Decorin-expressing adenovirus decreases collagen synthesis and upregulates MMP expression in keloid fibroblasts and keloid spheroids

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Short title: Decorin alters keloid extracellular matrices

ABSTRACT

Background: Decorin is a natural transforming growth factor-β1 (TGF-β1) antagonist. Reduced decorin synthesis is associated with dermal scarring and increased decorin expression appears to reduce scar tissue formation. To investigate the therapeutic potential of decorin for keloids, human dermal fibroblasts (HDFs) and keloid-derived fibroblasts (KFs) were transduced with decorin-expressing adenovirus (dE1-RGD/GFP/DCN), and we examined the therapeutic potential of decorin-expressing Ad for treating pathologic skin fibrosis.

Method: Decorin expression was examined by immunofluorescence assay on keloid tissues. HDFs and KFs were transduced with dE1-RGD/GFP/DCN or control virus, and protein levels of decorin, epidermal growth factor receptor (EGFR), and secreted TGF-β1 were assessed by western blotting and ELISA. And collagen type I, III, and matrix metalloproteinase-1, 3 (MMP-1, 3) mRNA levels were measured by real-time RT-PCR. Additionally, we immunohistochemically investigated expression levels of the major...
extracellular matrix (ECM) proteins in keloid spheroids transduced with dE1-RGD/GFP/DCN.

**Results:** Lower decorin expression was observed in the keloid region compared to adjacent normal tissues. After treatment with dE1-RGD/GFP/DCN, secreted TGF-β1 and EGFR protein expression were decreased in TGF-β1-treated HDFs and KFs. Also, type I, III collagen mRNA levels were decreased and expression of MMP-1, 3 mRNA was strongly upregulated. In addition, expression of type I, III collagen, fibronectin, and elastin was significantly reduced in dE1-RGD/GFP/DCN-transduced keloid spheroids.

**Conclusion:** These results support the utility of decorin-expressing adenovirus to reduce collagen synthesis in KFs and keloid spheroid, which may be highly beneficial in treating keloids.

**Keywords:** Keloid, Gene therapy, Decorin-expressing adenovirus, Collagen, MMP, keloid spheroid

**Introduction**

Keloids and hypertrophic scarring are pathologic fibro proliferative skin disorders that represent abnormal wound healing resulting from excessive collagen production and abnormal collagen assembly, with severely disfiguring results for patients. Various mechanisms have been proposed to explain keloid pathogenesis (1-5). However, treatment of keloid scars is extremely difficult because keloids are highly recurrent after surgical excision and often spread beyond the original margin. Therefore, although numerous treatment modalities are available, none are fully effective (6-8). Transforming growth factor-β1 (TGF-
β1) is a well-studied growth factor that seems to play the main role in keloid pathophysiology (6,9-13). The excess collagen present in keloids may result from TGF-β1 overexpression (14,15), which decreases collagen degradation.

Decorin, a small leucine-rich proteoglycan, is a ubiquitous component of the interstitial matrix of the dermis and stroma of other tissues and preferentially associates with collagen fibrils. Decorin binds to collagen fibrils and regulates cell-matrix interactions. Decorin also delays the lateral assembly of individual triple-helical collagen molecules, resulting in decreased fibril diameter (16-18). Decorin also modulates interactions of extracellular matrix (ECM) components, such as fibronectin and thrombospondin, with cells (19).

Decorin may also affect the production of collagen and other ECM components by blocking TGF-β1 synthesis and activity (20-24). Thus, decorin is a physiological TGF-β1 inhibitor. Furthermore, decorin affects ECM remodeling by inducing the matrix metalloproteinase collagenase-1 (25,26). Because TGF-β1 complexes with decorin and causes an antifibrotic response, decorin has been explored as a potential antifibrotic in experimental kidney (27), lung (28), and human corneal fibroblast models (29). Low decorin levels are observed in keloid tissues compared with normal skin (15,30-32), and collagen bundles in keloid scars are thicker and more disorganized than that in normal scar tissue (33). Based on these previous findings, we hypothesized that lower decorin expression in keloid dermal layers results in upregulated TGF-β1 activity and increased collagen synthesis and deposition. We also speculated that decorin replacement may represent a therapeutic alternative for preventing keloid formation.
In this study, we have generated a decorin-expressing adenovirus (Ad) (dE1-RGD/GFP/decorin) and transduced it into human dermal fibroblasts (HDFs) and keloid-derived fibroblasts (KFs), to examine the therapeutic potential of decorin-expressing Ad for treating pathologic skin fibrosis. Gene therapy, which has been extensively examined for use in anti-cancer therapy (34), has great clinical potential in treating keloids (35,36) because it can express transgene expression for long periods, which cannot be achieved by protein therapy.

