Functional improvement in ligament scar tissue following antisense gene therapy: A model system for in vivo engineering of connective tissues

Review Article

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Summary

The present studies indicate that decorin antisense approaches and a HVJ-liposome delivery system can functionally impact on the mechanical properties of MCL scar tissue in a rabbit model. This is one of the first instances where such approaches have led to functional improvement in the tissue. While decorin antisense gene therapy has been shown to be effective at improving MCL scar tissue, the resulting tissue still remains deficient compared to normal tissue. Therefore, this study must be considered a first step towards tissue regeneration and additional experimentation is required to further move scar tissue down the path towards tissue regeneration.

I. Introduction

Overt injuries to joint tissues such as ligaments, tendons, menisci, and cartilage are major clinical problems which occur with significant frequency. In some instances, such as the extraarticular ligaments and tendons, the injured tissues heal with the formation of scar tissue (Frank, 1996; Frank et al, 1983,1994,1999). A number of the other injured tissues either heal poorly or not at all. The latter scenario may be due to the loss of a template to promote the healing process (ie: the ruptured anterior cruciate ligament (ACL) of the knee) or due to the fact that some of these tissues are either hypo- or aneural and are hypo- or avascular (cartilage, menisci). Even those tissues that do initiate scar formation following injury remain functionally compromised for extended periods of time due to the inferior mechanical and biochemical properties of the scar tissue (reviewed in Frank, 1996; Frank et al, 1983,1994,1999). Furthermore, rupture of ligaments such as the ACL often require reconstruction with autografts derived from other tissues (ie: patellar tendon) and these autografts gradually become infiltrated with scar tissue, a process which appears to lead to altered biomechanical function of the tissue (reviewed in Frank et al, 1999).

One of the consequences of compromised biomechanical integrity in the tissues discussed above is impaired function and increased risk of re-injury, induction of remodeling in other joint tissues, and development of diseases such as osteoarthritis. Therefore, development of new therapies to enhance repair or replace damaged joint tissues would have an impact on the quality of life for a large number of individuals of all ages.

Considerable effort is currently on-going to develop both in vivo and in vitro methods to either improve healing of connective tissues (ie: pleiotrophic stem cells, in vivo delivery of growth factors/cytokines, drugs) or to replace damaged tissues (ie: in vitro tissue engineering, in vitro expansion of endogenous cells). In recent years, this effort has involved the use of gene therapy approaches. While much of this effort has focussed on replacing damaged tissue, not as much effort has been concentrated on improving in vivo healing to prevent development of joint disease. In part, this is likely due to the difficulties inherent in delivering

effective concentrations of antisense ODN or transgenes to such hypocellular and dense connective tissues.

Our initiative in the area of employing gene therapy approaches to improve in vivo connective tissue healing has involved the well characterized rabbit medial collateral ligament (MCL) injury model (Boykiw et al, 1998; Frank, 1996; Frank et al, 1983, 1992, 1994, 1999; Sciore et al, 1998). We chose an antisense gene therapy approach to transiently modulate the expression of specific molecules during the early stages of healing where there is significant hypercellularity and the density of the scar tissue is less than that of normal tissue. We did not want to permanently alter the target tissue, but instead wanted to temporarily influence the scar tissue until it had regained more normal properties. This model has been subjected to multidisciplinary characterization (histology, biochemistry, molecular biology and biomechanical assessment) and therefore lends itself to complete evaluation of the functional impact of the gene therapy interventions.

In the present report, we present the results of studies using antisense ODN directed toward the small matrix proteoglycan, decorin. Decorin has been implicated in extracellular matrix formation and organization both directly via collagen fibril assembly (Vogel et al, 1984; discussed in Nakamura et al, 1998b) and indirectly through its ability to bind and functionally inactivate growth factors such as TGF-beta (Harper et al, 1984; Lysiak et al, 1995). The results indicate that is is possible to functionally improve the properties of ligament scar tissue using decorin-specific antisense ODN and a hemmaglutinating virus of Japan-liposome (HVJ-liposome) delivery system (discussed in Nakamura et al, 1996, 1998, 1998a, 1998b; Tomita et al, 1997).

