Dermatopontin interacts with transforming growth factor β and enhances its biological activity

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Dermatopontin, a recently found low-molecular-mass component of the extracellular matrix, was studied for its interaction with decorin and transforming growth factor β (TGF-β) and its influence on TGF-β bioactivity. Dermatopontin reacted with decorin with an apparent K_d of 100 nM in a solid-phase assay. Dermatopontin inhibited the formation of the decorin–TGF-β1 complex. Decorin also competed with dermatopontin for the binding of this cytokine. The dermatopontin–decorin complex bound 3-fold more TGF-β1 than did each component individually, and binding was inhibited more strongly by decorin preincubated with dermatopontin than by dermatopontin or decorin alone. Dermatopontin augmented the biological activity of TGF-β1, as analysed by the expression of luciferase in mink lung epithelial cells transfected with a plasminogen activator inhibitor–promoter–luciferase construct, although dermatopontin itself did not show apparent induction of luciferase. Dermatopontin showed weak inhibitory activity on the proliferation of mink lung epithelial cells, and it enhanced the growth-inhibitory activity of TGF-β on these cells. Thus dermatopontin increases the cellular response to TGF-β. These findings strongly suggest that dermatopontin modifies the behaviour of TGF-β through interaction with decorin in the microenvironment of the extracellular matrix *in vivo*.

Key words: cytokines, decorin, dermatopontin.

INTRODUCTION

We previously isolated a 22 kDa protein and identified it as bovine dermatopontin [1]. The molecule has five disulphide loops [2] and is composed of 183 amino acid residues; about half of its tyrosyl residues are sulphated [3]. The protein is reportedly expressed in skin, skeletal muscle, bone, cartilage and other tissues on the basis of Western blotting or Northern analysis [1,3,4]. Dermatopontin has been reported to interact with decorin [1,5], to accelerate collagen fibrillogenesis and to modify the diameter of collagen microfibrils [6]. However, the functions of this protein are still poorly understood despite its abundance in the extracellular matrix (ECM).

Transforming growth factor β (TGF-β) modulates the behaviour of many cell types by interacting with its receptors and by transducing signals to the cell nucleus through the successive phosphorylation of intracellular messenger molecules. This cytokine inhibits the proliferation of epithelial and endothelial cells, modulates the differentiation of many cell types, and up-regulates ECM proteins including collagen and proteoglycans [7–9]. Although the function of this cytokine is important in tissue metabolism, the means of regulation of its activity in the pericellular milieu is unclear.

One of the ECM components and a member of the small leucine-rich proteoglycans is decorin, which is abundantly present in the ECM; decorin interacts with several types of collagen [10–12] and has a strong inhibitory effect on type I and II collagen fibrillogenesis [13–16]. This molecule is thought to have a role in the formation of the ECM architecture. However, recombinant decorin was recently shown to bind to TGF-β through a core protein and to cancel its biological effects [17]. However, it also was reported that bone matrix decorin binds to TGF-β and enhances its inhibitory effect on the proliferation of osteoblastic cells [18]. At present, only decorin is known to modulate the function of TGF-β in the ECM. Other dynamic functions of this ECM molecule are currently being elucidated [19–21].

The observation that decorin binds to TGF-β and to dermatopontin prompted us to speculate that dermatopontin modulates the interaction between decorin and TGF-β, thereby regulating the latter's biological activities. Here we examine the interaction between dermatopontin, decorin, TGF-β and their complexes, and the influence of dermatopontin on the biological activities of TGF-β by using mink lung epithelial cells (MLECs).

MATERIALS AND METHODS

Materials

^{125}I-TGF-β1 was purchased from NEN Life Science Products (Boston, MA, U.S.A.), human TGF-β1 from Pepro Tech EC (London, U.K.), geneticin (G-418 sulphate) from Gibco–BRL (Grand Island, NY, U.S.A.), peroxidase-conjugated anti-(rabbit IgG) antibodies from Cappel (Aurora, OH, U.S.A.), and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) from Wako (Osaka, Japan). MLECs transfected with plasminogen activator inhibitor promoter–luciferase construct (PAI/L MLECs) were prepared as described previously [22].