**Materials and Methods**

**Human dermal fibroblast, keloid tissue, and keloid-derived fibroblast cells**

HDFs and KFs were obtained from the ATCC (American Type Culture Collection, Manassas, VA). After obtaining informed consent according to a protocol approved by the Yonsei University College of Medicine Institutional Review Board, keloid tissues were obtained for fibroblast culture, histologic, and immunohistochemical analysis with excision. Keloid fibroblasts were obtained from both the central dermal layer of keloids (Supplementary table 1). All experiments involving humans were performed in adherence to the Helsinki Guidelines. Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 μg/mL).
Generating decorin-expressing adenoviral vectors

To generate an Ad expressing GFP and decorin at the E1 and E3 regions, respectively, pdE1-RGD/GFP (37) was linearized by SpeI digestion and co-transformed into *Escherichia coli* BJ5183 with the *Pvu*I-digested pSP72-E3/CMV-DCN E3 shuttle vector (38) for homologous recombination, generating a pdE1-RGD/GFP/DCN adenoviral vector. The propagation, purification, and titration of Ad were performed as described previously (39,40).

Immunofluorescence assay

Keloid tissue sections (n=4) were deparaffinized, rehydrated, blocked with 5% goat serum, and incubated with anti-decorin antibody (ab54728; Santa Cruz Biotechnology, CA) overnight at 4°C. Sections were then washed with phosphate-buffered saline (PBS) and incubated with Texas Red-conjugated bovine anti-mouse IgG (sc-2788; Santa Cruz Biotechnology) secondary antibody for 2 hr at room temperature. Tissues were mounted on slides using Vectashield® mounting medium containing the nuclear stain DAPI (Vector Laboratories, Burlingame, CA), and viewed by confocal microscopy (LSM700, Olympus, Center Valley, PA). The expression levels of decorin were semi-quantitatively analyzed using MetaMorph® image analysis software (Universal Image Corp., Buckinghamshire, UK). Results are expressed as the mean optical density for six different digital images.

Transduction efficiency analysis

HDFs and KFs were seeded into a 24-well plate and cultured to 60% confluence 1 day prior to the transduction assay. Cells were transduced with dE1/GFP or dE1-RGD/GFP. Different MOIs were applied to HDFs (1, 2, 5, 10, 20 and 100 MOI) and KFs (1, 5 and 20 MOI). The This article is protected by copyright. All rights reserved.
transduced cells were incubated for an additional 48 h. Cells were imaged using fluorescence microscopy (Olympus IX81; Olympus Optical, Tokyo, Japan), and the GFP expression levels were quantified using FACS analysis BD FACScan analyzer (Becton–Dickinson, San Jose, CA) and the CellQuest software (Becton-Dickinson). Data from 10000 events were collected and the mean ± SEM of three independent experiments were presented.

Preparation and adenoviral transduction of keloid spheroids

Keloid tissues were obtained from active-stage keloid patients (n=3). Keloid spheroids were prepared by dissecting keloid central dermal tissue into 2-mm diameter pieces with sterile 21-gauge needles. Explants were plated onto HydroCell® 24 Multi-well plates (Nunc, Rochester, NY) after which they were cultured for 4 h in IMDM (Isocove’s modified Dulbecco’s medium, Gibco BRL) supplemented with 5% fetal bovine serum, 10 mM l⁻¹ insulin and 1 mM l⁻¹ hydrocortisone. Each of the Ads (dE1/GFP, dE1-RGD/GFP, or dE1-RGD/GFP/DCN) at 1 x 10¹⁰ VP were added into the plates containing keloid spheroid, and incubated at 37 °C in 5% CO₂ incubator for 3 days. The transduced keloid spheroids were then fixed with 10% formalin, paraffin-embedded, and cut into 5-μm-thick sections. For the observation on penetration of Ads into the keloid spheroid, the keloid spheroids were embedded in O.C.T. compound and snap frozen. Keloid spheroids were imaged using fluorescence microscopy (Olympus IX81; Olympus Optical, Tokyo, Japan).
**Western blot analysis**

