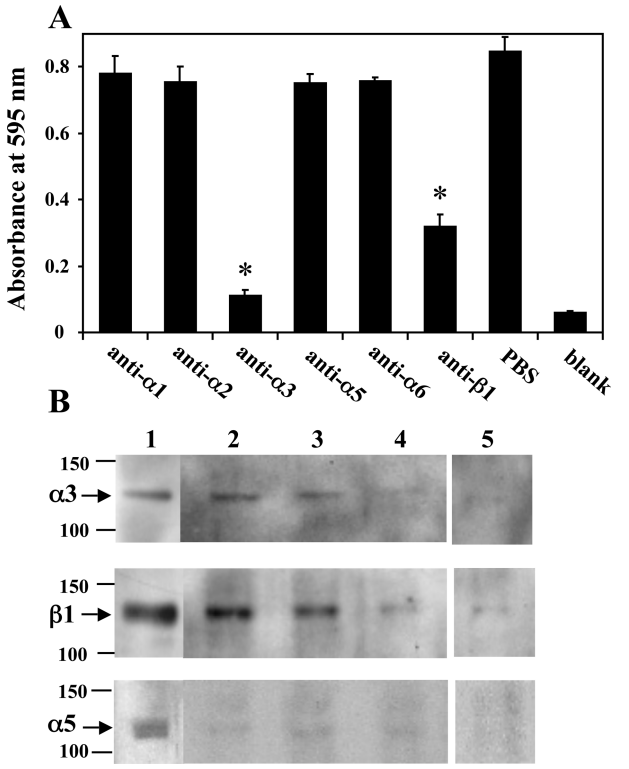

FIGURE 3: Identification of an integrin-type cell surface receptor for dermatopontin. (A) Inhibition of adhesion of cells to dermatopontin by anti-integrin functional blocking antibodies. Six antibodies against different integrin subunits were mixed with HaCaT cells in suspension, and the cell adhesion assay was performed. PBS was added to the cell suspension as a control. Antibody concentrations were 10 μg/mL for anti-α3 and 50 μg/mL for other antibodies. The incubation time was 30 min. Asterisks indicate $p < 0.01$. Data are expressed as means ± SD of triplicate results. Experiments were performed three times and gave similar results. (B) Dermatopontin affinity chromatography. The n-OG extract of HaCaT cells was passed over a dermatopontin column, and the eluted fractions were probed with anti-integrin α3 (top panel), β1 (middle panel), and α5 (bottom panel) subunit antibodies. Lane 1 contained the whole cell extract, and lanes 2−4 contained the antibody reaction mixtures in the second to fourth fractions, respectively, eluted from the dermatopontin column by 10 mM EDTA. Lane 5 contained the fraction eluted from the BSA column. Molecular masses (kilodaltons) are indicated at the left of each panel.

inactive in this assay. These results suggested that the DP-4 sequence is involved in adhesion of cells to dermatopontin.

Characterization of Adhesion of HaCaT Cells to the DP-4 Peptide. Next, we characterized adhesion of cells to the DP-4 peptide. A scrambled peptide DP-4S (AVRVSQVPHGV) was used as a control. The DP-4 peptide exhibited a dose-dependent cell adhesion with a plateau at 3−5 μg/mL (Figure 5, top panel), while DP-4S did not exhibit any activity. The scrambled peptide also did not inhibit adhesion of cells to dermatopontin (data not shown). These data suggest that the activity of DP-4 is sequence specific. As observed with dermatopontin, some of the cells that adhered to DP-4 at concentrations of > 3 μg/mL were spread, but the cells remained round when attached to the DP-4 peptide at lower concentrations (Figure 5A−E). The attached and spread cells had fewer actin fibrils as shown in Figure 5F, compared to those that attached to dermatopontin.