Preparation of bovine dermatopontin and decorin

Bovine dermatopontin was extracted, as described previously [1], from newborn calf dermis with 4 M guanidinium chloride and was then purified by gel-filtration, ion-exchange and reverse-

Abbreviations used: ABTS, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid); DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; FCS, fetal calf serum; MLEC, mink lung epithelial cell; PAI-1, plasminogen activator inhibitor type 1; PAI/L, plasminogen activator inhibitor promoter–luciferase construct; TGF-β, transforming growth factor β.

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phase chromatographies. Bovine dermal decorin was prepared by using the method of Nakamura et al. [23].

Preparation of polyclonal antiserum against dermatopontin

Polyclonal antiserum was raised against purified bovine dermatopontin by the subcutaneous injection of Japanese White rabbits with 100 μg of dermatopontin emulsified in a 1:1 mixture of complete Freund's adjuvant six times at intervals of 2 weeks.

Binding assay of ^{125}I-TGF-β1 to immobilized decorin or dermatopontin

A solid-phase assay was performed as described previously [24]: 100 μl of protein solution in 0.14 M NaCl/30 mM sodium phosphate (pH 7.3) was added to wells of a 96-well microtitre plate (Sanko Chemicals, Tokyo, Japan) and incubated overnight. After the wells had been rinsed with 0.15 M NaCl/5 mM sodium phosphate (pH 7.4), non-specific binding was blocked with 1% BSA in the same buffer for 6 h. Each well was rinsed with 0.14 M NaCl/30 mM sodium phosphate/0.05% (v/v) Tween 20 (pH 7.3), followed by incubation with 50 μl of ^{125}I-TGF-β1 in the same buffer. After 12 h the wells were rinsed; bound ^{125}I-TGF-β1 was solubilized with 0.3 M NaOH/1% (v/v) Triton X-100 for counting. All procedures were done at 18–22 °C. In some experiments, various concentrations of dermatopontin or decorin, or a mixture of the two, were added to wells.

Solid-phase decorin–dermatopontin binding assay

The interaction between decorin and dermatopontin was assayed by using essentially the same procedures as above. Microtitre wells were coated with 10 μg/ml decorin in 200 μl of buffer. After blocking with BSA, 200 μl of dermatopontin solution was added to the immobilized decorin and incubated for 12 h. After washing, 200 μl of anti-dermatopontin antiserum at a dilution of 1:500 was added and incubated for 2 h and washed, then incubated with 100 μl of peroxidase-conjugated anti-(rabbit IgG) at a dilution of 1:1000 for 2 h. The colour reaction was begun by incubation with 150 μl of 1 mM ABTS in 57 mM citric acid/86 mM sodium phosphate (pH 4.3) at room temperature in the dark and quenched by adding an aliquot of 0.1 M citric acid containing 1.5 mM sodium azide; the absorbance at 414 nm was measured.

Cell culture

PAI/L MLECs were cultured as a monolayer in 25 cm^2 carrels at 37 °C in an incubator under an air/CO_2 (19:1) atmosphere. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum (FCS) and 400 μg/ml geneticin until use in experiments.

PAI/L MLEC assay

PAI/L MLEC assay was performed as described by Abe et al. [22], with some modifications. This bioassay for TGF-β is based on the ability of TGF-β to stimulate plasminogen activator inhibitor type I (PAI-1) expression. PAI/L MLECs (2×10^4 per well) in DMEM containing 10% (v/v) FCS were inoculated into 96-well tissue culture dishes (Iwaki Glass, Funabashi, Japan) and left to attach overnight at 37 °C in an incubator under an air/CO_2 (19:1) atmosphere. The medium was replaced with DMEM/0.1% BSA containing TGF-β or dermatopontin, or a mixture of the two, and incubated for 15 h at 37 °C. Cell extracts were prepared and assayed for luciferase activity with the luciferase assay kit (Promega, Madison, WI, U.S.A.) in accordance with the manufacturer's instructions. Luciferase activity was analysed with a Lumat luminometer (Berthold, Bad Wildbad, Germany).