To examine the decorin, epidermal growth factor receptor (EGFR), Akt, Erk 1/2, phospho Erk 1/2, and HER2/ErbB2 expression, HDFs and KFs were transduced with dE1-RGD/GFP/DCN or control virus (dE1-RGD/GFP) at 100 and 200 MOI. At 48 h after transduction, cells were lysed in 50 mM Tris-HCl (pH 7.6), 1% Nonidet P-40 (NP-40), 150 mM NaCl, and 0.1 mM zinc acetate in the presence of protease inhibitors. Protein concentration was determined by the Lowry method (Bio-Rad, Hercules, CA), and 30 μg of each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins on the gel were electrotransferred to polyvinylidene fluoride membrane, incubated with the primary human anti-decorin antibody (AF143; R & D Systems, Minneapolis, MN), rabbit anti-EGFR antibody (#2232; Cell Signaling Technology, Beverly, MA), rabbit anti-Akt antibody (#9272; Cell Signaling Technology), rabbit anti-Erk 1/2 antibody (#4370S; Cell Signaling Technology), rabbit anti-phospho Erk 1/2 antibody (#9121; Cell Signaling Technology), rabbit anti-HER2/ErbB2 antibody (#2242; Cell Signaling Technology), or rabbit anti-β-actin antibody (Sigma, St Louis, MO), and then incubated with the HRP (horseradish peroxidase)-conjugated secondary antibody (6160–05; Southern Bio Technology Associates, Inc., Birmingham, AL). The expression patterns were revealed using the ECL detection kit (sc-2004; Santa Cruz Biotechnology), and the expression levels of decorin, EGFR, Akt, Erk 1/2, phospho Erk 1/2, and HER2/ErbB2 were semi-quantitatively analyzed using ImageJ software (National Institutes of Health, Bethesda, MD).
Enzyme-linked immunosorbent assay (ELISA) for secreted decorin and TGF-β1 expression

HDFs (2×10^5 cells) in 6-cm culture dishes were transduced with either dE1-RGD/GFP or dE1-RGD/GFP/DCN at MOIs of 100 to 200. At 2 days post-infection, supernatants were collected by centrifugation at 15,000×g for 10 min at 4°C, and secreted decorin and TGF-β1 protein was assessed using an ELISA kit (R&D Systems, Minneapolis, MN).

qRT-PCR analysis of expression levels of type I and III collagen, MMP-1, and MMP-3 mRNAs

HDFs and KFs (5×10^5 cells) were transduced with dE1-RGD/GFP/DCN or control virus (dE1-RGD/GFP) in the absence or presence of TGF-β1 (10 ng/mL) at 100 multiplicity of infection (MOI). At 3 days post-transduction, total RNA was prepared with TRIzol® reagent (Gibco BRL, Grand Island, NY), and complementary DNA was prepared from 0.5 μg total RNA by random priming using a first-strand cDNA synthesis kit (Promega Corp., Madison, WI), under the following conditions: 95°C for 5 min, 37°C for 2 h, and 75°C for 15 min. Taqman® primer/probe kits [assay ID: Hs00164004_m1 (collagen type I), Hs00164103_m1 (collagen type III), Hs00233958_m1 (MMP-1), and Hs00233962_m1 (MMP-3)] were used to analyze mRNA expression levels with an ABI Prism® 7500 HT Sequence Detection System (primer kits and instrument from Applied Biosystems, Foster City, CA). Target mRNA levels were measured relative to an internal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control (assay ID: Hs99999905_m1, Applied Biosystems). For cDNA amplification, AmpliTaqGold® DNA polymerase (Applied Biosystems) was activated by 10 min incubation at 95°C; this was followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C for each cycle. To measure cDNA levels, the threshold cycle at which fluorescence was first detected above this article is protected by copyright. All rights reserved.
baseline was determined, and a standard curve was drawn between starting nucleic acid concentrations and the threshold cycle. Target mRNA expression levels were normalized to GAPDH levels, and relative quantization was expressed as fold-induction compared with control conditions in each cell type.

**Histology and immunohistochemistry**

Representative sections were stained with hematoxylin and eosin (H & E), picrosirius red or masson’s trichrome and then examined by light microscopy. Keloid spheroid sections were incubated at 4°C overnight with mouse anti-collagen type I (ab6308; Abcam, Ltd., Cambridge, UK), mouse anti-collagen type III (C7805; Sigma), mouse anti-elastin (E4013; Sigma), mouse anti-fibronectin (sc-52331; Santa Cruz Biotechnology), rabbit anti-TGF-β1 (ab9758; Abcam), mouse anti-EGFR (Ab-1; Oncogene Research Products, Calbiochem), or rabbit anti-Erk 1/2 (#4370S; Cell Signaling Technology) primary antibody, and then incubated at room temperature for 20 min with the Dako Envision™ Kit (Dako, Glostrup, Denmark) as secondary antibody. Diaminobenzidine/hydrogen peroxidase (Dako) was used as the chromogen substrate. All slides were counterstained with Meyer’s hematoxylin. The expression levels of TGF-β1, EGFR, Erk 1/2, type I and III collagen, elastin, and fibronectin were semi-quantitatively analyzed using MetaMorph® image analysis software. Results are expressed as the mean optical density for six different digital images.
Statistics

Results are expressed as the mean ± standard error of the mean (SEM). Data were analyzed by a repeated-measures one-way ANOVA. Two sets of independent sample data were compared using a paired t-test; \( p \)-values < 0.05 was considered indicative of statistically significant differences.

