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Effect of Age on Collagen Fibril Diameter in Rabbit Patellar Tendon Repair

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The effect of aging on soft tissue repair is poorly understood. We examined collagen fibril diameter in repairing patellar tendons from young adult and aging rabbits. We hypothesized that repairing tendons from older (geriatric) rabbits would have similar diameter fibrils compared with the younger (young adult) rabbits. Full-length, full-thickness, central-third (2.5 to 3 mm) patellar tendon injuries were made by cutting out the center of the tendon in twelve 1-y-old and thirteen 4- to 5.5 (average, 4.25)-y-old female New Zealand White rabbits. The contralateral tendon served as an unoperated control. The rabbits were euthanized at 6, 12, and 26 wk after surgery. The collagen fibril diameter was examined by electron microscopy at the patellar end, middle, and tibial end of the patellar tendon. There was no significant decline in collagen fibril diameter at any location in the aging rabbit healing patellar tendons compared with those of the 1-y-old rabbits. This study found that collagen fibril diameter was not altered with increasing age in the healing rabbit patellar tendon.

Abbreviations: MMP-1, matrix metalloprotease-1

With the aging population (people older than 65 y) in 2030 estimated to be 70 million in the United States,[16] there is an increasing need to provide quality orthopedic care to the elderly. It is generally believed that tissue repair in the geriatric patients occurs more slowly and incompletely than in the adolescent or even the young adult.[1,13] Unfortunately many of the studies on tissue repair are confounded by the presence of other diseases in the elderly, such as diabetes, vascular diseases, and ultraviolet ray exposure.[21] Therefore, the effect of aging on soft tissue repair in the absence of other diseases is poorly understood.

A common human orthopedic procedure performed is cruciate ligament repair. During this procedure the patellar tendon is often used to replace the cruciate ligament. This practice results in an open gap wound in the patellar tendon. It is not known whether the defect created in the patellar tendon heals better in young versus older individuals, nor is it known how other tendon injuries repair in the elderly human.

To examine the effect of age on tendon and ligament structure and repair, we and others have used the rabbit as a model of the geriatric human.[5-7, 23] Geriatric is defined as the age in which a population has reached 50% mortality (in humans, life expectancy at birth is 77.6 y[11]). In a small group of specific pathogen-free rabbits, we observed nearly a 50% mortality when housing rabbits from 1 to 4 y of age.[5] The mortality was attributed to a variety of neoplasms, or remained undetermined. No evidence of an infectious disease was found in these rabbits. In a controlled wild population of rabbits (*Oryctolagus cuniculus L.*), the average life expectancy of adult male and female rabbits (excluding mortality at a preweaning age) was 28.2 and 31.7 mo, respectively.[22] The authors speculated that the cause of mortality in most cases was intestinal coccidiosis. Although in general wild animals do not have as long a lifespan as animals held in captivity, this study highlights the natural short lifespan of the rabbit. On the basis of these 2 studies, 4- to 5.5-y-old rabbits are considered geriatric and can be used as a model for geriatric humans.

Although many parameters are involved in healing, one of the key components to re-establishing tendon strength is the deposition of properly aligned collagen fibers into the repair site. Studies by our group and others have demonstrated that changes in rabbit and human tendon strength parallel changes in fibril diameter.[7,18] Tendon strength is correlated most closely with the size of the type I collagen fibril, with larger fibrils and better packing providing greater strength. Repairing tendons in the young adult rabbits regain <40% of normal uninjured strength and have smaller collagen fibrils.[2] The decline in the biomechanical properties of healing tendon strength in aging rabbits could be due to significant reduction in the collagen fibril diameter, similar to what we have shown in young adult rabbits. However, this possibility had not been previously examined.

Several factors can lead to alterations in collagen deposition and fibril diameter in the healing tissues of elderly human patients. For example, aged rat fibroblasts on type I collagen matrices have decreased migration and increased concentrations of matrix metalloprotease-1 (MMP-1) and tissue inhibitors of MMP-1.[19] There is also a decrease in collagen turnover, as collagen synthesis and collagenolytic activity diminish with aging.[12,14,17] Other more generalized changes, such as reduced oxygenation of cells in the elderly, may contribute to reduction in collagen turnover and synthesis. Despite these alterations in collagen synthesis and

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turnover with aging, it is not known whether these changes have a biologic impact on repair.