II. The model

The rabbit bilateral MCL gap injury model has been well characterized over the past 15 years using multidisciplinary methodology (reviewed in Frank et al, 1983, 1994, 1999). In the present study, skeletally mature female animals had 4 mm gap injuries to their MCL surgically induced at T = 0. Two weeks later, each MCL was injected with either a decorin antisense ODN (10 uM ODN in HVJ-liposomes; 5'-GGA-TGA-GAG-TTG-CCG-TCA-TG-3'), a decorin sense ODN (10 uM ODN in HVJ-liposomes; 5'-CAT-GAC-GGC-AAC-TCT-CAT-CC-3'), or was "poked" with a needle the same number of times as the antisense and sense treated animals (injection controls for reinjury to the scar tissue). The optimal sequence and concentration of ODN was determined in preliminary experiments with rabbit MCL scar cells in vitro and the delivery system was that reported previously (Nakamura et al, 1998b). Four weeks post-treatment (6 weeks post-injury), the animals were sacrificed and the scar tissue assessed using semiquantitative RT-PCR

for decorin, decorin protein levels in the tissue, morphologic methods (light and transmission electron microscopy), and well established biomechanical techniques to measure both low and high load behavior of the scar tissue (reviewed in Frank et al, 1999).

In a second set of experiments, rabbits with bilateral MCL injuries were exposed chronically (21 days, 2.5 ng TGF-beta/day) to exogenous human TGF-beta (Dr. Paul Gladstone, Bristol Myers-Squibb, Seattle, WA) or saline delivered via implanted ALZET pumps from day 0 to day 21, or to a single bolus injection of 7 µg TGF-beta delivered to the scar tissue at 3 weeks post-injury. Animals were sacrificed at 6 weeks post injury and assessed as above.

III. Influence of decorin antisense ODN on decorin mRNA and protein levels in MCL scar tissue in vivo

Total RNA was isolated from MCL tissue by the TRIspin method (Reno et al, 1997) and assessed for decorin mRNA levels by semiquantitative RT-PCR as described previously (Sciore et al, 1998, Boykiw et al, 1998, Reno et al, 1998). Treatment of MCL scar tissue with decorin antisense ODN led to significant depression of decorin mRNA levels compared to those in the sense ODN treated samples and the injection controls ($p < 0.025$). There was no significant difference between decorin mRNA levels in the sense ODN treated samples and the injection controls.

Protein was extracted from MCL scar tissue, separated by SDS-PAGE, transblotted and assessed semiquantitatively using Western Blot analysis and an anti-decorin antibody (Dr. Paul Scott; University of Alberta; Edmonton, AB). Decorin protein levels were also signficantly depressed in the antisense treated scar tissue compared to the sense ODN treated samples ($p = 0.045$). Thus, the decorin antisense ODN treatment led to a persistant depression in decorin mRNA levels even at 4 weeks post-injection of the tissue and this was correlated with a depression in decorin protein levels in the scar tissue.

IV. Effect of decorin antisense ODN on collagen fibril diameters in MCL scar tissue

Collagen fibril diameters were assessed by transmission electron microscopy and image analysis as described previously (Cunningham et al, 1999; Frank et al 1992). Normal MCL tissue from skeletally mature animals express a bimodal distribution of both large and small diameter collagen fibrils (reviewed in Frank et al, 1992, 1994, 1999). MCL scar tissue expresses a unimodal population of small diameter collagen fibrils and these persist out to approximately 2 years post-injury when a few large diameter fibrils are again detected (Frank et al, 1992).

Figure 1. Distribution of collagen fibril diameters in MCL tissues. Fibril diameters were assessed by TEM.