Results

Decorin expression is decreased in keloid tissues versus adjacent normal tissues

After H&E staining, we observed that keloid tissue had a dense and excessive collagen deposition that extended over the clinical keloid margin into the extra-lesional dermal tissue (Fig. 1a-c). To evaluate decorin protein expression patterns in keloid tissue, immunofluorescence staining was performed. Compared to extra-lesional normal tissue (Fig. 1d), markedly decreased decorin immunoreactivity was noted in central and peripheral keloid region (Fig. 1e). The reduced expression of decorin was semi-quantitatively measured with MetaMorph® image analysis software (**\( p < 0.01 \)) (Fig. 1f).

Construction of decorin-expressing adenovirus and expression pattern of decorin

Normal tissues express low levels of the coxsackie-adenoviral receptor (CAR), which is a primary receptor for Ad’s cell entry. Therefore, we constructed a fiber-modified Ad, which alters the virus’ cell recognition domain, to efficiently transduce normal HDFs and KFs. We inserted the Arg-Gly-Asp (RGD) motif in the HI loop of the Ad fiber, generating a dEl-RGD/GFP Ad expressing GFP. We also generated a replication-incompetent decorin-

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expressing Ad (dE1-RGD/GFP/DCN) (Fig. 2a). Both HDFs and KFs were highly susceptible to the RGD-modified Ad (dE1-RGD/GFP). The RGD-modified Ad vector led to 105-fold increase GFP expression in HDFs compared with unmodified Ad at a multiplicity of infection (MOI) of 100, and 31-fold increase GFP expression in primary KFs at 20 MOI (Fig. 2b and c) (**p<0.01). Moreover, RGD-modified Ad elicited significantly enhanced GFP expression on the keloid spheroid compared to control Ad (Fig. 2d and e), demonstrating the superiority of RGD-modified Ad for gene transfer efficacy in keloid fibroblasts. Of note, both whole image (Fig. 2d) and cross-sectioned image (Fig. 2e) of spheroids transduced with dE1-RGD/GFP/DCN demonstrated that the distribution of GFP expression was much more extensive as GFP-positive cells were observed throughout the entire spheroid, whereas GFP expression was not detected in the core region of spheroids transduced with dE1-RGD/GFP. These results demonstrate that decorin-expressing adenovirus (dE1-RGD/GFP/DCN) spread to the core of the spheroid with higher efficiency than the control vector dE1-RGD/GFP adenovirus in the three-dimensional (3-D) structure of keloid spheroids. Based on these results, we transduced HDFs using RGD-modified Ad to evaluate decorin expression levels. Immunoblotting results showed that decorin protein levels were increased in HDFs and KFs in a dose-dependent manner at 2 days after transducing with dE1-RGD/GFP/DCN versus dE1-RGD/GFP (Fig. 2f). Moreover, ELISA results showed that secreted decorin protein levels were increased in a dose-dependent manner at 2 days after transducing with dE1-RGD/GFP/DCN versus dE1-RGD/GFP (Fig. 2g) (**p<0.01).
Decorin-expressing adenovirus downregulates type I and III collagen mRNAs in HDFs and KFs

Type I and III collagen mRNA levels were examined in HDFs and KFs by qRT–PCR. Type I collagen mRNA in TGF-β1-treated (10 ng/mL) and decorin-transduced HDFs and KFs was significantly decreased by 83% and 30%, respectively, versus control virus-treated cells (Fig. 3a), showing that decorin reduces collagen type I mRNA in HDFs and KFs. Similarly, mRNA expression of type III collagen was significantly reduced by 94% and 85%, respectively compared with control virus-treated HDFs and KFs (Fig. 3b). Further, markedly reduced type I and III collagen mRNA was observed in primary keloid-derived fibroblasts treated with decorin-expressing Ad compared to untreated or control dE1-RGD/GFP-transduced fibroblasts (n=4, Figure S3). These results suggest that decorin expression attenuates collagen type I and III mRNA levels in TGF-β1-treated HDFs and KFs, which have upregulated collagen expression.