Determination of the Essential Amino Acid Sequence in the DP-4 Peptide Required for Cell Adhesion. To determine the minimum active sequence of the DP-4 peptide needed to induce cell adhesion, deletion peptides of DP-4, named

Table 1: Synthetic Dermatopontin Peptides[a]

peptide	sequences	location
DP-1	QYGDYGYSYHQY	1-12
DP-2	YHQYHDYSDDGWV	9-21
DP-3	DGWVNLNRQGFSYQ	18-31
DP-4	PHGQVVVAVRS	33-43
DP-5	AVRSIFNKKEGS	40-51
DP-6	KEGSDRQWNYA	48-58
DP-7	MPTPQSLGEPTE	60-71
DP-8	WWEEINRAGMEWYQT	73-87
DP-9	SNNGLVAGFQSRYFE	89-103
DP-10	RYFESVLDREWQFY	100-113
DP-11	WLTTEYPGHYGEE	126-138
DP-12	YGEEMDMISYNYD	135-147
DP-13	YNYDYYMRGATT	144-155
DP-14	GATTTFSAVERD	152-163
DP-15	VERDRQWKFIM	160-170
DP-16	RMTDYD	172-177

[a]Peptide sequences are expressed in the one-letter code, and the locations of the peptides are expressed as the numbers of amino acids within the bovine dermatopontin sequence. Cysteine residues have been omitted, and short peptides interrupted with cysteines were not synthesized (from amino acid 114 to 125).

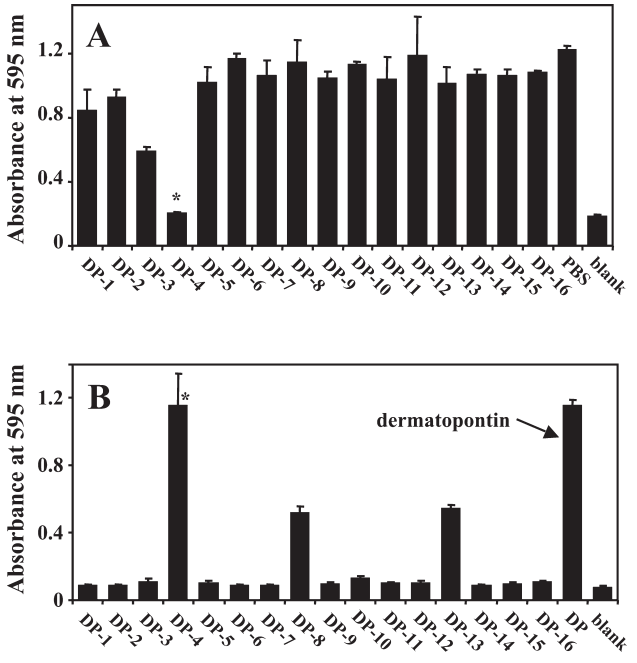

FIGURE 4: Determination of the cell adhesion site on dermatopontin. (A) Inhibitory activity of synthetic peptides for adhesion of cells to dermatopontin. HaCaT cells were incubated with the synthetic peptides (200 μg/mL) listed in Table 1 on a dermatopontin-coated plate, and adhesion was measured. (B) Cell adhesion activity of synthetic peptides. Synthetic peptides were coated at 4 μg/mL, and cell adhesion was measured. The coating efficiencies of the peptides were 6−10% (DP-1−5, DP-10, DP-11, DP-15,and DP-16), 11−15% (DP-6, DP-7, DP-9,and DP-13), and 18% (DP-12). Because DP-4 was poorly reactive with fluorescamine, the calculation was done using DP-4a as shown in Figure 6. The coating efficiencies of DP-8 and DP-14 were not available because of a poor reactivity with fluorescamine. In panels A and B, the incubation time was 30 min, and asterisks indicate $p < 0.01$. Data are expressed as means ± SD of triplicate results. Experiments were performed three times and gave similar results.

