Decerin Antisense Gene Therapy Improves Functional Healing of Early Rabbit Ligament Scar with Enhanced Collagen Fibrillogenesis \textit{In Vivo}


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\textbf{Summary:} Injured ligaments heal with scar tissue, which has mechanical properties inferior to those of normal ligament, potentially resulting in re-injury, joint instability, and subsequent degenerative arthritis. In ligament scars, normal large-diameter collagen fibrils have been shown to be replaced by a homogenous population of small collagen fibrils. Because collagen is a major tensile load-bearing matrix element and because the proteoglycan decorin is known to inhibit collagen fibrillogenesis, we hypothesized that the restoration of larger collagen fibrils in a rabbit ligament scar, by down-regulating the proteoglycan decorin, would improve the mechanical properties of scar. In contrast to sense and injection-treated controls, \textit{in vivo} treatment of injured ligament by antisense decorin oligodeoxynucleotides led to an increased development of larger collagen fibrils in early scar and a significant improvement in both scar failure strength (83-85\% improvement at 6 weeks; \(p < 0.01\)) and scar creep elongation (33-48\% less irrecoverable creep; \(p < 0.03\)) under loading. This is the first report that \textit{in vivo} manipulation of collagen fibrillogenesis improves tissue function during repair processes with gene therapy. These findings not only suggest the potential use of this type of approach to improve the healing of various soft tissues (skin, ligament, tendon, and so on) but also support the use of such methods to better understand specific structure-function relationships in scars.

Ligament injuries are one of the most common injuries to joints, but the optimal conditions for promoting their healing remain unknown. Previous studies have shown that ligaments heal with scar that is similar in some respects to scar elsewhere in the body (skin, tendon, and so on) but mechanically inferior to normal tissue (11). Specifically, healing of a rabbit medial collateral ligament reaches only 30-40\% of normal material strength at 1 year after injury (8,11). Furthermore, some studies suggest that healing ligaments and some ligament grafts may also stretch out to an abnormal functional length as a result of normal daily activity (12,18). Irrecoverable creep is the engineering term for this type of permanent elongation after constant or repetitive loading. Because a major function of ligaments is to stabilize joints and because weak or loose ligaments can lead to re-injury, joint instability, and degenerative arthritis (4), inferior biomechanical qualities of ligament scar are a serious clinical concern.

Of the various components of connective tissue, collagen plays a key role as the major tensile load-bearing element. Therefore, qualitative and quantitative alterations of the collagenous matrix during scar formation are likely sources of altered tissue mechanical properties after injury. Previous studies have shown that soft-tissue scars have common alterations in biochemical properties compared with normal tissues, such as changes in collagen types (decreased type I and elevated types III and V) and abnormal collagen crosslinking (9,11,17). Morphologically, soft-tissue scars also demonstrate similar changes in the organization of their collagen. With scarring, normal large-diameter collagen fibrils in soft tissues are replaced by relatively small collagen fibrils (10), although a gradual gain in collagen fibril size has been reported in skin scars within weeks (14). However, ligament scars contain only homogenous small fibrils for months to years after injury (10). Previous studies have suggested that the presence of large-diameter collagen fibrils in normal and healing soft tissues could correlate with their mechanical strength (6,23). Therefore, production of larger collagen fibrils in soft-tissue scars could potentially improve their mechani-
cral properties. However, no studies regarding the in vivo manipulation of the diameter of newly synthesized collagen fibrils during tissue repair processes have been reported.

The interaction of collagen molecules with proteoglycans is one mechanism implicated in collagen fibrillogenesis. Members of the small leucine-rich proteoglycans, decorin, fibromodulin, and lumican have been suggested to inhibit collagen fibrillogenesis (15). Specifically, the results from in vitro binding experiments, molecular and biochemical analyses of tendon development, and knockout mouse studies collectively suggest the involvement of decorin in collagen fibril assembly (5,30). Furthermore, decorin mRNA (1) and protein (19) have been observed in ligament scar. On the basis of this background, we hypothesized that inhibition of decorin expression during early stages of ligament healing would potentially enhance the assembly of newly synthesized collagen fibrils and thus improve the quality of ligament scar tissue.