Decorin-expressing adenovirus decreases secreted TGF-β1 protein and EGFR protein by HDFs and KFs

To examine the mechanism underlying the suppression of collagen mRNA levels by decorin-expressing Ad, secreted TGF-β1 and EGFR were investigated in both HDFs and KFs using ELISA and Western blotting, respectively. As shown in Figure 3c, dE1-RGD/GFP/DCN Ad decreased the secretion of TGF-β1 in both HDFs and KFs compared with dE1-RGD/GFP control virus (*p<0.05, **p<0.01), suggesting that decorin expression decreases collagen mRNA level by inhibiting TGF-β1 expression. Previous reports showed that TGF-β triggers upregulation of ERBB ligands and activation of cognate receptors via the canonical SMAD pathway in fibroblasts. In agreement with these previous findings, TGF-β1 treatment elevated

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the expression of EGFR by 2.7-fold in HDF cells (Fig. 3d). Further, dE1-RGD/GFP/DCN elicited attenuated expression of EGFR in KFs and HDFs compared with control dE1-RGD/GFP Ad, demonstrating that decorin expression can decrease the expression of EGFR through the suppression of TGF-β1, a key regulator of fibrogenesis. The decreased expression of TGF-β1, EGFR, and Erk 1/2 were also observed in keloid spheroids treated with dE1-RGD/GFP/DCN in comparison to those with control dE1-RGD/GFP (Fig. 3e and f). To examine the intracellular effect of decorin expression on EGF/EGFR signaling, Akt, Erk 1/2, and phospho Erk 1/2 protein expression were investigated. As shown in Fig. 3g, dE1-RGD/GFP/DCN Ad decreased the expression levels of Akt, Erk 1/2, and phospho Erk 1/2 in HDFs compared with untreated or HDFs transduced with dE1-RGD/GFP control virus (100 and 200 MOI) regardless of exogenous EGF treatment (10 ng/mL). These results suggest that dE1-RGD/GFP/DCN-mediated decorin overexpression attenuates EGF/EGFR signaling pathways, which have a role in keloid pathogenesis.

Decorin-expressing adenovirus increases MMP-1 and -3 mRNA expressions in HDFs and KFs

We examined the effect of decorin-expressing Ad on MMP-1 and -3 mRNA expressions in KFs and HDFs by qRT-PCR (Fig. 3h and i) (*p<0.05, **p<0.01). MMP-1 mRNA levels were significantly increased by 3.6- and 10.4-fold in KFs and HDFs, respectively, following transduction with dE1-RGD/GFP/DCN versus dE1-RGD/GFP in KFs and TGF-β1-stimulated HDFs (Fig. 3h). Similarly, MMP-3 mRNA levels were also significantly increased by 4.4- and 3.1-fold, respectively, in KFs and TGF-β1-stimulated HDFs after decorin transduction (Fig. 3i; p<0.01 in both cell types). Moreover, both MMP-1 and MMP-3 mRNA levels were significantly increased by 8.9- and 4.7-fold (**p<0.01), respectively, in primary

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keloid-driven fibroblasts (n=4, Supplementary Figure S3). These results suggest that decorin upregulates expression of both MMP-1 and MMP-3 expression, which are major factors in collagen degradation.

**Decorin-expressing adenovirus decreases collagen type I and III, elastin, and fibronectin protein expression in primary human keloid spheroids**

Keloid spheroids derived from active-stage keloid patients (n=3) were cultured and transduced with either dE1-RGD/GFP or dEl-RGD/GFP/DCN. The effect of decorin on expression of major ECM components of keloid was evaluated histologically. Masson’s trichrome and picrosirius red staining of keloid sections revealed that collagen deposition was decreased in spheroids transduced with dEl-RGD/GFP/DCN versus control virus (Fig. 4a). In addition, dense and coarse collagen bundles structure were replaced by thin and shallow collagen bundles. Image analysis of immunohistochemical staining also revealed significantly reduced type I collagen, type III collagen, elastin, and fibronectin in decorin-overexpressing keloid spheroids, by 24%, 28%, 22%, and 34%, respectively, versus control virus-transduced spheroids (*p*<0.05 in all cases; Fig. 4b and c). Taken together, these data strongly suggest that expressions of the major ECM components such as collagen type I and III, elastin, and fibronectin are significantly decreased by decorin overexpression in primary keloid tissues. These results imply that decorin plays a prominent role in ECM remodeling during keloid development.
DISCUSSION