MLEC proliferation assay

Aliquots (50 μl) of TGF-β or dermatopontin, or a mixture of the two, dissolved in DMEM containing 0.4% (v/v) FCS were placed in a 96-well plate containing 2×10^4 MLECs in 50 μl of the same culture medium and incubated for 44 h at 37 °C in an incubator under an air/CO_2 (19:1) atmosphere. TetraColor ONE (Seikagaku Kogyo, Tokyo, Japan) (10 μl) was added to each well and incubated for an additional 4 h. The absorbance at 450 nm was monitored. The absorbance was proportional to the number of biologically active cells.

RESULTS

Interaction of dermatopontin with decorin

In the solid-phase system, a dose-dependent curve was obtained when immobilized decorin was incubated with increasing concentrations of dermatopontin (Figure 1). Scatchard analysis of the binding of dermatopontin to immobilized decorin produced a straight line with an apparent K_d of 100 nM (Figure 1, inset).

Binding of TGF-β1 to decorin and/or dermatopontin

The radioactivity of ^{125}I-TGF-β1 bound to the solid phase was proportional to the concentration of the decorin or dermatopontin solutions immobilized on wells; this reached a plateau at a concentration of 5–10 μg/ml (Figure 2A). The amount of bound ^{125}I-TGF-β1 was proportional to the amount of ^{125}I-TGF-β1 added to wells in which dermatopontin or decorin was immobilized (Figure 2B). The total binding of ^{125}I-TGF-β1 to the immobilized decorin or dermatopontin reached approx. 10% of the applied radioactivity. The binding of ^{125}I-TGF-β1 to

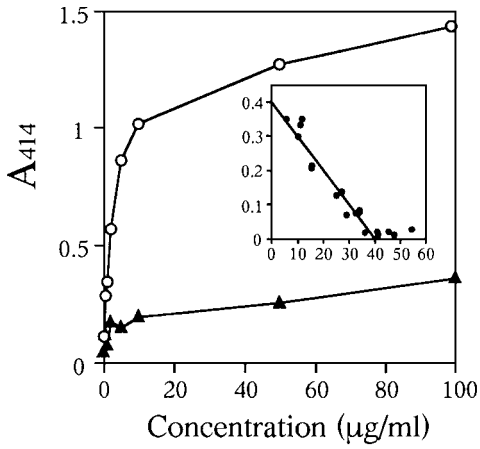

Figure 1 Solid-phase analysis of the interaction between dermatopontin and decorin

Microtitre wells in which decorin had been immobilized were incubated with increasing concentrations of dermatopontin; and bound dermatopontin was detected immunologically (○). BSA was immobilized and incubated with dermatopontin (▲). The *y*-axis is the absorbance at 414 nm and the *x*-axis is the concentration of dermatopontin. Data points are means for duplicate determinations. Inset: Scatchard analysis of binding of dermatopontin to immobilized decorin. The *y*-axis is the ratio of bound dermatopontin to free dermatopontin and the *x*-axis is the concentration of bound dermatopontin (nM).