The antisense method, which blocks the transcription or translation of specific genes by binding of oligodeoxynucleotides to target mRNA (31), was used for this purpose. To increase the efficiency of gene transfer, a highly efficient hemagglutinating virus of Japan (HVJ)-liposome method has been developed (7,16). HVJ promotes fusion of the liposome with target cells and delivers the oligodeoxynucleotides into the cells (22). We previously reported that this method can be applied to healing ligament by a direct injection of HVJ-liposomes into the ligament scar (19,21). We then established an efficient model for in vivo oligodeoxynucleotide delivery into rabbit medial collateral ligament scar and confirmed the specific suppression of decorin at both mRNA and protein levels over 4 weeks after introduction of antisense decorin oligodeoxynucleotides (20). In the present study, we report the morphological and biomechanical effects of this antisense treatment on early healing of medial collateral ligament using the same model.

**MATERIALS AND METHODS**

**Synthesis of oligodeoxynucleotides and selection of sequence targets:** The sequences of oligodeoxynucleotides for rabbit decorin were antisense, 5'-GGATGAGTTTGGCCCATG-3' and sense, 5'-CCATGACGCGAACCTCTCC-3' (−1 to +19 of rabbit sequence). The antisense oligodeoxynucleotides specifically inhibit decorin mRNA in primary cultured cells from rabbit medial collateral ligament scar (20). All the oligodeoxynucleotides and polymerase chain reaction primers were synthesized and purified by the University of Calgary Regional DNA Synthesis Laboratory.

**Preparation of HVJ-liposomes:** HVJ-liposomes were prepared as described previously (20). Phosphatidyicholine, phosphatidylserine, and cholesterol were mixed in a weight ratio of 1:4.8:2, and dried lipid was rehydrated in balanced salt solution containing sense or antisense oligodeoxynucleotides. The mixture was agitated and sonicated to prepare unilamellar liposomes, which were then incubated with HVJ that had been previously inactivated with ultraviolet irradiation to allow for the formation of HVJ-liposome complexes. The mixture was incubated at 4°C for 10 minutes, and the volume was adjusted to 4 ml and incubated for 60 minutes with gentle shaking at 37°C. Free virus was removed from the complexes by sucrose density gradient centrifugation prior to the in vivo injection.

**In vivo transfer of oligodeoxynucleotides:** Standardized bilateral medial collateral ligament gaps were surgically created in 39 12-month-old female New Zealand White rabbits by a technique previously described in detail (1). The four corners of the cut ends of the gap were marked with sutures to identify the scar edge for tissue sampling. After 2 weeks of healing, the animals were randomly placed in one of three groups (n = 13 per group): antisense or sense-treated and an injection control group. Transfected scars received 500 μl of 10 μM oligodeoxynucleotide complexes by a previously standardized technique (19), and the scar tissue of the controls was needle-poked without solution. The animals were allowed to return to normal cage activity after treatment. All the animals were handled according to established ethical guidelines approved by the local animal care committee and were killed with a phenobarbital overdose (Ethanyl 275 mg/kg; MTC Pharmaceuticals, Cambridge, Ontario, Canada) 4 weeks after the injection (6 weeks after the initial injury). Six rabbits from each group were assigned for morphological assessment and reverse transcription-polymerase chain reaction analysis, and the remaining seven rabbits of each group were assigned for biomechanical analyses.

**Tissue sampling:** Scar tissue between the marking sutures was isolated and then divided into specific portions for the following analyses: transmission electron microscopy (1 × 1 × 3 mm), sampled from the center area; histochemistry, sampled from each parasagittal area; and reverse transcription-polymerase chain reaction analysis, including all remaining tissue. Six normal rabbit medial collateral ligaments were sampled similarly for morphological analyses.

**Histochemistry:** Specimens were mounted in embedding medium (Tissue-Tek; Sakura Finetek, Torrance, CA, U.S.A.), and cryosections (6 μm thick) were prepared. Hematoxylin and eosin staining was used to assess gross matrix organization and tissue crimp (29) with a polarizing microscope.

**Transmission electron microscopy:** The specimens were fixed in modified Karnovsky’s fixative followed by 1% osmium tetroxide in the same buffer, stained en bloc overnight with 0.5% uranyl acetate in 50% ethanol, dehydrated in graded ethanol, and embedded in Spurr’s epoxy resin. Ultrathin sections of 50-90 nm were stained with uranyl acetate and lead citrate for 15 minutes and analyzed with an H-600 electron microscope operating at 50 kV (11). For quantitative studies, collagen fibril diameters were measured on photographic negatives with a calibrated final magnification of ×30,000. A total of 30,921 randomly selected collagen fibrils (20,246 from six antisense-treated scars, 4,465 from six sense-treated control scars, 4,054 from six injection controls, and 2,156 from six normal medial collateral ligaments) were measured, and histograms were generated by an image analysis system (Kontron Elektronik, Munich, Germany).

