The Natural Interface between Bone and Tendon: SEM

Observations of the Enthesis in an Ovine Model

by

Willie Mae Reese

Submitted to the Department of Materials Science and Engineering in partial fulfillment of the requirements for the degree of Bachelor of Science at the MASSACHUSETTS INSTITUTE OF TECHNOLOGY

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Abstract

The present study investigates the naturally occurring interface between bone and tendon using scanning electron microscopy. The micrographs revealed a cartilaginous layer, 100 to 400 μm thick apposing bone, that contained cells varying in size and shape as a function of their location in this cartilaginous layer. Further investigation is required to conclude whether these cells are undergoing further differentiation during development of this graded layer. This study found the interface between bone and the cartilaginous layer to be interdigitated, which may explain why injuries at the bone-tendon interface are comparatively rare. Also, the cartilaginous layer was revealed to be substantially mineralized. A somewhat higher concentration of calcium and phosphorus was observed near the interface with the apposing bone that gradually diminished into the cartilaginous layer. These findings support the four zone description of the bone-tendon interface established by others using histological methods. However, further research is suggested to resolve other questions about the observed sub-micrometer morphologies of the bone-tendon interface.
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<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Introduction and Background</td>
<td>9</td>
</tr>
<tr>
<td>1.1. Morphology and Anatomy of Bone</td>
<td>9</td>
</tr>
<tr>
<td>1.2. Morphology and Anatomy of Cartilage</td>
<td>10</td>
</tr>
<tr>
<td>1.3. Morphology and Anatomy of Tendon</td>
<td>10</td>
</tr>
<tr>
<td>1.4. Histological Studies of the Enthesis</td>
<td>11</td>
</tr>
<tr>
<td>1.5. Tendon Rupture and Repair</td>
<td>14</td>
</tr>
<tr>
<td>2. Methods and Materials</td>
<td>16</td>
</tr>
<tr>
<td>3. Results</td>
<td>17</td>
</tr>
<tr>
<td>3.1. Scanning Electron Microscopy Results</td>
<td>17</td>
</tr>
<tr>
<td>3.2. X-ray Energy Dispersive Spectroscopy Results</td>
<td>20</td>
</tr>
<tr>
<td>4. Discussion</td>
<td>25</td>
</tr>
<tr>
<td>4.1. Cell Type Differentiation</td>
<td>25</td>
</tr>
<tr>
<td>4.2. Morphological Aspects of the Interfaces</td>
<td>25</td>
</tr>
<tr>
<td>4.3. Calcified and Uncalcified Fibrocartilage Basophilic Line</td>
<td>26</td>
</tr>
<tr>
<td>5. Conclusions</td>
<td>27</td>
</tr>
<tr>
<td>6. References</td>
<td>28</td>
</tr>
<tr>
<td>7. Appendices</td>
<td>31</td>
</tr>
</tbody>
</table>
List of Figures

1. Four zones of tendon (after Benjamin et al.) ........................................ 12
2. Collagen Fibers in Tendon and Fibro-cartilage (after Benjamin et al.) ...... 13
3. Cartilaginous layer formed between tendon and bone ............................ 18
4. Interface between cartilaginous layer and bone ..................................... 19
5. Interface between cartilaginous layer and tendon .................................... 20
6. Higher magnification secondary-electron image of cartilaginous layer apposing bone ........................................ 21
7. XEDS calcium and phosphorus map of cartilaginous layer apposing bone  22
8. XEDS calcium and phosphorus map of bone apposing cartilaginous layer, and tendon ........................................ 23
9. XEDS calcium and phosphorus map of interface of cartilaginous layer and tendon ........................................ 24
List of Appendices

A. Additional SEM Images and XEDS Maps 31
B. Discussion of Growth Plates 47
1. Introduction and Background

An enthesis is the insertion site of ligament, tendon, or joint capsule in bone. The bone-tendon junction has been the subject of many research studies. However, most of this research is focused on the healing process after traumatic tendon rupture. The literature that does report the natural enthesis is limited in its understanding of the bone-tendon interface largely due to primary use of light microscope-based histological methods to characterize it only well above the micrometer-scale length. Understanding this interface is essential to developing and improving new technologies to aid in the healing process. Traumatic injuries to healthy tendon insertions into bone only rarely occur at the bone-tendon junction, which suggests that the interface is strong or protected by its sub-micrometer structure. Once the morphology of the natural enthesis is more thoroughly understood at all length scales, methods and techniques can be improved to aid the body in regenerating this morphology during tendon repair.