To examine the effects of age on tendon repair, we surgically resected the middle 3rd of the patellar tendon in 1-y-old and 4- to 5.5-y-old rabbits. We examined these tendons in 3 different locations along the tendon at 6, 12, and 26 wk after surgery. We hypothesized that the diameters of the collagen fibrils in the repairing tissues from aging rabbits would be similar to those of younger rabbit tissues. We further hypothesized that there would be no significant differences in the fibril diameter at different locations in the repairing tendon.

Materials and Methods

We purchased twelve 1-y-old and thirteen 4- to 5.5 (average, 4.25)-y-old specific pathogen-free New Zealand White rabbits from Myrtle's Rabbitry (Thompson Station, TN). The rabbits were negative for the following pathogenic agents: *Clostridium piliforme*; *Treponema paraluis–cuniculi*; *Encephalitozoon cuniculi*; and *Pasteurella multocida.* Their care and usage were approved by the University of Cincinnati Institutional Animal Care Use Committee. Water and food (Rabbit Chow Lab Diet 5326, High-Fiber Maintenance Formula, Purina, St. Louis, MO) were given ad libitum. The rabbits were housed in 5-ft^2 (0.464 m^2) cages with metal slat floors. The rooms were maintained at 19 to 23 °C on a 12:12-h light:dark cycle.

The rabbits were anesthetized with an intramuscular injection of a mixture of ketamine hydrochloride (25 mg/kg body weight) and acepromazine (0.7 mg/kg body weight) and were given a subcutaneous injection of buprenorphine (0.5 to 0.75 mg/kg body weight) pre-emptively as an analgesic. The hair on 1 leg was shaved, and the skin was prepped using povidone–iodine solution (Betadine, Henry Schein, Indianapolis, IN) and alcohol. The rabbits were maintained under general anesthesia by using a mixture of oxygen and isoflurane, administered to effect by mask. Full-length, full-thickness, central-3rd (2.5 to 3 mm) patellar tendon injuries were created by excising the center of the tendon with a #15 scalpel blade. A suture was tied in the center of each of the margins of the adjacent normal tendon to identify the limits of the wound site during subsequent dissection. The skin then was sutured closed. Upon recovery from the anesthesia the rabbits were ambulatory on the day of surgery. The contralateral tendon served as an unoperated control. At 6, 12, or 26 wk after surgery, rabbits were euthanized with an overdose of pentobarbital, and their patellar tendons were excised.

Ultrastructural analysis. We collected sections measuring 1 mm × 1 mm × 2 mm from the center of the defect and at the patellar and tibial attachment sites. Similar pieces were cut from the control (unoperated) patellar tendon. The sections were immediately fixed on ice in 2% glutaraldehyde for 4 h and then transferred to 100 mM cacodylate buffer (pH 7.4). The tendons then were post-fixed with osmium tetroxide and thin sections (70 to 80×) were stained with lead acetate. The sections were examined and photographed with an electron microscope (H600, Hitachi, Tokyo, Japan). Five photographs were taken of collagen fibrils in cross-section from 5 different grid areas at ×30,000 magnification (final magnification, 1 mm = 0.0201 μm). The selection process was performed blinded to both the source of the material and selection of the grid location. Any fibrils showing a banding pattern were not sampled because they likely were not cut in cross-section. Eccentrically shaped fibrils were assumed to be cut obliquely. Minimum fibril diameter then was used to estimate the actual fibril diameter.[9]

Developed negatives were digitally photographed, and minimum fibril diameter was computed using The Image Processing Tool Kit (Reindeer Graphics, Raleigh, NC), an Adobe Photoshop compatible plug-in. A 'watershed' function in the program was used to distinguish individual fibrils from fibril clusters. Images then were reviewed manually to ensure that the watershed function had not split or distorted fibrils and thus skewed the results. Minimum fibril diameter was computed using NIH Image v1.62 (http://rsb.info.nih.gov/nih-image/) and histograms were created with 10 nm bin sizes. Differences in the size, number of fibrils of each size, and in the distribution/range of the fibril cross sections were calculated and statistical analysis was performed.

An average of 724 fibrils was analyzed for each tendon section. The mean collagen fibril diameter was compared between the different specimens; analyzing differences with age (1- and 4-y-old), time post-surgery (6, 12 and 26 wk), normal and repairing tissue, and location in the tendon (patellar end, middle, or tibial end). Group size for all measurements was either 4 or 5 rabbits.

Statistical analysis. Four-factor mixed model ANOVA analyses were conducted for mean observations. The models fit correspond to a split-split plot design in which the fixed whole plot factors are Age and Time, the split plot factor is Injury, and the split-split plot factor is Location. SAS PROC MIXED was used for the analysis. A subsequent pair-wise contrast analysis was also performed on the main effect of each factor as well as the various combinations of these 4 factors' levels. Significance was set at $\alpha \leq 0.05$.