**Reverse transcription-polymerase chain reaction analysis:** Semi-quantitative reverse transcription-polymerase chain reaction analysis was performed on RNA extracted from the scar tissues as previously reported in detail (1,25) with use of primer sets specific for rabbit decorin and a housekeeping gene, glyceraldehyde-3-phosphate-dehydrogenase. The rabbit decorin 5' primer (nucleotides 780-800) was 5' - TGT-GGA-CAA-TTG-TTC-TCT-GG-3' and the 3' primer (nucleotides 1239-1250) was 5' - CCA-CAT-TGC-AGT-TAG-GTT-CC-3'. The rabbit glyceraldehyde-3-phosphate-dehydrogenase 5' primer (nucleotides 211-230 of rabbit sequence)
FIG. 1. Hematoxylin and eosin staining of A: normal medial collateral ligament, C: 6-week injection control scar, E: sense-treated control scar, and G: antisense-treated scar. B, D, F, and H are polarizing microscopy in the corresponding field to A, C, E, and G. Relatively well aligned collagenous matrix (G) with limited but distinct restoration of crimp pattern (H) is observed in the antisense-treated scar, whereas only disorganized collagenous matrix with a micro-crimp pattern is observed in the injection (C and D) and sense-treated (E and F) control scars (×250).

was 5'-TCA-TCT-TCC-AGG-AGC-GA-3' and the 3' primer (nucleotides 488-507) was 5'-CAC-AAT-GCC-GAA-GTG-GTC- GT-3'. Reaction mixtures were assessed by 2% agarose gel electrophoresis and ethidium bromide staining and were then quantitated with a Masterscan Interpretive Densitometer (CSPI, Billerica, MA, U.S.A.). The normalization of decorin mRNA to glyceraldehyde-3-phosphate-dehydrogenase mRNA in each group was calculated from triplicate experiments. To test the correlation between the mean collagen fibril diameter and normalized decorin mRNA levels in each scar (n = 18), analysis of variance (ANOVA) and Pearson's correlation coefficient were used with a significance level of p < 0.05.

Biomechanical analysis: Dissected femur-medial collateral ligament-tibia complexes were mounted on a specialized closed-loop, servohydraulic material testing system (MTS Systems, Minneapolis, MN, U.S.A.) at a joint angle of 70° of flexion. These complexes were first cycled between 2 and −5 N at 1 mm/min to determine ligament zero, which was identified as the crosshead position where the ligament began to take up 0.1 N load. Next, the ligament length and tissue cross-sectional area were measured (26). The specimens were then enclosed in an environmental chamber (37°C and 99% relative humidity). The creep protocol (28) involved 30 cycles of cyclic loading at 1 Hz to a stress level of 2.2 MPa (cyclic creep), followed by 20 minutes of being held at a constant stress of 2.2 MPa (static creep). This stress level is approximately 2% of the failure strength of a normal rabbit medial collateral ligament and represents the estimated normal tensile loads carried by it in vivo (33). The creep test load for each ligament scar was calculated on the basis of cross-sectional area, which was not statistically different among these groups (antisense: 7.64 ± 0.90 mm², sense: 9.40 ± 1.69 mm², and injection control: 7.98 ± 1.72 mm²). Strain was defined as the measured deformation of the ligament divided by the undeformed ligament length measured at ligament zero. Total creep strain (resulting from both the cyclic and static creep tests) was defined as the strain at the end of the static creep test minus the strain at the peak of the first cycle of the cyclic creep test. After creep testing, the ligament was allowed to recover at 0 N load for 20 minutes. The residual strain after this period was defined as irrecoverable creep. After the recovery period, tensile failure tests were performed at a crosshead displacement rate of 20 mm/min. To assess the material properties of tissue, failure strength was calculated as the failure load divided by the measured cross-sectional area of the scar. All of the mechanical testing was performed in a blinded fashion, and outcomes were analyzed statistically by ANOVA (single factor) followed by Bonferroni cor-

FIG. 2. A: Transmission electron micrographs of normal medial collateral ligament, B: 6-week injection control scar, C: sense-treated control scar, and D-F: antisense-treated scar. Although both the injection control and sense-treated control scar contain only small collagen fibrils (B and C), antisense-treated scars contain large fibrils (D and E), some of which are comparable with those in normal ligament (A and D) (×30,000).
rejection for multiple comparisons with a significance at p < 0.05.