1.1 Morphology and Anatomy of Bone

Bone is a calcified aggregation of type I collagen fibers (20 wt. %), crystallized calcium phosphate crystals similar to hydroxyapatite (70 wt. %), and water and other proteins. There are several cells within bone that are of particular importance in this study: osteoblasts, osteocytes, and osteoclasts. Osteoblasts are the cells that are responsible for creating bone. These cells secrete collagen and the extracellular matrix that compose bone. They are also responsible for calcifying the matrix. Osteocytes are osteoblasts that have become completely surrounded by matrix. They maintain their ability to create matrix, but also can resorb the matrix. Through these cells and others,
bones have the dynamic ability to grow larger and smaller through signaling processes.1 For instance, larger loads on bones signal these cells to excrete more matrix. Osteoclasts are large multinucleate cells that break down the matrix by releasing lysosomes, organic acids, and hydrolytic enzymes.

1.2 Morphology and Anatomy of Fibrocartilage

There are three known types of cartilage: hyaline (the most dominant), elastic cartilage, and fibrocartilage.8 In this study, fibrocartilage is the most interesting, as light microscope-based histological methods have revealed its presence in the bone-tendon interface.3 The primary cells of cartilage are chondrocytes and fibroblasts. Chondrocytes are cells which secrete and regenerate the extracellular matrix of cartilage.9 The extracellular matrix is an amorphous water-based matrix rich in proteoglycans and other non-collagenous proteins such as link proteins, calcium-binding proteins and matrix glycoproteins.8 Fibroblasts are responsible for also synthesizing extracellular matrix and collagen. The two main types of collagen found in fibrocartilage are Type I and Type II. The concentrations of the two types are known to vary between different bone joints or tendon-bone interfaces. They may also vary within a particular layer of cartilage. Other types of collagen found in fibrocartilage may include Type V, VI, IX, XI and M.8

1.3 Morphology and Anatomy of Tendon

Tendon is a highly oriented fibrous tissue also composed of collagen bundles, mostly type I collagen.10,11 The bundles are supported by a proteoglycan-water matrix with
embedded collagen and elastin. Some of the primary cells in tendons are called tenocytes. These cells excrete procollagen building blocks of proteins and extracellular matrix. Tenocytes are flat and tapered and distributed sparingly throughout the tendon around the collagen bundles. Tendon has a hierarchical structure beginning with collagen fibrils that are grouped into collagen fibers, which are grouped into primary fiber bundles (subfascicles) that are grouped into secondary bundles (fascicles) that are grouped into tertiary bundles. Several tertiary bundles are then wrapped in epitenon, a loose connective tissue sheath that encase tendon proper. The epitenon contains vascular, lymphatic, and nerve supplies.

1.4 Histological Studies of the Enthesis

Benjamin, Evans and co-workers performed an extensive study, using light-microscope based histological methods, on the enthesis of the tendon in man. They and others have divided the enthesis into four zones: (1) tendon, (2) uncalcified fibrocartilage, (3) calcified fibrocartilage, and (4) bone. They noted that collagen bundles extended from the tendon zone into the uncalcified fibrocartilage zone. These bundles of collagen fibers then crossed into a matrix of chondrocytes and cartilage in the uncalcified fibrocartilage zone (Figure 1). Between the uncalcified and calcified fibrocartilage lies a distinct basophilic line, called the tide mark. Basophilic refers to the dark hemotoxylin staining of microscopic sections in light-microscopy based histology. Chondrocytes are more numerous on the side of the tidemark within the uncalcified fibrocartilage and are arranged in short rows. Thick plugs of calcified fibrocartilage then meet the hard surface.
of the bone, often apposing the bone surface at near normal incidence (Figure 2). These mineralized fibers are called Sharpey fibers.