In the sense ODN treated scars, as well as the injection control scars, only a unimodal population of small diameter collagen fibrils were detected (**Figure 1**). In contrast, some large diameter collagen fibrils were detected in 6 week scar tissue exposed to the decorin antisense ODN (**Figure 1**). Collagen fibril diameter analysis demonstrated that the average fibril diameter in antisense treated scars, sense treated scars, injection controls and normal MCL tissue was, 104.7 +/- 51.1 (n = 20246), 74.8 +/- 11.0 (n = 4465), 78.2 +/- 11.9 (n = 4054) and 189.1 +/-104 (n = 2156), respectively. Approximately 38% of the fibrils in normal MCL tissue had diameters >125 nm, while 14% of the fibrils in the antisense treated scars had diameters >125 nm. In contrast, collagen fibril diameters > 125 nm were not detected in the sense ODN treated scars or the injection control scars.

To determine whether there was a correlation between mean collagen diameter and mRNA levels for decorin, a correlation analysis was performed. There was a significant inverse correlation between the mean collagen fibril diameter and the expression level of decorin mRNA (p = 0.000073). Therefore, the antisense-mediated depression in decorin mRNA levels was significantly correlated with the observed changes in collagen fibril diameter.

V. Influence of decorin antisense ODN on the biomechanical properties of MCL scar tissue

To evaluate the functional impact of the depression in decorin mRNA and the increase in large diameter collagen fibrils observed in the antisense treated scar tissue, ligament scar creep (low load behavior) (Thornton et al, in press) and stress at failure (high load behavior) (discussed in Frank et al, 1983, 1999) were assessed using established methods in an MTS material testing machine (MTS Systems, Inc.; Minneapolis, MN). Normal MCL tissue exhibits significantly less creep than does early MCL scar tissue (Thorton al, in press). As shown in **Figure 2**, treatment of the MCL scars with decorin antisense ODN resulted in the tissue exhibiting significantly less creep than either the sense treated scars or the injection controls. Such a decrease in creep behavior could indicate an early scar tissue which would be more resistant to "stretching out" (ie: more normal ligament behavior). As this viscoelastic/creep behavior is likely related to matrix proteoglycans and tissue organization, these observed changes following antisense treatment may relate to decorin-mediated alterations in the mixture and organization of these important molecules.

Furthermore, the antisense treated scars also exhibited a significantly higher stress at failure than did the sense treated scars or the injection controls (**Figure 2**). As this high load behavior is, at least partially, likely related to collagen

organization and collagen fibril diameters, this increase in high load behavior is consistant with the observed increase in the large diameter collagen fibrils observed in the antisense treated scars (discussed above).

These results indicate that the antisense treatment led to a functional improvement in the MCL scar tissue at 6 weeks post-injury. Preliminary experiments assessing scar function at 14 weeks post-injury and 12 weeks post-antisense treatment have indicated that the scar tissue continues to be functionally better than the sense treated or the injection controls.

VI. Influence of exogenous TGF-beta on MCL scar tissue

The above decribed effects of decorin antisense therapy could be a direct influence of decorin on collagen fibril assembly, and subsequent alteration in tissue biomechanics. Alternatively, since decorin has a number of biological activities, the observed changes in collagen assembly could be an indirect effect of decreasing decorin levels in the scar tissue. Thus, a decrease in decorin levels could lead to a "cascade" effect in which the activity of other biologically important molecules were modified and the antisense therapy effect due to these molecules rather than decorin itself. One possibility in this regard is the growth factor transforming growth factor beta.

As mentioned earlier, decorin binds TGF-beta and can functionally decrease the availability of this growth factor in tissues (Giri et al, 1997; Harper et al, 1984; Lysiak et al, 1995; Peters et al, 1997; Stander et al, 1999).

Administration of decorin to the lungs of bleomycin treated rats can inhibit development of TGF-beta mediated pulmonary fibrosis (Giri et al, 1997). Therefore, it was possible that decreasing decorin levels in the antisense treated scars led to increased levels of functional TGF-beta, and the observed functional effects were indirect and actually mediated by TGF-beta. It is known that TGF-beta mRNA levels are elevated in MCL scar tissue at 3 weeks post-injury (Sciore et al, 1998) and therefore, there is likely an increased supply of this growth factor in early scar tissue.

