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Type V collagen is increased during rabbit medial collateral ligament healing

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Abstract To understand the reparative process of medial collateral ligament (MCL), fibrillar collagen and their relative ratios in healing MCL with anterior cruciate ligament (ACL) reconstruction were analyzed. Skeletally mature New Zealand white rabbits were subjected to a mop-end tear of MCL without repair with ACL reconstruction. Rabbits were killed 6 and 52 weeks after injury. Ligamentous tissues from the injury site and sham controls were soaked in 0.5 M acetic acid for 24 h, minced, and treated with pepsin to solubilize collagen. Pepsin solubilized about 80% of the total collagen as determined by hydroxyproline analysis of the pepsin residues. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of the solubilized collagen revealed presence of fibrillar collagen types I, III, and V. Densitometric scanning of the protein bands corresponding to types I, III, and V collagen indicated that in sham controls types III and V collagen represented about 8% and 12%, respectively, of the type I collagen whereas the healed MCL ligaments at 6 weeks showed significant increase in type III and V collagen to about 19% and 24%, respectively. By 52 weeks type III collagen in the healed MCL had returned to that of sham controls while type V collagen remained elevated at approximately 18%. These data suggest that presence of type V collagen in high concentration in healing ligaments may have an influence on collagen fibril diameters seen in healed ligament and should be included in the analysis when evaluating ligament healing.

Keywords Medial collateral ligament · Ligament healing · Collagen · Fibril diameter

Introduction

Attempts to accelerate and improve ligament repair are currently an active area of investigation. Several studies from different laboratories using animal models of ligament repair have shown that medial collateral ligament (MCL) has potential to heal without intervention, but that the healed tissue remains mechanically, structurally, and materially inferior to normal ligaments [6, 22, 24]. The failure of the ligament to heal and reach the quality of that of normal ligament is not clearly understood. Several parameters have been examined to try to understand the reparative process of MCL; these include the amount of collagen present [1, 24], collagen fibril diameters [7], level of collagen cross-links [8], amount of type III collagen present relative to type I collagen [9], and proteoglycan content [11]. No correlation has been established between any of the parameters and the mechanical properties of the ligament [19]. The collagen fibril diameters of healing ligaments have, however, been shown to remain relatively smaller than those of normal ligaments even up to 2 years after injury [7]. Studies on wound healing have suggested that tensile strength, toughness, and organiza-

tion of the repaired tissue is related to the collagen fiber diameter [4]. These findings suggest that the healed ligaments with smaller collagen fibrils are mechanically weaker than those with the normal collagen fibrils.

Collagen fibril diameters are controlled by other molecules that associate with the major fibrillar forming collagens. Among these type V collagen has been implicated in the regulation of type I collagen fibril diameters [13, 15, 16]. We have previously shown that normal MCL is composed of six genetically distinct types of collagen [17]. Type I collagen is the major fibrillar collagen of the MCL and types III and V collagen are quantitatively minor components. The role of types III and V collagen in ligaments has not been clearly defined. Previous studies on ligament healing have demonstrated that during early phases of ligament healing type III collagen is highly elevated relative to type I collagen [9]. A high concentration of type III collagen relative to type I is suspected to result in smaller collagen fibrils [1]. No other collagen types have however, been examined during ligament healing.

In this study we examined changes in fibrillar collagen ratios in a mop-end tear of MCL injury model without repair so as to gain insight into the reparative process of MCL. We report here that, in addition to type III collagen increasing, type V collagen levels increase relative to type I collagen following ligament injury and remain elevated even up to 1 year after injury. The implication of the presence of high concentration of type V collagen relative to type I collagen is discussed in the context of the suspected role for type V collagen.

Materials and methods

Twenty-four skeletally mature New Zealand white rabbits were subjected to a combined injury of the ACL with reconstruction and a mop-end tear of the MCL without repair, as described previously [24]. Briefly, the MCL substance was ruptured to create a mop-end by pulling a rod medially beneath the ligament; the ends of the ruptured MCL were opposed but not repaired [21]. The ACL was transected at its tibial insertion, and the ligament was detached. The ACL was reconstructed using a flexor tendon allograft which was trimmed to approximately 4 mm in width and 6 cm in length at the time of surgery [24]. Contralateral knees received a sham operation by opening the skin and facia and passing a rod beneath the MCL without rupturing. The rabbits were divided into three groups of eight animals for each healing time interval, i.e., 6, 12, and 52 weeks. The analyses that were performed on the MCL of the rabbits of each group have been previously reported [23, 24]. In the present study the collagen type ratios were determined from the MCL harvested from the rabbit knees of the 6- and 52-week groups. Postoperatively all animals were allowed unrestricted cage activity.