Figure 1 - The four zones described by Benjamin et al.: tendon (T), uncalcified fibrocartilage (FC), calcified fibrocartilage (C-FC) and bone (B). Blood vessels (BV) are absent from the fibrocartilage zones. The tidemark (TM) between the calcified and uncalcified zones of articular cartilage (AC) is continuous with that at the insertion of the tendon. Transmission light microscopy stained with haematoxylin and eosin. Shown here at total magnification of 9X. (Reproduced from Benjamin, Evans et al.)
Benjamin reports that others have suggested that the two-layer system of uncalcified and calcified fibrocartilage acts as a padding to protect the bone from shearing effects. It must be noted that the exact thickness and maturation of the three zones depend on many specific factors such as which tendon attachment is being discussed and the average stresses on those zones over time of development. Bone is a dynamic organ and may change its size and density depending on its function and load.
1.5 Tendon Rupture Repair

A common approach to aid in tendon repair is to develop synthetic polymer scaffolds to facilitate the regeneration of normal, healthy tissue. A somewhat different approach is to capitalize on the body’s own intrinsic repair mechanisms and direct or encourage their own role in injury repair. Other researchers have been working specifically on designing ways to replace torn ligament with tendon (one approach to anterior cruciate ligament injury in the knee) or to reconnect torn or severed tendon (a common rotator cuff shoulder injury), both of which entail rebonding tendon (a fibrous connective soft tissue) to bone (a fibrous but mineralized hard tissue). A promising approach has been to implant a small biodegradable anchor or interference screw, used to put the soft and hard tissues into apposition for long time periods (months to a year) in order for bonding between the hard and soft tissues to occur. As the implant degrades, bonding occurs through 1) normal bone tissue replacing the screw and 2) formation and mineralization of intermediary tissue to provide a graded junction between the hard and soft tissues.

In in vivo studies that have focused on tendon-bone interface regeneration, researchers have noted a layer of fibrous cellular tissue that matures and reorganizes during the healing process. Oguma et al., using scanning electron microscopy, suggest that this layer is the result of many collagen fibril bundles from scar tissue that weave themselves into the bone, even as early as two weeks after trauma. As this layer develops, they claim that the number of collagen bundles increases and the bundles begin to deeply
penetrate into the layer of bone.\textsuperscript{2,4} Using mechanical tests, a positive correlation between the strength of the interface and the degree of bone ingrowth was noted.\textsuperscript{2} More mature samples also showed evidence of collagen fibers that connected tendon to bone. These collagen fibers were both calcified and uncalcified, separated by a basophilic line, the tidemark, that became more evident with a longer healing time.\textsuperscript{2}

Microscopy studies, using scanning (SEM) and transmission electron microscopy (TEM), have documented the progress and mechanisms of bone regrowth as the implant resorbs and also followed the formation of a partially mineralized graded interface that appears to involve three cell types: osteoblasts, tenocytes, and chondocytes\textsuperscript{7}.

The present SEM-based study was carried out to characterize the morphology and mineralization of a natural tendon-bone interface at higher length scale than light-microscopy based histology and to assess mineralization directly, rather than by inference, using micro-chemical analysis employing X-ray energy dispersive spectroscopy (XEDS). Through comparison to the studies previously cited, most of which used light histology on a much larger length scale, it was hoped to identify more definitively the key features of the natural interface that allow it to withstand greater mechanical loads than post-trauma regenerated interfaces and establish a micro-morphological benchmark against which to measure regenerate interface morphologies and chemistries.
2. Methods and Materials

Animal Samples

All samples were supplied by Smith & Nephew LLC, Endoscopy Division, Mansfield, Massachusetts. Healthy, uninjured patellar tendons still naturally attached to bone were collected from male 2 year old sheep and prepared for scanning electron microscopy (SEM). The samples were all of the patellar tendon and its intersection with the tibia.

Preparation for Scanning Electron Microscopy

All ovine samples for SEM were first trimmed and then fixed, embedded, and polished using the following procedures.