Results

There was no significant difference in the weight of the rabbits at the time of surgery (1-y-old rabbits weighed 4.8 ± 0.37 kg and 4-y-old rabbits weighed 4.9 ± 0.47 kg). The rabbits were euthanized, and sections from 1- and 4-y-old normal and surgically dissected patellar tendons at 6, 12, and 26 wk postsurgery were collected (Table 1). Three locations in the tendon were examined: near the patella, in the middle of the tendon, and at the tibial end of the patellar tendon. The data were evaluated comparing these different variables.

Fibril diameter changes in the healing patellar tendon compared with normal patellar tendon. We compared mean collagen fibril diameter between the contralateral control and repairing tendons at 6, 12, and 26 wk postsurgery (Table 1). When location was not included as a variable (location data was pooled), there was a significant reduction (P, < 0.0001 to 0.018) in the mean fibril diameter in the repairing tendons at all time points (mean difference, 60 nm; range, 37.4 to 93.9 nm) in 1- and 4-y-old rabbits.

When location was included in the analyses, significant differences (P, <0.0001 to 0.018) in mean collagen fibril diameter were found in most of the patellar and middle sections (mean average difference = 78 nm; range, 34.3 to 150.5 nm). Two exceptions were found at these sites. There was no significant difference in the fibril diameter at the patellar end of the tendon in the 1-y-old rabbits at 26 wk postoperatively (P = 0.125) nor in the middle tendon sections from the 4-y-old rabbits at 12 wk postoperatively (P = 0.127).

Surprisingly, there was only 1 significant difference in mean collagen fibril diameter in tibial sections from normal and repairing tissue. A significant decrease (P = 0.046) in fibril diameter (difference, 63 nm) was seen in repairing tendons in the tibial section for only the 1-y-old rabbits at 6 wk postoperatively compared with the control tendons.

Table 1. Collagen fibril diameter (in nm; mean ± 1 standard deviation) in rabbit patellar tendons

Age[a]	Treatment[b]	Location	Time postinjury[c]		
			6 wk	12 wk	26 wk
1	Control	P	121.65 ± 16.5	174.83 ± 10.95	125.30 ± 58.33
1	Control	M	182.04 ± 48.85	220.73 ± 115.82	132.45 ± 61.67
1	Control	T	117.12 ± 10.27	87.98 ± 8.74	103.38 ± 28.18
1	Defect	P	61.91 ± 4.54	62.39 ± 3.58	84.4 ± 61.62
1	Defect	M	61.77 ± 2.94	70.26 ± 10.18	64.78 ± 6.43
1	Defect	T	67.2 ± 3.88	69.1 ± 8.85	83.77 ± 36.53
4	Control	P	136.03 ± 42.97	132.36 ± 34.81	165.08 ± 38.9
4	Control	M	146.73 ± 53.03	121.29 ± 39.31	152.43 ± 17.78
4	Control	T	103.05 ± 18.1	107.88 ± 23.6	108.03 ± 14.58
4	Defect	P	72.78 ± 16.79	76.82 ± 22.03	78.49 ± 25.93
4	Defect	M	76.4 ± 20.49	87.2 ± 32.55	77.22 ± 28.55
4	Defect	T	124.22 ± 40.93	70.75 ± 8.83	78.51 ± 19.15

M, middle of the patellar tendon; P, patellar end of the patellar tendon; T, tibial end of the patellar tendon.
[a]1 indicates 1-y-old animals; 4 indicates 4- to 5.5 (mean, 4.25)-y-old rabbits.
[b]Control indicates the unoperated contralateral tendon; defect indicates tendons in which full-length, full-thickness, central-3rd (2.5 to 3 mm) injuries were made by cutting out the center of the tendon.
[c]n = 4 for all time points, except for the 4-y-old, 12-wk time points (n = 5).

Aging changes in the fibril diameter in the healing patellar tendon. While controlling the factors of location and time postsurgery, we examined the effects of age on collagen fibril diameter in the repairing tendon (Table 1). Mean collagen fibril diameter in repairing tendons of 4-y-old rabbits was significantly larger ($P = 0.024$) at the tibial end at 6 wk postoperatively compared with that of repairing tendons in 1-y-old rabbits (difference, 57 nm). In addition, fibrils at the tibial end of the patellar tendon in 4-y-old rabbits at 6 wk postsurgery were significantly larger than healing fibrils from all other locations, time points, and populations (average difference, 51 nm; range, 37 to 63 nm). No differences were found in collagen fibril diameter between the 1- and 4-y-old rabbits in any other tendon section at any time point.