Collagen isolation and analysis

Ligaments were dissected from rabbit knee joints following mechanical testing and were suspended in saline containing protease inhibitors. Four ruptured and four sham-operated ligaments at 6 weeks, and five ruptured and five sham-operated ligaments at 52 weeks were used for collagen analysis. The harvested MCLs were washed in saline containing protease inhibitors, after which tissue samples from the injury site were dissected out, washed in distilled water, and dried. Approximately 2 mg each of the dry ligament was suspended in 1 ml of cold 0.5 M acetic acid and allowed to soak for 24 h. Softened tissues were minced with a scalpel blade and resuspended in 1 ml of 0.5 M acetic acid. To this, pepsin was added at 1:30 by weight (enzyme:tissue) and stirred at 4°C for 24 h. After 24 h the pepsin concentration was raised to 1:10, and tissue digestion with pepsin was continued further for 24 h at 4°C with stirring. Pepsin digests were clarified by centrifugation in a microfuge at the maximum speed of 14,000 rpm for 10 min. The supernatants were removed, and the pepsin residues were reextracted in 0.5 M acetic acid for 24 h and then centrifuged. The first and second supernatants were combined and neutralized with 0.5 M NH_4HCO_3. Aliquots of the samples were dried and analyzed by gel electrophoresis using the methods of Laemmli [12]. To resolve type III collagen chains from α1(I) chains, interrupted gel electrophoresis was performed as described [20]. Quantitation of collagen bands was performed by densitometric scanning of protein bands corresponding to α1(I), α1(III) and α1(V) chains on a Biorad G670 imaging densitometer (Biorad Laboratories, Hercules, Calif., USA). To determine collagen type relative ratios the area under the peaks for α1(III) or α1(V) chains was divided by the area under the α1(I) peak of the same sample. The relative percentage was obtained by multiplying the ratio obtained by 100. Relative ratios of α1(III) or α1(V) from each sample were pooled with ratios obtained from different samples within the experimental or sham group. Statistical analysis was performed using Student's *t* test for paired samples with significance set at $P<0.05$.

Hydroxyproline analysis

To determine the amount of collagen solubilized by pepsin digestion, the residues remaining after pepsin digestion were hydrolyzed in 6 M HCl at 108°C for 24 h. Samples were dried, and aliquots of the hydrolyzates were subjected to hydroxyproline analysis as described previously [23]. The amount of total collagen solubilized from each sample was determined by assuming that 90% of the tissue dry weight is collagen.

Results

Pepsin solubilized approximately 70–80% of total collagen in each of the ligaments as determined from the hydroxyproline analysis. The collagen solubilization was about equal for both injured and sham controls. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the collagen solubilized by pepsin digestion indicated the presence of fibrillar collagen types I, III, and V.

Figure 1 shows SDS-PAGE of collagen solubilized by pepsin from sham and ruptured MCL after 6 and 52 weeks. After 6 weeks of healing prominent protein bands corresponding to α1(III), α1(V), and α2(V) chains are evident from the collagen solubilized from the ligament of the experimental group (lane E). The same protein bands are less intense in the collagen solubilized from sham controls (lane S). After 52 weeks of healing only a faint protein band corresponding to the α1(III) chain can be seen. In contrast, α1(V) and α2(V) chains are evident from the collagen solubilized from the ligaments of the 52-week experimental group. In sham controls α1(V) and α2(V)