RESULTS

Matrix organization and crimp analysis: In the normal uninjured medial collateral ligament, collagen was well aligned in bundles along the longitudinal axis of the ligament with an organized crimp pattern (Fig. 1A and B). In all injection control and sense-treated control scars, the collagenous matrix was disorganized with a relatively homogenous micro-crimp pattern (Fig. 1C-F) as previously reported in tendon scars (29). In contrast, most antisense-treated scars contained detectable bundles of aligned collagen with some restoration of a more normal crimp pattern. Four of six antisense-treated scars contained these collagen bundles; two of these scars had collagen bundles throughout the length of the scar (Fig. 1G and H) and the other two had them only in some areas (data not shown). Two antisense-treated scars were indistinguishable from injection control or sense-treated control scars (data not shown).

Transmission electron microscopic analysis: Normal medial collateral ligament contained a bimodal distribution of both large and small-diameter collagen fibrils, whereas injection control scars contained uniformly small collagen fibrils (Fig. 2A and B). Collagen fibrils in sense-treated control scars also contained only small fibrils (Fig. 2C). However, some larger collagen fibrils were seen in five of six antisense-treated scars. The degree of collagen fibril increase varied according to the location (Fig. 2D and E), and no larger fibrils were observed in some areas (Fig. 2F). Furthermore, only small collagen fibrils were observed in one antisense-treated scar (data not shown). These results suggest that collagen fibril assembly was not improved in every scar or in the whole area of each scar.

FIG. 4. Correlation between the normalized levels of decorin mRNA and mean collagen fibril diameters among all the ligament scar specimens (n = 18). The inverse correlation between the expression levels of decorin mRNA and collagen fibril diameters was significant (p < 0.000073). GAPDH = glyceraldehyde-3-phosphate-dehydrogenase.
by this single antisense treatment. Analysis of collagen fibril diameter demonstrated that the average size in antisense-treated scars, sense-treated control scars, injection control scars, and normal medial collateral ligament was 104.7 ± 51.1 nm (n = 20,246), 74.8 ± 11.0 nm (n = 4,465), 78.2 ± 11.9 nm (n = 4,054), and 189.1 ± 104.0 nm (n = 2,156), respectively. Notably, antisense-treated scars exhibited a wider range; profiles ranged from 25 to 300 nm, similar to those of normal ligament. Approximately 38% of the tissue from normal medial collateral ligament contained collagen fibrils with diameters greater than 125 nm, whereas 14% of the collagen fibrils in the antisense-treated scars had diameters greater than 125 nm. In contrast, sense-treated control and injection control scars contained nearly identical profiles, which ranged from 25 to 125 nm (Fig. 3).

Correlation between suppression of decorin mRNA and increase in collagen fibril size in antisense-treated scar: With the same animal model, we previously reported that this antisense decorin treatment resulted in a significant suppression of decorin mRNA and protein levels over a period of 4 weeks after treatment (20). To evaluate a more precise correlation between the suppression of decorin and the change of collagen fibril size, we performed a correlation analysis using mean collagen fibril diameter and decorin mRNA levels in each scar specimen that was examined. There was a significant inverse correlation between the mean collagen fibril diameter and the expression levels of decorin mRNA (R² = 0.6365, p < 0.000073) (Fig. 4).

Effect of decorin antisense therapy on mechanical properties of healing ligament: To evaluate the functional effects of these morphological changes, two major mechanical properties of scars were quantified. Creep and failure properties were assessed because the irrecoverable creep of a ligament scar may increase its functional length in a joint, and scar weakness is a potential contributor to the risk of reinjury. First, we quantified the elongation (creep) of ligament scars subjected to a low stress (2.2 MPa). Antisense-treated scars were significantly less susceptible to creep than sense-treated control and injection control scars (by 18-22%) (Fig. 5A). Second, we measured the residual elongation after loading had been removed and the ligament scar was allowed a period of recovery. Antisense-treated scars showed 33-48% less irrecoverable creep than sense-treated control or injection control scars (Fig. 5A). Finally, we performed failure testing to quantify scar failure strength. Antisense-treated scars failed at 14.9 ± 6.62 MPa on average, which was significantly higher (by 83-85%) than sense-treated control (8.07 ± 3.45 MPa) and injection control (8.16 ± 3.86 MPa) scars (Fig. 5B). Normal medial collateral ligaments, however, fail at approximately 70-80 MPa (8,10).

DISCUSSION

In this study, we demonstrate a novel therapeutic strategy to improve the quality of early ligament scar by in vivo introduction of decorin antisense oligodeoxynucleotides into the scar tissue. To our knowledge, this is the first demonstration of in vivo manipulation of collagen fibrillogenesis during the repair processes of soft connective tissue, as well as the first report that such a treatment can improve the functional properties of scar. Scarring is the ubiquitous process by which all soft connective tissues (e.g., skin, ligament, tendon, and so on) heal; therefore, this observation may have considerable significance.