To address this issue, two sets of experiments were performed. In the first, MCL scar tissue was exposed to 2.5 ng TGF-beta/day from day 0 to day 21 via implanted ALZET pumps. The purpose of this experiment was to assess the effect of TGF-beta on very early scar development. However, when the MCL tissues were assessed at 6 weeks post-injury, the TGF-beta exposure had no effect on either collagen fibril diameters or the biomechanical properties of the tissue (both low and high load behavior).

In the second set of experiments, animals were exposed to a bolus of 7 μg of TGF-beta injected into the MCL scar tissue at 3 weeks post-injury and then assessed at 6 weeks post-injury. The rationale for this experiment was that increases in biologically active tissue TGF-beta levels would likely occur by 1 week post-exposure to the decorin antisense ODN and that this was, therefore, the interval when exogenous TGF-beta may also be seen to have a positive effect on healing of the tissue. However, when assessed at 6 weeks, there was again no detectable effect of the TGF-beta on either the biomechanical properties of the scar tissue or collagen fibril diameters.

Figure 2. Creep strain (total and irrecoverable) (Panel A) and stress at failure (Panel B) for antisense, sense and injection control tibia-MCL scar-femur complexes. All scars failed in the scar tissue.

From these experiments one can conclude that the influence of decorin antisense on MCL scar tissue is apparently not an indirect effect via TGF-beta. Furthermore, the experiments also indicate that endogenous levels of TGF-beta in the MCL scar tissue of healthy skeletally mature adult rabbits are optimal for its role in extraarticular ligament healing.

VII. Current studies

While the studies discussed above demonstrate that decorin antisense gene therapy can functionaly impact on MCL scar tissue at multiple levels, the outcome still remains a tissue with less than normal properties and characteristics. Therefore, additional experimentation is required to continue development of a regenerated tissue. This will require assessment of additional molecular targets for the gene therapy and possibly, development of an antisense "cocktail" consisting of antisense ODN directed towards a number of specific targets [ie: matrix molecules (proteoglycans, collagens), proteinases and inhibitors, regulatory enzymes) or pleiotropic targets (ie: growth factors, transcription factors and other intracellular regulators) that can influence a number of cellular molecules. We are currently evaluating a number of possibilities (minor collagens, specific proteinases, inflammation regulated transcription factors, etc). Obviously, the timing for delivery of such a combination of modifiers of multiple cellular targets will have to be optimized.

A second area where new developments are required is that of the delivery system. The present delivery system is functional and leads to prolonged alterations in the transfected cells, but it has limitations with regard to the uniformity of liposome delivery to all relevant cells in the tissue and clinical utility (an important consideration if the methodology is to be translated to clinical practice). While early scar tissue is more cellular (Frank et al, 1983) and more vascularized (discussed in Frank et al, 1994) than normal tissue, delivery via the regional vascularity is limited (Nakamura et al, 1998b). Therefore, new methods will be required to effect delivery in a clinically acceptable manner. A critical element is the diffusion of the liposomes within the scar matrix. We are currently exploring additional methods which overcome some of these limitations. As such limitations are not restricted to MCL scars, improvements in this area will likely influence application to other connective tissues.

VIII. Future directions

In the clinical realm, MCL healing is not as critical as healing/repair of other ligaments such as the ACL, or other connective tissues such as tendons and menisci. A question of critical importance is whether the effectiveness of antisense gene therapy in the MCL model can be extrapolated to these other connective tissues. Some studies have indicated that this liposome approach can be used in models of patellar tendon healing (Nakamura et al, 1996, 1998a) and can be used to affect gene transfer to cartilage (Tomita et al, 1997). Therefore, it is likely that the approach and methodology will have application in these tissues, as well. However, it remains to be determined whether generalizations can be made for healing/regeneration of most connective tissues or whether each tissue will have individual, or specific requirements in this regard because of unique mechanical environments, unique cellular aspects, or tissue-specific biochemical requirements.

Acknowledgements

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