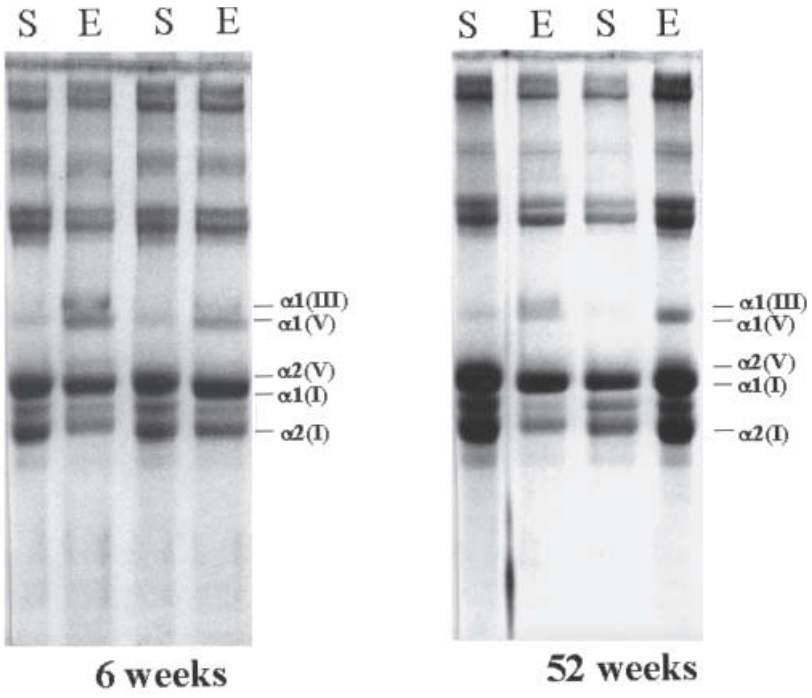

Fig. 1 SDS-PAGE of collagen solubilized from sham (*S*) and experimental (*E*) MCL at 6 and 52 weeks. Individual α chains were identified based on purified collagen standards type I, III, and V (not shown). Samples were electrophoresed under reducing conditions using the delayed reduction technique. The protein band between α1(I) and α2(I) chains is truncated α1(I) due to extensive pepsin digestion. Gels were stained in Coomassie blue and destained in methanol/acetic acid/water 1:1:8

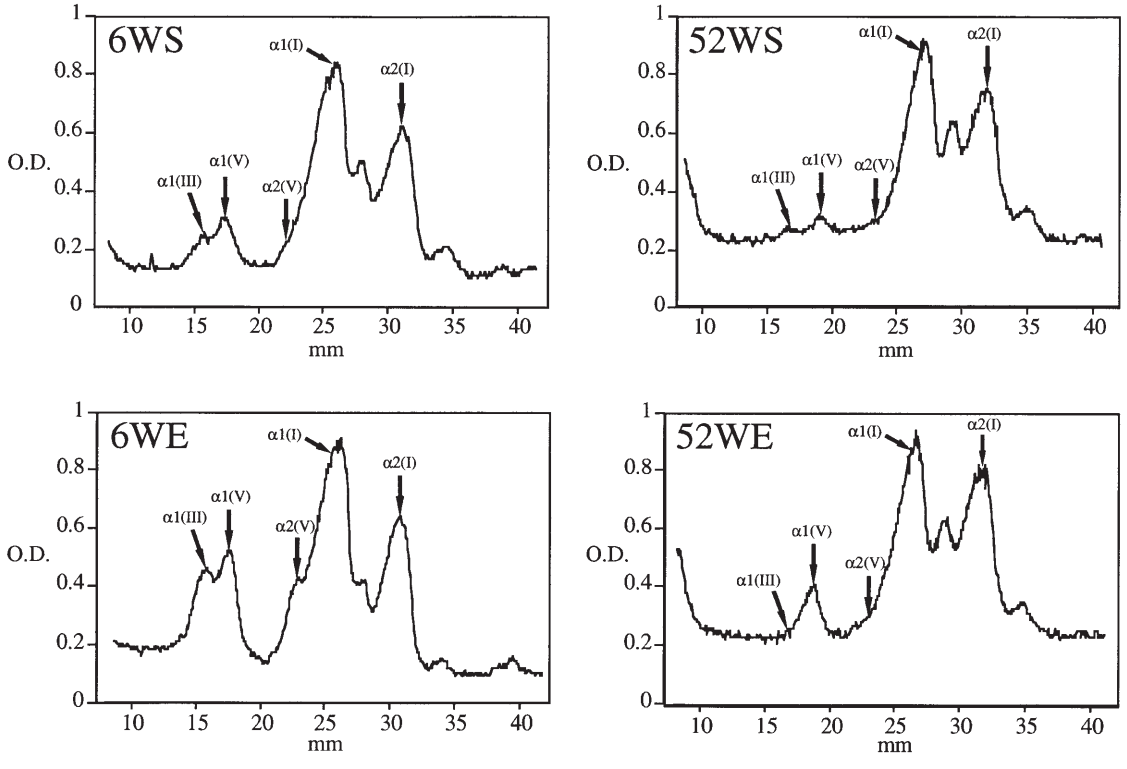

Fig. 2 Densitometric scans of SDS gels of the collagen solubilized from sham ligaments at 6 weeks (*6WS*) and 52 weeks (*52WS*) and experimental ligaments at 6 weeks (*6WE*) and 52 weeks (*52WE*). The α chains corresponding to individual peaks are identified

Table 1 Collagen type ratios in heating MCL

	Type III/I		Type V/I	
	6 weeks (n=4)	52 weeks (n v=5)	6 weeks (n=4)	52 weeks (n=5)
Sham	7.9±1.8	7.2±2.1	13.5±3.5	11.1±1.5
Experimental	19.0±5.3	8.5±3.9	24.9±3.6	18.1±4.9
P	0.0118	0.5446	0.0105	0.0327

chains are present as faint bands. These data indicate that type V collagen is present in higher concentration in the ligaments of the experimental group. The protein band migrating between α1(I) and α2(I) is truncated α1(I) generated by pepsin digestion [3].