Specifically, in this experiment a single dose of decorin antisense treatment in the animal model studied resulted in the suppression of decorin (20) and the development of larger diameter collagen fibrils in ligament scars within 4 weeks of the treatment. To in-
vestigate a more precise correlation between the suppression of decorin and changed size of collagen fibrils, we compared the expression level of decorin mRNA with the mean collagen fibril diameter in scars and found a significant inverse correlation. Although we lack the data on the specific decorin protein level compared with collagen fibril size in the samples reported here, our previous work has shown that decorin mRNA and protein were both suppressed at 4 weeks following the same antisense treatment (20). We therefore speculate that there may also be a relationship between decorin expression level and collagen fibril size in vivo during early healing processes. Also, this result supports previous work that has suggested some role of decorin in the regulation of collagen fibril size (5,30).

In contrast to the results of a decorin knockout study in mice (5), extremely large fibrils (>600 nm) with multiple and concurrent lateral fusion of fibrils were not observed in antisense-treated scars at 4 weeks after treatment. Instead, only moderately large fibrils (125-300 nm) were observed. Such discrepancies could be due to the fact that the knockout was obtained in the mouse and not in the rabbit and that the lack of decorin in the former occurred throughout the entire embryonic development and not in a small window of time in the adult animal. Spatial differences in the enlargement of collagen fibril sizes in antisense-treated scars could similarly be explained by variable down-regulation of decorin between cells within the scar, possibly due to nonuniform transfection with the antisense oligodeoxynucleotides.

To test for a functional effect of these morphological changes, we performed both creep and failure strength analyses. The results demonstrate that the antisense treatment leads to increases in strength in early medial collateral ligament scar in the rabbit. This result also supports the hypothesis of a correlation between tensile strength and the presence of large-diameter collagen fibrils in soft tissues (6,23). In vivo, ligaments are likely repetitively loaded to a constant low stress during ambulation (13), and therefore repeated loads could create conditions favorable for creep in ligament scars. The results of creep tests showed significantly less creep and significantly better creep recovery by antisense-treated scars than by sense or injection controls. Therefore, antisense decorin therapy could potentially help protect early ligament scars from stretching out under repeated stress. If optimized, improvements in creep resistance and strength may allow earlier or more aggressive rehabilitation of an injured joint with less risk of reinjury. Although the mechanisms responsible for these improved creep behaviors resulting from the antisense treatment are not yet clear, they may include changes in the ultrastructure or organization of collagen fibrils.

Unexpectedly, although it varied somewhat among the rabbits within the group, the development of collagen bundles was observed in some antisense-treated scars with a partially restored tissue crimp pattern. As far as we know, there have been no directly supportive data in the literature to explain the mechanisms underlying this event. Interestingly, decorin is known to associate with type-VI collagen in vivo (27), and this collagen has been found in relatively large amounts in the interfibrillar spaces of ligaments co-localized with chondroitin sulfate proteoglycan (2). Decorin also interacts with fibronectin (24), one of the major noncollagenous matrix proteins in healing soft tissue (32). A recent study has suggested that decorin/fibronectin binding might be involved in the regulation of interfibrillar bonding of collagens (3). Collectively, loss of decorin in scar tissue might somehow alter those interactions, thereby enhancing the packing of collagen fibrils into more highly ordered structures and altering the interaction between collagen fibrils and the interfibrillar matrix.

Before attributing all of the changes observed to decorin suppression alone, it must be pointed out that the ratio of decorin to other matrix molecules might be a critical factor in vivo. In fact, although the oligodeoxynucleotides were designed to be specific for decorin on the basis of the available evidence, it is possible that secondary regulation of other molecules could be involved in vivo. Further characterization will be required before concluding that the results were due only to modulation of decorin expression.

Finally, this technique has not yet been fully optimized for this application. In part due to the likely limited transfection efficiency of a single injection in vivo (20), scars were not uniformly transformed by the oligodeoxynucleotides. Subsequently, the enlargement of collagen fibril sizes was not observed in every scar or in the entire area of antisense-treated scars. Perhaps not surprisingly, therefore, the recovery of biomechanical properties was only partial. With further optimization of the dosage, timing, and delivery techniques, this antisense therapy appears to have great potential not only to contribute to the better understanding of the structure-function relationships in connective tissue scar but also to significantly enhance the quality of soft-tissue healing in vivo.

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