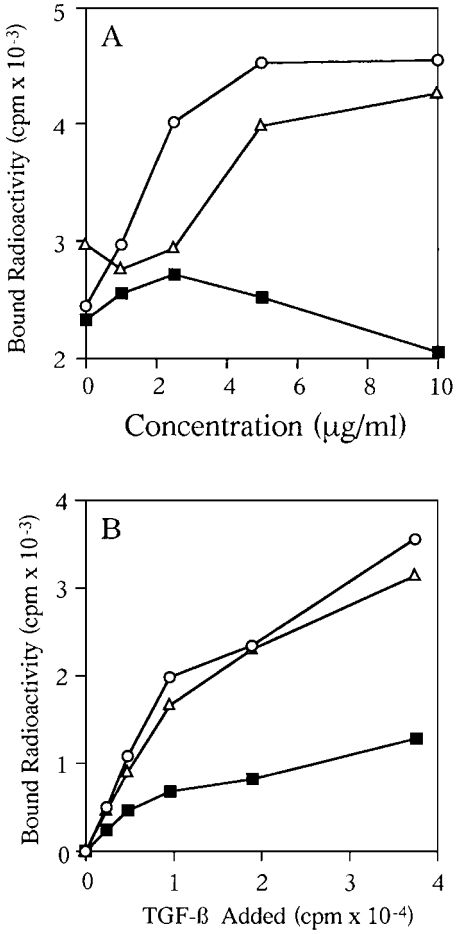

Figure 2 Interaction between dermatopontin, decorin and TGF-β1

(**A**) Binding of TGF-β1 to immobilized dermatopontin or decorin. ^{125}I-TGF-β1 (4.4×10^4 c.p.m.) was added to wells containing increasing concentrations of immobilized dermatopontin (○), decorin (△) or BSA (■). Bound radioactivities were determined. The *y*-axis is the bound radioactivity of ^{125}I-TGF-β1 and the *x*-axis is the concentration of the immobilized proteins. Data points are means for three separate experiments. (**B**) Microtitre wells coated with 10 μg/ml dermatopontin (○), decorin (△) or BSA (■) in 100 μl of buffer were incubated with increasing amounts of ^{125}I-TGF-β1, and bound radioactivities were determined. The *y*-axis is the bound radioactivity of ^{125}I-TGF-β1 and the *x*-axis is the amount (c.p.m.) of added ^{125}I-TGF-β1.

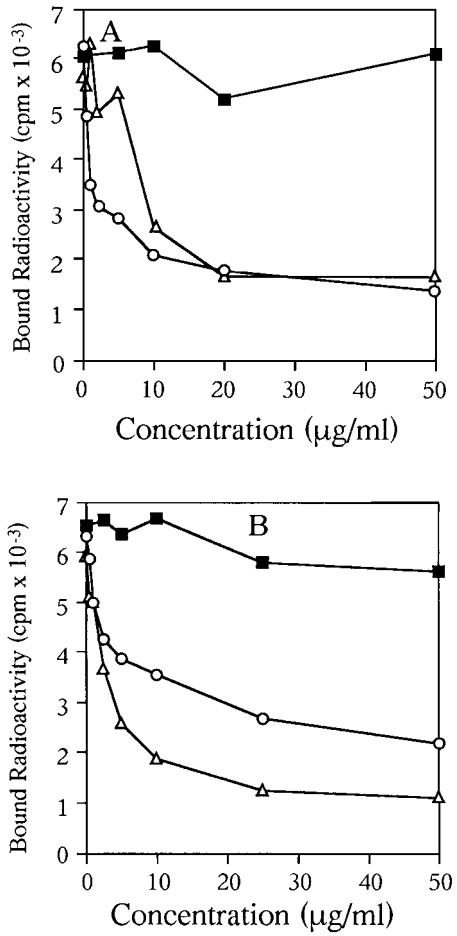

Figure 3 Competition assays

(**A**) Competition experiment for the interaction of dermatopontin–TGF-β1 with decorin. Microtitre wells coated with 10 μg/ml dermatopontin in 100 μl of buffer were incubated with 5×10^4 c.p.m. of ^{125}I-TGF-β1 mixed with increasing concentrations of dermatopontin (○), decorin (△) or BSA (■); bound radioactivities were determined. (**B**) Competition experiment for the interaction of decorin–TGF-β1 with dermatopontin. Microtitre wells coated with 10 μg/ml decorin in 100 μl of buffer were incubated with 5×10^4 c.p.m. of ^{125}I-TGF-β1 mixed with increasing concentrations of dermatopontin (○), decorin (△) or BSA (■); bound radioactivities were determined. All data points are means for two separate experiments.

immobilized dermatopontin was decreased to the background level by increasing concentrations of either dermatopontin or decorin, in a dose-dependent manner (Figure 3A). Decorin or dermatopontin produced a dose-dependent inhibition of binding of ^{125}I-TGF-β1 to immobilized decorin (Figure 3B), but binding was not fully inhibited by dermatopontin. When dermatopontin was preincubated with immobilized decorin, the bound radioactivity in ^{125}I-TGF-β1 was proportional to the concentration of preincubated dermatopontin and reached a plateau at a concentration of 10 μg/ml (Figure 4A). At this concentration, the bound radioactivity in ^{125}I-TGF-β1 was 3-fold that which bound to immobilized decorin or dermatopontin alone. The interaction between ^{125}I-TGF-β1 and the decorin–dermatopontin complex was inhibited by decorin or dermatopontin in a dose-dependent manner. When decorin was preincubated with 2.5–5 μg/ml dermatopontin, then mixed with ^{125}I-TGF-β1 and added to the immobilized decorin–dermatopontin complex, the degree of inhibition was twice that of decorin or dermatopontin alone at similar concentrations; this difference in inhibition remained unchanged at higher concentrations (Figure 4B).