Figure 2 shows the representative densitometric scans of the collagen solubilized from ruptured ligaments and sham controls after 6 and 52 weeks. The scans show the relative intensities of each of the collagen chains. After 6 weeks of healing the α1(III), α1(V) and α2(V) chains are evident. After 52 weeks of healing α1(V) and α2(V) chains are evident in the experimental group while the α1(III) chain is barely detectable, again suggesting that type V collagen is elevated in the collagen isolated from the ligaments of the experimental group.

Analysis of the areas under the peaks for α1(III) and α1(V) chains from sham and injured ligaments and their relative ratios to the area under α1(I) chains of the same sample revealed that in control ligaments types III and V collagen comprised about 8% and 12%, respectively, of type I collagen (Table 1). The injured ligaments after 6 weeks showed a significant increase in type III collagen to 19%. By 52 weeks type III collagen levels in the experimental group returned to that of the sham group or were barely detectable on SDS-PAGE (Table 1). Similarly, after 6 weeks the injured ligaments showed a significant increase in type V collagen to about 24% (Table 1). In contrast to type III collagen, however, by 52 weeks type V collagen in the MCL of experimental group remained elevated at about 18%, which although reduced from the 6-week group is still significantly higher than that of the sham control. Although the data reported here cannot be taken as absolute values, they suggest that type V collagen remains elevated even up to 52 weeks of ligament healing.

Discussion

Previous studies on MCL healing using cyanogen bromide digestion have shown that type III collagen levels increase in early phases of ligament healing [6]. In the present study we demonstrate that, in addition to type III collagen, type V is also increased during ligament healing in relation to type I collagen. Although pepsin solubilization of collagen from tissues is usually not efficient, we were able to solubilize about 80% of the total collagen of the ligamentous tissues when using the pepsin solubilization procedure described above. In addition, consecutive pepsin extractions of the same samples showed relatively constant ratios between α1(III) or α1(V) chains to α1(I) chains (data not shown). We have previously demonstrated that normal bovine MCL is comprised of fibrillar collagen types I, III, and V [17]. The role of type III collagen in ligaments or other tissues is not clear, but this collagen may associate with type I collagen fibrils and may regulate the fibril diameters of type I collagen [18]. Studies have, however, shown that type III collagen may associate with large collagen fibrils as well as small collagen fibrils [10]. The present investigation demonstrates that type III collagen returns to normal levels by 52 weeks of healing, but that type V collagen remains elevated in relation to type I collagen over the same period.

Studies on type V collagen in chick cornea have shown that type V collagen may be involved in the regulation of corneal collagen fibril diameters. Type V collagen accounts for about 20% of the total collagen in chick cornea; the thin fibrils of uniform diameter present in the cornea are believed to result from the high concentration of type V collagen in this tissue [13].

The tissue form of type V collagen has been shown to retain amino terminal extension peptides [5, 15]; these peptides are believed to play a role in the regulation of type I collagen fibrils in a heterotypic assembly. A model of the way in which type V collagen regulates type I collagen fibrils has been proposed [13]. In this model type V collagen helical domain is buried in the interior of the heterotypic collagen fibrils; the amino-terminal domains retained on the tissue form of type V collagen α chains project out on the surface of the collagen fibrils, presumably through the hole zone. In this assembly the retained amino-terminal domains act as steric hindrance for further addition of type I collagen molecules thereby regulating the lateral growth of collagen fibrils. Indeed studies on intermolecular cross-linking of bone type V collagen have demonstrated covalent cross-links between types I and V collagen [15].

Further evidence for the role of type V collagen in the regulation of collagen fibril diameters has been obtained from studies on transgenic mice with an in-frame deletion of exon 6 of the collagen α2(V) chain [2]. In these mice the skin was found to be extremely fragile and to contain disorganized fibrils of variable diameters, resembling those seen in some forms of Ehlers-Danlos syndrome. Furthermore, linkage of the α1(V) gene to Ehlers-Danlos syndromes I and II has been demonstrated [14]. All together, these data strongly demonstrate that type V collagen is involved in the organization and regulation of type I collagen fibril diameters. Although the present study was performed on ligaments obtained from the combined injury model, the same findings may apply to isolated MCL injury.

In view of the ascribed role for type V collagen, together with the correlation drawn between collagen fibril size and tissue strength in wound healing, it will be of interest to determine whether the smaller collagen fibril diameters seen in healed ligament are due to the presence of high levels of type V collagen. Thus the presence of type V collagen in high concentration should be considered among the parameters when evaluating ligament healing.

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