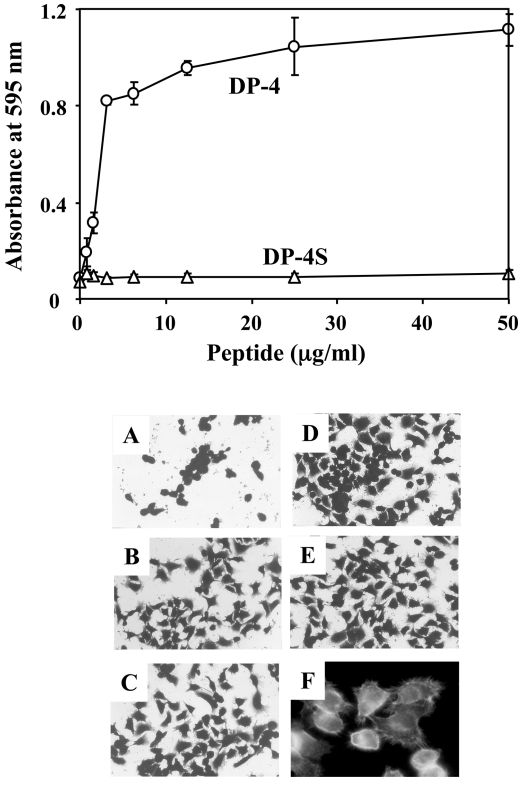

FIGURE 5: Adhesion of cells to the DP-4 peptide. In the top panel, the DP-4 peptide was coated as indicated on the horizontal line, and cell adhesion was performed. For a control, scrambled peptide DP-4S was coated. Experiments were performed three times and gave similar results. (A−E) Stains of adhered cells. DP-4 concentrations were (A) 1.56, (B) 3.13, (C) 6.25, (D) 12.5, and (E) 25 μg/mL. The magnification was 200×. (F) Staining of phalloidin. The magnification was 400×.

DP-4a−4i, were produced as indicated in Figure 6. The inhibitory ability of the deletion peptides with respect to adhesion of cells to dermatopontin was examined (Figure 6, left panel). DP-4a, which lacks one amino-terminal amino acid, retained the inhibitory activity. DP-4b, which lacks two amino acids, exhibited a reduced inhibitory effect. DP-4c, which lacks four amino acids, was inactive. DP-4e, which lacks one carboxyl-terminal amino acid, retained its inhibitory ability, whereas DP-4f, which lacks two amino acids, was inactive. The inhibition experiments using deletion peptides revealed that the GQVVVAVR sequence is critical for cell adhesion activity.

We also determined direct cell attachment activity using the deletion peptides. Only three peptides, DP-4a, DP-4b, and DP-4e, were active for cell adhesion when coated directly on the wells (Figure 6, right panel). These data show that peptides active in blocking adhesion of cells to dermatopontin are also active for direct cell adhesion. Thus, the minimum amino acid sequence in the cell binding domain of dermatopontin was determined to be GQVVVAVR, which corresponds to amino acids 35−42 of bovine dermatopontin.

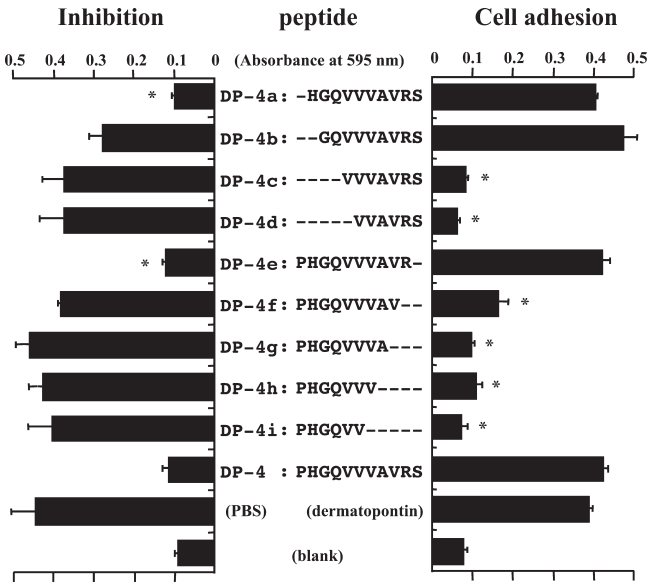

FIGURE 6: Determination of the minimal active sequence of dermatopontin needed for cell adhesion. Inhibition of adhesion of cells to dermatopontin by deletion peptides of DP-4 (left). Deletion peptides of DP-4 shown in the center were mixed with suspended HaCaT cells at a concentration of 200 μg/mL, and the mixtures were incubated for 30 min on a dermatopontin-coated plate. The DP-4 peptide and PBS were used as positive and negative controls, respectively. Cell adhesion activities of deletion peptides (right). Deletion peptides were coated at a concentration of 4 μg/mL, and cells were incubated for 1 h. Asterisks indicate $p < 0.01$. Data are expressed as means ± SD of triplicate results. Experiments were performed twice and gave similar results.