Aberrant synthesis, accumulation, and organization of ECM molecules such as collagens, microfibrillar proteins, and proteoglycans, play significant roles in abnormal scar tissue formation. Excessive ECM accumulation resulting from an imbalance between ECM protein synthesis and degradation can lead to hypertrophic scars and keloids. TGF-β1 is a potent fibrogenic growth factor that plays a main role in keloid pathophysiology (6,9,10). Therefore, TGF-β1 represents an attractive therapeutic target for pharmacologic intervention of keloids. Inhibiting TGF-β1-dependent signaling, either by TGF-β1/TGF-β receptor neutralizing antibodies, truncated receptor, antisense oligonucleotides, and Smad2-/Smad3-specific siRNAs decrease procollagen gene expression and inhibit fibrosis progression (8,10,41-44). This implies that modulating TGF-β1 expression or signaling is a potential therapeutic approach for inhibiting keloid formation.

Decorin, a small proteoglycan with one dermatan sulfate sugar chain, is normally prevalent in the dermal ECM and has been proposed to be a physiological TGF-β1 inhibitor (24,27,29,41,45,46). Decorin interacts with collagen and influences collagen fibrillogenesis, resulting in thinner fibrils (19,28,45,47,48). Lower decorin levels exist in keloid tissues compared with normal skin (15,30-32); therefore, collagen bundles in keloid scars are thicker and more disorganized versus normal scar tissue (33). In agreement with this knowledge, our immunofluorescence assays also demonstrated markedly lower decorin expression in keloid tissues versus the adjacent normal tissues. Given the role of decorin as a natural neutralizer of TGF-β1, we hypothesized that reduced decorin expression in keloids stimulates TGF-β1 activity, resulting in dense and disorganized collagen in keloid tissues. Notably, our data showed that decorin overexpression by dE1-RGD/GFP/DCN viral vector...
transduction reduced secreted TGF-β1 protein in HDFs and collagen type I and III mRNAs in TGF-β1-treated HDFs. In addition, decorin expression significantly reduced collagen type I and III, elastin, and fibronectin protein expression in KF spheroids.

In addition to affecting TGF-β1 pathways, decorin also directly affects multiple signaling pathways that regulate cell growth, differentiation, and apoptosis (49,50). Decorin is also a ligand for modulating signals initiated by epidermal growth factor receptors (EGFR) (51-53), insulin-like growth factor-I receptors, and Met receptors (46,50,54). Among these, the EGF/EGFR signaling pathway plays important roles in keloid pathogenesis (55,56). EGF is a mitogen that also increases keloid fibroblast collagen and matrix protein levels (55,56), and induces cell motility and migration during normal wound repair. Decorin also reduces EGFR-regulated intracellular calcium mobilization (50-52). Decorin binds EGFR and induces receptor dimerization that leads to lysosomal degradation (50). Based on these previous studies and our results, we speculate that dE1-RGD/GFP/DCN-mediated decorin expression attenuates EGF/EGFR signaling pathways, which might be another potential mechanism underlying decorin’s therapeutic effect on keloids.

The major effectors of ECM degradation and remodeling are structurally-related MMP enzymes. The MMP family is composed of more than 20 Zn-dependent metalloproteinases that are responsible for cell adhesion, migration, proliferation, and differentiation (57-59). Among them, MMP-1 degrades the collagen triple helix, making this ECM component susceptible to proteolysis by gelatinases (MMP-2). Thus, increased MMP-1 and -2 productions likely play pivotal roles in eliminating abnormal or unfolded collagen during wound healing (14,60). In line with previous results (25,26), in our experiments, MMP-1 and
MMP-3 mRNA expression was significantly upregulated by decorin-expressing Ad in KFs, TGF-β1-stimulated HDFs, and keloid-derived fibroblasts. These results suggest that gene therapy using decorin-expressing Ad may therapeutically reverse the pathologic fibrosis of keloids. Particularly, this approach could decrease the postoperative keloid recurrence rates by increasing MMP-1 and -3 expressions, thereby decreasing abnormal collagen accumulation after surgical excision.