Effect of dermatopontin on MLECs

To examine whether the binding of dermatopontin and TGF-β modulates the biological activity of TGF-β, we examined the influence of dermatopontin on the ability of TGF-β to induce luciferase in PAI/L MLECs. TGF-β alone induced a dose-dependent expression of luciferase in cells, whereas dermatopontin alone did not exhibit any apparent effect on the expression of luciferase (Figure 5, upper panel, inset). When PAI/L MLECs were cultured with TGF-β and increasing concentrations of dermatopontin, the expression of luciferase was enhanced 2-fold compared with TGF-β alone at a dermatopontin concentration of 100 μg/ml (Figure 5, upper panel). The enhanced luciferase expression to dermatopontin was decreased to background levels when 20 μg/ml anti-(TGF-β) neutralizing antibody (Genzyme,

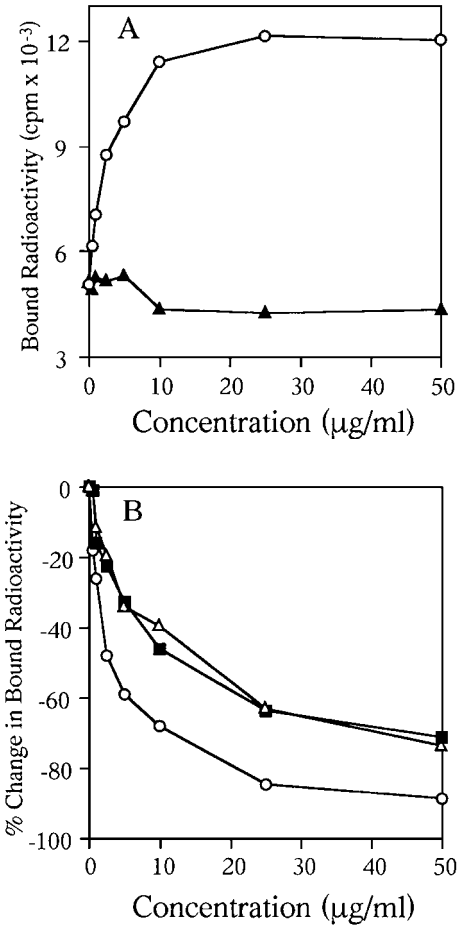

Figure 4 Interaction between dermatopontin–decorin complex and TGF-β1

(**A**) Binding of TGF-β1 to the dermatopontin–decorin complex. Microtitre wells coated with 10 μg/ml decorin in 100 μl of buffer were incubated with increasing concentrations of dermatopontin (○) or BSA (▲) for 12 h. After the unbound fraction had been rinsed, 5×10^4 c.p.m. of ^{125}I-TGF-β1 was added to each well and incubated for 12 h; bound radioactivities were then determined. (**B**) Competition experiment for the interaction between TGF-β1 and decorin–dermatopontin complex. Dermatopontin or decorin at the indicated concentrations were preincubated with 5 μg/ml decorin or dermatopontin respectively for 12 h at 4 °C and mixed with 5×10^4 c.p.m. of ^{125}I-TGF-β1. The solutions then were added to wells on which decorin–dermatopontin complex had been formed by incubating the same amount of immobilized decorin as in (**A**) and 100 μl of dermatopontin at 10 μg/ml. Dermatopontin was incubated with 5 μg/ml decorin (○) and with the same concentration of dermatopontin itself (△). Decorin was incubated with 5 μg/ml decorin (■). The *y*-axis is the percentage change in bound radioactivity compared with wells without any premixed proteins; the *x*-axis is the concentration of premixed decorin or dermatopontin. All data points are means for two separate experiments.