Samples were first fixed in 10% neutral buffered formalin solution at 4 °C for 24 hours, with gentle shaking. Samples were then rinsed with buffer solution (phosphate buffered solutions, PBS) three times, and soaked in the same buffer solution at 4 °C for 24 hours, with gentle shaking in order to remove formalin. Next, samples were dehydrated through a series of ethanol baths: 50%, 75%, 85%, 95%, 100%, 100%. Soaking time for each bath was 30 minutes.

The embedding process began by infiltrating the sample with a 1:2 volume ratio of fresh LR White™ resin to ethanol and then a 1:1 ratio. Each embedding step was done under vacuum at 4 °C for 24 hours. The last step of embedding was placing samples in pure LR White™ resin at 60 °C for 24-48 hours in a closed container to avoid interaction with oxygen.
The samples were polished using abrasive silicon carbide papers (grit: 400, 800, 1200). The polished samples were then sputter-coated with a 15 nm layer of carbon.

Two scanning electron microscopes were used to generate micrographs and elemental analysis maps: a JEOL Model JSM 5910 conventional SEM and an FEI/Philips Model XL30 FEG ESEM.

3. Results

3.1 Scanning Electron Microscopy Results

Scanning electron micrographs revealed a 100 to 300 micrometer layer between bone and tendon (Figure 3), deduced to be cartilaginous.

In this layer, chondrocytes were identified by their size (~10 μm in diameter), their "D-"shape, and their typical characteristic pairing. These features suggested that the layer was made of cartilage. Near the interface of the cartilaginous layer and bone, the chondrocytes were extremely large (Figure 4) compared to those described in other studies.\textsuperscript{14}
Figure 3 - Cartilaginous layer interposed between bone and tendon. This layer varied in thickness along the interface.

Also, the chondrocytes near or at the interface had bone (or heavily mineralized tissue) surrounding them on three sides, except for the side facing the cartilaginous layer (Figure 4). Larger cells within the bone were identified as osteocytes.

Near the interface of the cartilaginous layer and tendon, the cells changed from the typical chondrocyte "D-"shape to more elongated and also smaller cell shapes (Figure 5). These smaller elongated cells morphologically resembled tenocytes. In the layer, there was a gradual transition from the larger chondrocyte-like cells (near bone) to the smaller tenocyte-like cells (near tendon).
Figure 4 - Cartilaginous layer containing many large chondrocytes cells near the interface with bone.

Unlike in other studies mentioned earlier, the individual collagen bundles could not be distinguished even at high resolution. In a previous study⁵ that utilize SEM, the collagen of the intermediate layer was decalcified and revealed the individual collagen bundles.
Figure 5 - Presumed chondrocyte cells, of typical size but flatter, near the interface of the cartilaginous layer and tendon.

3.2 X-ray Energy-dispersive Spectroscopy Results

X-ray Energy-dispersive Spectroscopy (XEDS) both identified and spatially located bone present in the samples. XEDS revealed an apparent abrupt change in calcium (Ca) and phosphorus (P) concentrations at the interface between bone and the cartilaginous layer viewed at low magnification. However, the change in concentrations was more gradual when viewed at higher magnification (≈750X, Figures 6 and 7), with noticeably higher levels (but still substantially lower than in bone) of Ca and P in the first 20-40 μm of the cartilaginous layer apposing bone.
Figure 6 – High magnification (750X) micrograph of bone and cartilage interface. At the higher magnification, the interface observed is to be diffuse.
Locating a “tidemark” was not possible using XEDS maps; however, somewhat higher levels (still well below those in bone) of Ca and P were observed in the cartilage layer within 20-40 μm of the cartilage-bone interface. Further into the cartilaginous layer, there was no observed abrupt transition from calcified to uncalcified fibrocartilage. In histological studies, a distinct tidemark was visible by employing haematoxylin stains.\textsuperscript{2,3}

Figure 8 shows XEDS Ca and P elemental maps superimposed onto the secondary electron image of the same area as Figure 3. The map shows a high concentration of
phosphorus and calcium in the area identified as bone. In most of the cartilaginous layer, the signals from calcium and phosphorus were uniform, extending at these same levels into the tendon. These signals are believed to be at least partly attributable to proteins that contain phosphorus and calcium.