Despite positive results in animal models (23,27,28), decorin has not reached clinical testing yet. One of the biggest hurdles in translating these experimental results into the clinical setting is the short half-life of decorin protein and difficulty in producing clinical-grade decorin. To abrogate this limitation, we have generated and characterized a decorin-expressing and fiber-modified Ad (dE1-RGD/GFP/DCN) for treating pathologic fibroproliferative disorders such as keloids and hypertrophic scar. We think that gene therapy deserves much more positive attention and has great clinical potential for treating keloids, because Ad-mediated transgene expression can last up to 14 days without permanently altering dermal tissue (62,63). Thus, we assert that gene therapy using decorin-expressing Ad could have a therapeutic effect on keloids by inhibiting type I and III collagen and increasing MMP-1 and -3 expression in dermal tissues. Despite this role, the potential therapeutic use of decorin expressing adenovirus possesses limitations, such as a transient effect, which is not desirable to chronically persistent keloids, acute inflammatory responses, and the innate immune response. Exhaustive research efforts has prompted the development of novel strategies to overcome these limitations (64). Therefore, we think that further refinement studies will be needed for more efficient and safe gene transfer in patients.

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References


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Figure Legends

Figure 1. H&E staining and immunofluorescence staining of decorin in human keloid tissues.

Keloid tissue had a dense and excessive collagen deposition that extended over the clinical keloid margin into the extra-lesional dermal tissue (a-c). Decorin (red) stained intensely in the ECM of normal skin adjacent to the keloid (d) but was markedly decreased in keloid tissue (e). (f) The reduced expression of decorin was semi-quantitatively measured. Marked reduction of decorin expression in keloid region versus extra-lesional normal tissue (**p<0.01).

Figure 2. Construction of decorin-expressing adenovirus and enhanced gene transfer expression mediated by RGD-incorporated adenovirus. (a) Schematic representation of the decorin-expressing adenoviral vectors. The RGD-incorporated adenovirus was generated by inserting RGD motif between HI-loop of the fiber knob (star). (ITR = inverted terminal repeat; Ψ = packaging signal; pA = polyA sequence; IX = protein IX; and DCN = decorin) (b) GFP expression in human dermal fibroblasts (HDFs) and keloid fibroblasts (KFs) after transduction with dE1/GFP or dE1-RGD/GFP. Original magnification: x50. (c) The marked enhancement of GFP expression mediated RGD-incorporated adenovirus. The RGD-incorporated Ad vector led to 105-fold increase of GFP expression in HDFs compared with unmodified Ad at a multiplicity of infection (MOI) of 100, and 31-fold increase of GFP expression in primary KFs at 20 MOI. (d, e) Superiority of RGD-incorporated Ad for gene transfer.
transfer efficacy in keloid fibroblasts to control Ad. Whole image (Fig. 2d) and cross-sectioned image (Fig. 2e) of spheroids transduced with dE1/GFP, dE1-RGD/GFP, or dE1-RGD/GFP/DCN. (f) Western blot analysis of decorin protein in HDFs and KFs at 2 days after transduction with either dE1-RGD/GFP or dE1-RGD/GFP/DCN. Decorin levels were increased in HDFs and KFs transduced with 100 MOI and 200 MOI of decorin-expressing virus compared to control dE1-RGD/GFP. (g) Efficient secretion of decorin from HDFs transduced with dE1-RGD/GFP/DCN. Secreted decorin levels were increased in a dose-dependent manner at 2 days after transducing with dE1-RGD/GFP/DCN versus dE1-RGD/GFP (*p<0.01)

Figure 3. Transduction of decorin-expressing Ad is associated with decreased type I and III collagen mRNA levels and increased MMP-1 and MMP-3 mRNA expression in HDFs and KFs. (a) qRT–PCR analysis of type I collagen mRNA levels in KFs and HDFs treated with or without TGF-β1 (10 ng/mL, 2 days). Significantly reduced type I and III collagen mRNA was observed in cells treated with dE1-RGD/GFP/DCN (*p<0.05, **p<0.01) compared with control Ad -treated cells. (b) qRT–PCR analysis of type III collagen mRNA levels in KFs and HDFs treated with and without TGF-β1 (10 ng/mL, 2 days). Type III collagen mRNA was significantly reduced by decorin overexpression in both cell types (*p<0.05, **p<0.01). (c) Reduced TGF-β1 expression by decorin-expressing Ad. HDFs and KFs transduced with dE1-RGD/GFP/DCN showed decreased TGF-β1 expression compared with dE1-RGD/GFP-transduced or non-transduced control cells (*p<0.05 for both comparisons). Data are expressed as mean ± SEM of three experiments. (d) Decorin-expressing Ad decreases EGFR expression in HDFs and KFs. HDFs with/without pretreatment of TGF-β1 (10 ng/mL, 36 h) and KFs transduced with dE1-RGD/GFP/DCN (200 MOI) showed decreased EGFR expression compared with dE1-RGD/GFP-transduced or non-transduced control cells. Data This article is protected by copyright. All rights reserved.
shown are representative of three independent experiments. (e, f) Reduced expression of TGF-β1, EGFR, and Erk 1/2 protein in primary keloid spheroids by decorin-expressing adenovirus. The decreased expression of TGF-β1, EGFR, and Erk 1/2 were observed in keloid spheroids treated with dE1-RGD/GFP/DCN in comparison to those with control dE1-RGD/GFP (**p<0.01). Original magnification: x400. (g) Effect of decorin on EGF/EGFR signaling. dE1-RGD/GFP/DCN Ad decreased the expression levels of Akt, Erk 1/2, and phospho Erk 1/2 in HDFs compared with untreated or HDFs transduced with dE1-RGD/GFP control virus (100 and 200 MOI) regardless of exogenous EGF treatment (10 ng/mL). (h, i) Enhanced expression of MMP-1 and MMP-3 by decorin-expressing adenovirus in KFs and HDFs with or without treatment of TGF-β1 (10 ng/mL, 2 days). Both MMP-1 and MMP-3 mRNA levels were significantly increased in the cells treated with dE1-RGD/GFP/DCN compared with dE1-RGD/GFP (*p<0.05 and **p<0.01). Data are expressed as mean ± SEM of three experiments.