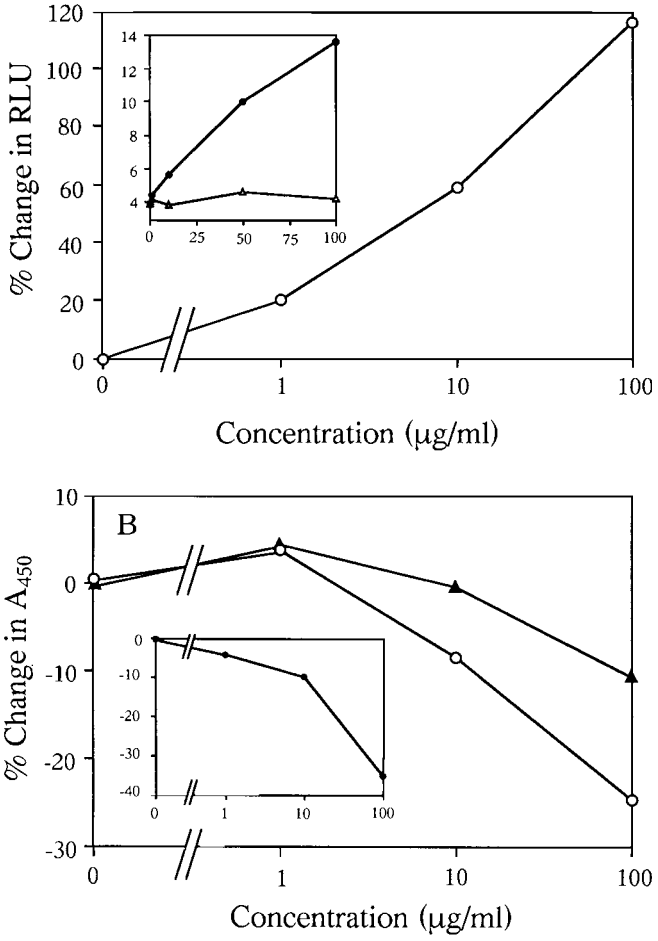

Figure 5 Effects of dermatopontin on TGF-β1-mediated cellular events

Upper panel: PAI/L MLEC assay. TGF-β1 (50 pM) was incubated with increasing concentrations of dermatopontin (○) for 1 h at room temperature and added to wells containing 2×10^4 PAI/L MLECs. After 15 h of incubation, the cells were extracted and luciferase activities were determined. The luciferase activity is expressed as relative light units (RLU). The *y*-axis is the percentage change in RLU; the *x*-axis is the concentration of dermatopontin. Data points are means for three separate experiments. Inset: wells containing 2×10^4 PAI/L MLECs were incubated with increasing concentrations of TGF-β1 (●) or dermatopontin (△) for 15 h, and luciferase activities were determined. The *y*-axis is RLU $\times 10^{-3}$; the *x*-axis is the concentration of TGF-β (pM) or dermatopontin (μg/ml). Data points are means for four separate experiments. Lower panel: proliferation assay with MLECs. Various concentrations of dermatopontin were incubated with 50 pM TGF-β1 for 1 h at room temperature and added to wells containing 2×10^4 MLECs (○). In another experiment, wells containing 2×10^4 cells were incubated with increasing concentrations of dermatopontin alone (▲). After 48 h, the absorbance at 450 nm was determined. Results are expressed as the percentage change in absorbance compared with control wells without dermatopontin. The *y*-axis is the percentage change in absorbance; the *x*-axis is the concentration of dermatopontin. Inset: wells containing 2×10^4 cells were incubated with increasing concentrations of TGF-β1. Results are expressed as the percentage change in absorbance compared with control wells without TGF-β1. The *x*-axis is the concentration of TGF-β1. All data points are means for three separate experiments.

Cambridge, MA, U.S.A.) was added (results not shown). To confirm the enhancement of TGF-β bioactivity by dermatopontin, the effect of TGF-β on cell proliferation was analysed. In the MLEC proliferation assay, TGF-β caused a dose-dependent growth suppression of MLECs (Figure 5, lower panel, inset). Dermatopontin alone decreased cell proliferation by approx. 10%. However, in the presence of TGF-β, cell proliferation was decreased in proportion to the concentration of dermatopontin, reaching 25% inhibition at a concentration of 100 μg/ml (Figure 5, lower panel).