Discussion

In this study we examined the effect of aging on fibril diameter in repairing patellar tendons in rabbits. In general, there was no significant difference in fibril diameter in the repairing patellar tendon in 4-y-old rabbits compared with 1-y-old rabbits at 6, 12, or 26 wk post injury or in the patellar, middle, or tibial end of the tendon. A significant increase in fibril diameter occurred at 1 time point in the tibial end of tendons of 4-y-old animals compared with all other locations and times in 1- and 4-y-old rabbits. This increase in repairing fibril diameter is difficult to explain. It may be attributed to a problem with the collection technique and possible harvesting of uninjured tissue, leading to false elevation of the fibril diameter. The absence of change in fibril diameter with increasing age for most samples argues against an in vivo age-related effect on fibril synthesis during tendon repair.

These results also parallel others from another of our laboratory's studies, which examined the effect of age on the biomechanical properties of repairing patellar tendons after similar injury.[6] Repairing patellar tendons of 1- and 4- to 5-y-old rabbits were examined, and we found no significant differences in the biomechanical properties between 1- and 4- to 5-y-old rabbits at 6 and 12 wk postinjury. At 26 wk increases in the modulus and maximal stress of 4-y-old rabbit patellar tendons were found. Thus, these 2 studies demonstrate neither an ultrastructural nor biomechanical decline in repairing patellar tendon with age.

We also compared the results of the current study to previous reports in the literature. There are only a few reports of aging effects on soft tissue repair.[8,15,20,21] Most conclusions on tendon healing have been extrapolated from studies examining the decline in skin repair with advancing age, because the strength of both tissues is correlated with their collagen content. One of these studies demonstrated that less force is required to disrupt wounds in the skin of aged people compared with younger adults,[10] thus supporting the concept of a decline in collagen matrix strength with increasing age. Another study showed that dehiscence occurs more frequently in geriatric persons compared with young adults.[20] In yet another comprehensive study of more than 1000 patients, age was the leading factor in progressive degeneration of the rotator cuff and was considered the single most important contributing factor in the pathogenesis of rotator cuff tears.[8] Complicating these studies and others on wound repair were the effects of other diseases on healing. In other words, reductions in tissue behavior may occur not only as a result of aging but also due to confounding factors such as ultraviolet ray exposure, diabetes, and vascular disease.[21] Another problem faced when using epidemiologic studies of healing is the differences in the injuries experienced between the aging and young adult population. Mansat and others found a decreased success rate in the repair of injured rotator cuffs in the elderly. However, it may have been due to the larger injuries present in aging patients.[15] Using animal models in controlled environments helps prevent some of these complications and thus is useful in examining the direct effects of aging on tendon repair. The rabbit, combining a reasonably short lifespan and large body size, offers a good model for examining aging effects on tendon repair.

Another possibility for the reduction in repair strength shown in previous studies in the elderly[8,20] is a change in the organization of the fibrils. Although we found no differences in the diameters of individual fibrils, the packing density or cross-linking between fibrils could play an important role in differences in tendon strength in repairing tissues.[3,4] Future studies to look at packing density and cross-linking are crucial to understanding aging changes in healing tissues.

It is interesting to note that no significant differences between collagen fibril diameters at the patellar, tibial, and middle portions of healing tendons were found. The mechanical signals driving collagen fibril synthesis are similar along the length of

the tendon. Although the normal tendon may be predisposed to rupture at the insertion sites, there appears to be little difference among the different locations of a healing tendon to predispose any one site to failure. Rather, the site of failure is most likely at the location of stress concentration, which is frequently the transition site between the tendon and bone or at sutured junctions. Of note in this study is the fact that the tibial end of the sample was readily harvested near the insertion site. The patellar end, however, represented a greater challenge to harvest because of the much broader and curved insertion area. Because of this difference, the tibial sections were harvested more consistently closer to the insertion site.

In summary, we found no change in collagen fibril diameter in the healing patellar tendon of the aging rabbit. This finding challenges the dogma that there is decreased healing potential in the tendons of aging animals, supporting contributions by other factors (disease and wound size) as the cause of decreased healing in aging humans. Future studies to examine collagen packing density should be performed to evaluate whether packing density and architectural change contribute to declines in tendon strength with age.[4]

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