Effect of EDTA and Heparin on Adhesion of Cells to DP-4. Adhesion of cells to DP-4 was inhibited by EDTA (Figure 7A), but the inhibition was not as strong as that with dermatopontin. In contrast, cell adhesion was strongly inhibited by heparin (Figure 7B), suggesting that adhesion of cells to DP-4 is largely heparin-dependent. Treatment of HaCaT cells with heparitinase I completely abolished adhesion to DP-4, while chondroitinase ABC treatment did not have any effect on adhesion (Figure 7C). In addition, adhesion of HaCaT cells to DP-4 was not inhibited by anti-integrin α1, α2, α3, α5, α6, or β1 subunit functional blocking antibodies (data not shown). These results strongly suggest that the receptor for DP-4 is a cell surface HSPG.

The DP-4 Peptide Interacts with Heparin and Syndecans. The binding of DP-4 with heparin was examined using biotinylated heparin. Biotinylated heparin bound to the DP-4-coated wells, while the scrambled peptides, DP-4S, did not interact (Figure 8A). Similarly, the DP-5 peptide that partially overlaps with DP-4 did not interact with heparin (Figure 8A). The interaction between the DP-4 peptide and biotinylated heparin was blocked by unlabeled heparin in a dose-dependent manner (Figure 8B). These data demonstrate that the dermatopontin-derived active peptide sequence binds to heparin, suggesting that a cell surface HSPG is the cellular receptor.

Syndecans are the most common receptor among cell surface HSPGs. Adhesion of syndecan-1-overexpressing ARH-77 cells to DP-4 was compared with that of cells transfected with an empty vector, because ARH-77 human lymphoid B cells express a low level of cell surface proteoglycans (*34*, *35*). The DP-4 peptide by itself induced cell adhesion of control vector-transfected ARH-77 cells; however, the extent of adhesion of syndecan-1-expressing ARH-77 cells to DP-4 was significantly greater than that of the control cells (Figure 8C). The other ARH-77 cells expressing syndecan-2 and -4 showed less adhesion to DP-4 (data not shown). On the other hand, neither the syndecan-expressing nor the control ARH-77 cells demonstrated adhesion to DP-5. Further, both DP-4 and DP-4S Sepharose 4B beads were

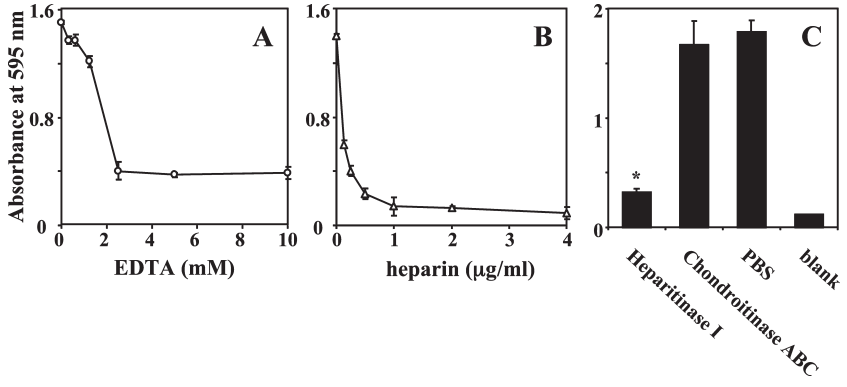

FIGURE 7: Inhibition of adhesion of cells to the DP-4 peptide by EDTA, heparin, and enzymatic treatment of cells. DP-4 was coated at a concentration of 4 μg/mL and incubated with EDTA (A) and heparin (B) in the same manner as described in the legend of Figure 2. Data are expressed as means ± SD of triplicate results. Experiments were performed three times and gave similar results. (C) HaCaT cells were treated with enzymes and added to the peptide-coated wells. PBS was added to the cells as a control. Data are expressed as means ± SD of triplicate results. The asterisk indicates $p < 0.01$.