DISCUSSION

We have previously shown, with the use of CsCl-density-gradient centrifugation, that dermatopontin binds to decorin [1]. The interaction between dermatopontin and decorin is now shown by a solid-phase assay in which bound dermatopontin was detected. Scatchard analysis showed that the binding of dermatopontin to decorin had an apparent K_d of 100 nM, which is higher than that between decorin and TGF-β (300 pM) [18]. These results indicate the presence of a single class of binding sites in these molecules, and that immobilized decorin binds TGF-β preferentially. The binding sites of these molecules are under investigation.

Dermatopontin binds to TGF-β. When the same amount of dermatopontin or decorin was immobilized, the bound amounts of TGF-β1 were almost equal. We speculate that dermatopontin and decorin compete with each other for binding TGF-β.

Decorin showed a dose-dependent and complete inhibition of the binding of TGF-β to immobilized dermatopontin, whereas dermatopontin inhibited the interaction between TGF-β and decorin only partly. When the dermatopontin–decorin complex was preformed by incubating dermatopontin with decorin before being reacted with TGF-β, the complex bound 3-fold more TGF-β than each component individually. This unexpected phenomenon was confirmed by a competition assay in which decorin that had been preincubated with dermatopontin inhibited the interaction between TGF-β1 and the decorin–dermatopontin complex more effectively than the same amount of either decorin or dermatopontin alone; this effect continued even at higher concentrations. This result indicates that decorin and dermatopontin form a complex with an ability to bind more TGF-β1 than either molecule alone. Furthermore the binding of dermatopontin to decorin in the liquid phase reached a plateau at a dermatopontin concentration of 2.5–5 μg/ml, which is consistent with the results of the binding assay between TGF-β and the immobilized decorin–dermatopontin complex. We speculate that within the dermatopontin–decorin complex, in addition to the binding site on each molecule for TGF-β, one or more novel binding sites for TGF-β are created by a conformational change. The inability of dermatopontin to completely inhibit the formation of the decorin–TGF-β1 complex might result from the formation of a small fraction of decorin–dermatopontin complex. Thus dermatopontin co-operates or competes with decorin on binding of TGF-β, depending on the conditions. The nature of dermatopontin seems to depend on the presence or absence of decorin. Although the conditions under which decorin forms a complex with dermatopontin are unclear, the complex is likely to occur *in vivo* because dermatopontin is co-purified with decorin [2]. Because both decorin and dermatopontin can bind to type I collagen fibrils [6,25], they might form a supramolecular complex with collagen and anchor TGF-β more effectively in the matrix, controlling the concentration of TGF-β in tissues.

The molecular profile of dermatopontin's interaction with TGF-β prompted us to examine the influence of dermatopontin on TGF-β activity. Dermatopontin enhanced both the inhibitory effect of TGF-β on cell proliferation and the activating effect of TGF-β on luciferase expression. In contrast, a TGF-β-receptor binding assay with ^{125}I-TGF-β1 [26] showed that the radioactivity bound to the surface of MLECs exhibited a minor change on the addition of dermatopontin (results not shown). These results indicate that by binding with TGF-β, dermatopontin preserves TGF-β and continues to supply the molecule to its receptor in an active form, thereby increasing the availability of TGF-β to cells. The subsequent sustained activation of the TGF-β signalling system results in an enhancement of the activation of the PAI-1 promoter and growth inhibition of MLECs.

The fact that dermatopontin enhances TGF-β-mediated activation of the PAI-1 promoter indicates that dermatopontin might have a role in the activation of latent TGF-β. At the time of vascular injury, plasminogen activator produced by the contact of endothelial cells with pericytes or smooth-muscle cells converts plasminogen to plasmin, which then produces active TGF-β from the latent form [27–29]. TGF-β induces PAI from endothelial cells, and PAI inhibits the proliferation and migration of activated endothelial cells [30]. The vascular endothelial cells control the excess activation of latent TGF-β and remain quiescent. We have recently found a truncated dermatopontin in endothelial cells [1] whose N-terminal 10 residues were absent (O. Okamoto and S. Fujiwara, unpublished work). Furthermore an immunoreactive molecule of molecular mass 22 kDa has been discovered in human and bovine serum (O. Okamoto and S. Fujiwara, unpublished work), and we speculate that this molecule is dermatopontin. It is possible that dermatopontin binds to TGF-β on the surface of endothelial cells and continues to produce PAI by activating its promoter through signals from TGF-β receptors, thereby maintaining vascular homoeostasis.

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