Figure 8 – Superimposed Ca and P XEDS elemental maps of bone, cartilage, and tendon layers (magnification 150x). (Same scale bar as Figure 3.) At such low magnification, there was little perceptible change in calcium or phosphorus concentrations across most of the cartilage layer and extending into the tendon.

At higher magnification (Figure 9, same area as Figure 5), their concentrations across the cartilage-tendon interface was confirmed to be uniform using XEDS.
Figure 9 – XEDS maps of Ca and P superimposed on the secondary electron image of either side of the interface between tendon and the cartilaginous layer (350x) (same scale bar as Figure 5). No substantial difference in concentrations of calcium or phosphorus was detected across the tendon-cartilaginous layer interface.

In all the XEDS elemental maps, the bone regions imaged mostly yellow, due to strong overlap of the signals of phosphorus and calcium (calcium in red and phosphorus in green). In the cartilaginous and tendon layers, the phosphorus and calcium signals were individually spatially more distinct (as well as weaker than in bone), supporting the inference that these signals originate in proteins, and likely different proteins for each elemental signal.

Additional secondary-electron images and superimposed and individual XEDS phosphorus and calcium maps are presented in Appendix A.
4. Discussion

4.1 Cell Type Differentiation

As seen in Figure 3, the cells within the cartilaginous layer varied in shape and size. Larger, "D-" shaped cells were present closer to the interface with bone, while smaller, elongated cells appeared closer to the interface with tendon. All the cell types in question (osteoblasts, chondrocytes, tenocytes) derive from similar mesenchymal stem cells.\(^{14}\)

One task for future research is then to establish the sequence of cell differentiation in this layer and to explain the apparent gradient in apparent cell type. If the cells can differentiate in some temporal sequence, the cells of the cartilaginous layer may go through a maturing process during development similar to that occurring in growth plates (see Appendix B). Another explanation for the gradation of cell type or cell shape and size is that the cells all start out as the same or similar type of cell and then select cells differentiate into different cell types depending on the signal or strength of a signal that they receive, which could be a function of their spatial location within the developing tissue matrix. Establishing the cell development sequences will require investigation of a developing natural interface.

4.2 Morphological Aspects of Interface

High magnification of the interface between cartilaginous layer and bone showed cells similar to chondrocytes almost surrounded by bone (Figures 3 and 4). The interface is interdigitated because of this unique feature. The interface between the cartilaginous layer and tendon does not exhibit this characteristic.
Interdigitation mechanically strengthens the interface. As noted before, injuries of
the tendon rarely occur at the interface with bone. The convoluted interface allows for
greater contact area between the bone and cartilage. A study that concentrates specifically
on the collagen fiber bundles could establish whether the collagen bundles were
continuous across the interface. The methods to decalcify the matrix used by Rodeo et
al.² would be necessary in order to image the fiber bundles using scanning electron
microscopy.

4.3 Calcified and Uncalcified Fibrocartilage Basophilic Line

Although a clear basophilic line was not visible in the micrographs of this study, a
gradual change in concentration of phosphorus and calcium was noted near the interface
of bone and cartilage (Figure7). If such gradual change in concentration happens over the
zone of calcified fibrocartilage, this study would show that the layer of fibrocartilage is
20 to 40 micrometers in width, varying along the interface.