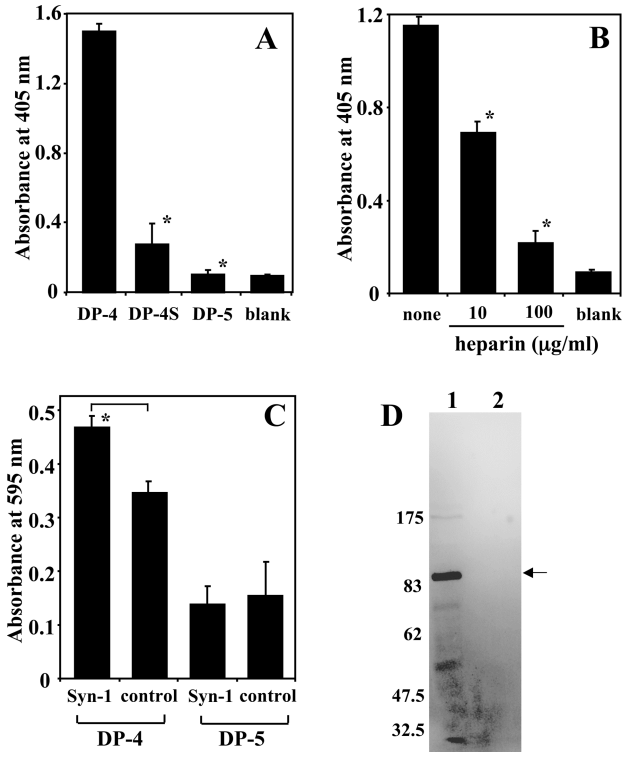

FIGURE 8: Identification of the HSPG-type cell surface receptor for the DP-4 peptide. (A) Peptides indicated under the horizontal line were coated on wells, and biotinylated heparin was added. Bound biotinylated heparin was probed with HRP-conjugated streptavidin. (B) Effect of heparin on binding of biotinylated heparin to the DP-4 peptide. Biotinylated heparin was mixed with unlabeled heparin and added to the DP-4-coated wells. Data are expressed as means ± SD of triplicate results. Asterisks indicate $p < 0.01$. (C) Adhesion of syndecan-expressing and control ARH-77 cells to DP-4. DP-4 and DP-5, partially overlapped with DP-4, were coated at a concentration of 4 μg/mL, and the cells were incubated for 1 h. Asterisks indicate $p < 0.01$. Data are expressed as means ± SD of triplicate results. (D) Affinity binding of syndecan-1 to DP-4-conjugated Sepharose 4B (lane 1, DP-4; lane 2, DP-4S) was examined by incubation of peptide beads with the HaCaT cell lysate. The beads were treated with heparitinase I and chondroitinase ABC for syndecan-1 elution and then were subjected to 7.5% SDS−PAGE and Western blotting. The arrow shows syndecan-1, and molecular masses (kilodaltons) are indicated at the left.

prepared for examination of the direct binding of DP-4 to syndecan (Figure 8D). The protein bound to the peptide beads was treated with heparitinase I and chondroitinase ABC to elute proteoglycans by sugar chain digestion, and syndecan-1 was detected by Western blotting of the DP-4 bead fraction. These data demonstrate that DP-4 binds to the syndecan-1 proteoglycan directly. We conclude that syndecan is involved in adhesion of cells to DP-4.

DISCUSSION

In this study, we found that dermatopontin promoted strong epidermal cell adhesion via an eight-amino acid active site. We also identified the cell surface receptors for dermatopontin. Although cell adhesion activity of dermatopontin had been described for fibroblasts (*18*), fibroblast cell adhesion required much higher concentrations of dermatopontin than the epidermal cells for maximal adhesion. These findings suggest that dermatopontin demonstrates stronger cell adhesion activity for epidermal HaCaT cells than for dermal fibroblasts. HaCaT cells not only adhere to dermatopontin but also spread on it, forming actin filaments. These findings indicate that the cells underwent morphological changes beyond adhesion on dermatopontin.

The cell adhesion sequence of dermatopontin was localized to eight amino acids in the DP-4 peptide in bovine dermatopontin and was located on the first loop structure. On the basis of hydrophobicity analysis using ProtParam of ExPasy, this peptide demonstrates a high hydrophobic score of 0.418. Although the DP-4 peptide is hydrophobic, when combined with adjacent peptides, DP-3−4 and DP-4−5 peptides possess hydrophilic scores (−0.304 and −0.216, respectively). The minimum active sequence of DP-4 was GQVVVAVR, and the arginine residue may play a critical role for the biological activity. While a precise analysis by X-ray diffraction is required for the determination of the location of the DP-4 peptide, it is possible that the DP-4 region containing the arginine residue is exposed on the molecular surface. We also examined the adhesion of HaCaT cells to the human DP-4 peptide (PQGQVIVAVRS), which differs by two amino acids from bovine DP-4 (PHGQVVVAVRS). The human DP-4 peptide promoted cell adhesion when coated on a dish and inhibited adhesion of cells to dermatopontin in a fashion similar to that of bovine DP-4 (data not shown). These data suggest that the biological activity of the DP-4 sequence is conserved in both human and bovine dermatopontin, and that this site is important for dermatopontin.