Figure 4. Immunohistochemical staining of keloid spheroid sections for type I and III collagen, elastin, and fibronectin protein from decorin-transduced keloid tissues. (a) Masson’s trichrome and Picosirius red staining of keloid spheroids. After dE1-RGD/GFP/DCN treatment of keloid spheroids, dense and coarse collagen bundles were replaced with thin and shallow collagen bundles. Original magnification: x400. (b) Reduced expression of ECM components including collagen type I and III, elastin, and fibronectin protein in keloid spheroids transduced with dE1-RGD/GFP/DCN compared to those in spheroids transduced with control dE1-RGD/GFP. Original magnification: 400×. (c) Semi-quantitative image analysis for type I and III collagen, elastin, and fibronectin protein expression. Significantly reduced type I collagen, type III collagen, elastin, and fibronectin

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were observed in keloid spheroids transduced with dE1-RGD/GFP/DCN versus spheroids transduced with control virus (*\(p<0.05\)).

### Table 1. Demographic information and description of the keloids from the patients who participated in this study.

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**Supplementary Figure Legends**

**Figure S1.** Significantly reduced decorin expression in three keloid patients. Compared to extra-lesional normal tissue, markedly decreased decorin immunoreactivity (red) was observed in keloid region. Original magnification: x400.

**Figure S2.** Complete membrane image to show decorin expression in HDFs at 2 days after transduction with either dE1-RGD/GFP or dE1-RGD/GFP/DCN.

**Figure S3.** Decorin-expressing adenovirus down-regulates type I and III collagen mRNAs, and increases MMP-1 and -3 mRNA expression in four primary keloid-derived fibroblasts.

**Figure S4.** (a) Masson’s trichrome and (b) Picosirius red staining in keloid spheroids. Significant reduced dense staining was observed in keloid spheroids transduced with dE1-RGD/GFP/DCN compared to dE1-RGD/GFP. Original magnification: 50x, 100x, and 400x.

**Figure S5.** Reduced HER2 expression in KFs and HDFs treated with or without TGF-β1 (10 ng/mL) by decorin-expressing adenovirus.
Figure 1

(f)

Total Integrated Optical Density

Adjacent normal region          Keloid region

0  10000  20000  30000  40000  50000  60000  70000
Figure 2

(c)

HDFs

GFP expression (mean)

KFs

GFP expression (mean)

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Figure 2

(g)

![Bar chart showing Decorin levels in different conditions.](chart.png)

- Untreated
- dE1-RGD/GFP
- dE1-RGD/GFP/DCN
Figure 3

(a) Collagen type-I

(b) Collagen type-III

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Figure 3

(g)

HDFs

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HDFs + TGF-β1

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1. Untreated 2. dE1-RGD/GFP (100 MOI) 3. dE1-RGD/GFP/DCN (100 MOI) 4. dE1-RGD/GFP (200 MOI) 5. dE1-RGD/GFP/DCN (200 MOI) 6. Untreated + EGF (10 ng/mL) 7. dE1-RGD/GFP (200 MOI) + EGF (10 ng/mL) 8. dE1-RGD/GFP/DCN (200 MOI) + EGF (10 ng/mL)
Figure 4

(c) Collagen type-I

Collagen type-III

Elastin

Fibronectin

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