It is suggested that further studies explore other histological methods, in addition
to the methods presented here on the samples derived from the same tendon insertion and
(if also possible) the same animal. Such a study could confirm that the location of the
basophilic line, observed in light-microscope histology, that marks the end of the
calcified fibrocartilage.
5. Conclusions

This study has augmented and supported the knowledge of other studies using light-microscope based histology. By employing SEM techniques, this study inferred an intermediate cartilaginous layer that establishes itself between bone and tendon. SEM images revealed that this layer exhibits a gradual change in cell type and size that might be indicative of a maturation sequence of cell differentiation during development of the tendon-bone interface. Sub-micrometer magnification also revealed the interdigitation of the cartilaginous layer and bone that creates greater surface area between the two layers, which could explain the rarity of bone-tendon ruptures occurring at this hard-tissue/softer-tissue interface. Although a distinct "tidemark" was not visible in the SEM images, XEDS maps did reveal a gradient in calcium and phosphorus concentrations across the interface between the cartilaginous layer and bone, extending 20-40 μm into the cartilaginous layer, a finding that supports the four layers found through histology. Further study of the interface gradient in cell type would provide a better understanding of this interface and its development.
6. References


7. Appendices

APPENDIX A: Secondary-electron SEM images and superimposed and individual XEDS Ca and P Elemental Maps.

The following images constitute a representative sampling of the images and elemental distribution maps obtained during this study. The first micrograph of each sample area is the SEM secondary-electron image of that area. The second micrograph is a composite of the secondary electron image, the calcium XEDS signal (red), and the phosphorus XEDS signal (green). The third micrograph of each sample area is the calcium signal of that area only, and the fourth micrograph of the phosphorus signal only.
Sample Area I-

SEM secondary-electron image of Sample Area I
Composite superimposed image and XEDS elemental maps of Ca (red) and P (green) of Sample Area I.

XEDS calcium signal image of Sample Area I
XEDS phosphorus signal image of Sample Area I
Sample Area II

SEM secondary-electron image of Sample Area II
Composite superimposed image and XEDS elemental maps of Ca (red) and P (green) of Sample Area II.
XEDS calcium signal image of Sample Area II.

XEDS phosphorus signal image of Sample Area II.
Sample Area III

SEM secondary-electron image of Sample Area III.
Composite superimposed image and XEDS elemental maps of Ca (red) and P (green) of Sample Area III.

XEDS calcium signal image of Sample Area III.
XEDS phosphorus signal image of Sample Area III.
SEM secondary-electron image of Sample Area IV.
Composite superimposed image and XEDS elemental maps of Ca (red) and P (green) of Sample Area IV.

XEDS calcium signal image of Sample Area IV.
XEDS phosphorus signal image of Sample Area IV.
Sample Area V

SEM secondary-electron image of Sample Area V.
Composite superimposed image and XEDS elemental maps of Ca (red) and P (green) of Sample Area V.

XEDS calcium signal image of Sample Area V.
XEDS phosphorus signal image of Sample Area V.
APPENDIX B: Discussion of Growth Plates

In a growth plate, there are seven different zones distinguished by cell size, shape and contents. The cells, which are chondrocytes, mature in the endochondral sequence and proceed through zones which represent a temporal stages in the sequence. The zones and the sequence are as follows:

Zone I - This zone is known as the "reserve or resting zone." Cells are sparse and surrounded mostly by extracellular matrix, although they may exist in pairs. These cells have low rates of proliferation and do not produce much type IIB collagen either. These cells store high contents of lipid bodies and vacuoles.

Zone II - This zone is called the "upper proliferative or columnar region." In this zone, chondrocytes are responsible for matrix production and cell proliferation, as the name suggests. These two activities result in longitudinal growth. The cells are flat and arranged in longitudinal columns.

Zone III - In this zone, cells are more mature, but still proliferate. They are morphologically no different from cells in Zone II, but they do not synthesize as much DNA as cells in Zone II.

Zone IV - This zone is known as the "upper hypertrophic zone." Here, cells are much larger than those in the previous zone and the columnar arrangement is disrupted and more sporadic. These cells do not proliferate, but do contain vacuoles. These cells are metabolically active and synthesize matrix three times faster than their predecessors.

Zone V - Here, the chondrocytes are terminal. The interface of this zone and Zone IV is marked by the last intact transverse cartilage septum. Here the matrix is calcified. These cells do produced type X and type II collagen.

Zone VI - Here the growth plate terminates and the bone begins. Before this zone chondrocytes lysis has occurred, and empty lacunae are left behind. In this region, osteoblasts lay down un-mineralized bone, called the osteoid. Bone then matures according to other processes.