One of the cell surface receptors for dermatopontin was determined to be $\alpha 3\beta 1$ integrin (Figure 3). Because the cell adhesion domain was identified as the DP-4 peptide, we examined if this peptide is the integrin binding site. Unexpectedly, adhesion of cells to DP-4 was not inhibited by anti-integrin antibodies (data not shown). These findings indicate that there is an integrin binding site somewhere in the dermatopontin molecule. It is likely that the integrin binding site requires more complex, longer peptides, or a domain with three-dimensional conformations. This site was not identified by the approaches using short peptides in this study.

We found via several approaches that a HSPG-type cell surface receptor is involved in adhesion of cells to the DP-4 peptide (Figures 7 and 8). Other studies have also suggested syndecans as cell surface receptors for several ligands (*38*−*40*). In fact, syndecan-expressing ARH-77-Synd-1 cells demonstrated a small but statistically significant enhancement of adhesion of cells to DP-4 (Figure 8C). On the other hand, ARH-77-Cont cells that do not express syndecan showed weak adhesion to the DP-4 peptide, but not to DP-5. Pull-down analysis using DP-4-conjugated beads also supported the direct binding activity of syndecan to DP-4 (Figure 8D). These findings suggest that the cells specifically adhere to DP-4 through HSPG and syndecan is a potential HSPG receptor, but it also suggests that other molecules on the ARH-77 cells may participate in the adhesion. The possible receptor molecular species was not examined further in this study. Syndecan-knockout mouse analysis revealed that this receptor plays important roles in wound healing via promoting cell adhesion and migration (*41*). With all the data taken together, we suggest that syndecan binding of dermatopontin via DP-4 may affect various biological events, such as wound healing.

In this study, it was revealed that HaCaT cells utilize multiple receptors for adhesion to dermatopontin, that is, $\alpha 3\beta 1$ integrin and HSPG-type receptors. Similar types of multireceptor systems composed of integrins and syndecans, or other receptors, are known (*38*, *42*−*45*). For example, syndecan and $\alpha 2\beta 1$ integrin are required for fibroblast spreading after adhesion to the laminin $\alpha 1$ LG4 module (*38*). Syndecan-4 and $\alpha 5\beta 1$ integrin promote

focal adhesion of melanoma cells and migration of melanoma cells on fibronectin (*42*). Although the mode and the fate of cooperation between syndecans and integrins on adhesion of cells to dermatopontin are not known, it is feasible that the cooperation results in certain biological activities beyond adhesion.

α3β1 integrin is expressed on the keratinocyte cell surface (*46*), and it is known to participate in keratinocyte adhesion and migration on an unprocessed form or on a specific module of laminin-332 (*47*, *48*). Syndecan is also expressed in keratinocytes, and the expression is enhanced around the wound edge (*49*). A growing body of evidence indicates that syndecan is involved in cell migration (*41*, *50*). Recently, it was reported that the interaction of syndecan-1 and the globular domain of laminin-332 is indispensable for keratinocyte migration on the unprocessed form of laminin-332 (*47*). Likewise, the involvement of both α3β1 integrin and syndecan in adhesion of cells to dermatopontin implies that dermatopontin has some roles in a wide range of biological events, including wound healing.

Dermatopontin is one of the noncollagenous components in the dermis, and it localizes around the collagen fibers (*2*, *3*). Dermatopontin-deficient mice exhibit a phenotype of Ehlers-Danlos syndrome, demonstrating poorly organized collagen microfibrils and susceptibility in the skin for mechanical shear stress (*51*). Thus, dermatopontin plays an indispensable role in maintaining functional tissue integrity. In skin injury, keratinocytes at the wound edge have direct contact with dermal components. At this time, dermatopontin in the newly exposed dermis may influence the behavior of epidermal cells by supplying a scaffold for the cells through its cell adhesion activity. In addition, the identification of the cell adhesion domain implies a possibility that this synthetic peptide may be used as a therapeutic to wounds.

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