1 **TITLE:**
2 A Protocol to Acquire the Degenerative Tenocyte from Humans
3
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33 **KEYWORDS:**
34 degenerative; tenocyte; tendinopathy; tendon; human; cell culture
35
36 **SUMMARY:**
37 In vitro use of degenerative tenocytes is essential when investigating the efficacy of novel
38 treatment on tendinopathy. However, most research studies use only the animal model or a
39 healthy tenocyte. We propose the following protocol to isolate human degenerative tenocytes
40 during surgery.
41
42 **ABSTRACT:**
43 Tendinopathy, a painful condition that develops in response to tendon degeneration, is on the
44 rise in the developed world due to increasing physical activity and longer life expectancy.

Despite its increasing prevalence, the underlying pathogenesis still remains unclear, and treatment is generally symptomatic. Recently, numerous therapeutic options, including growth factors, stem cells, and gene therapy, were investigated in hopes of enhancing the healing potency of the degenerative tendon. However, the majority of these research studies were conducted only on animal models or healthy human tenocytes. Despite some studies using pathological tenocytes, to the best of our knowledge there is currently no protocol describing how to obtain human degenerative tenocytes. The aim of this study is to describe a standard protocol for acquiring human degenerative tenocytes. Initially, the tendon tissue was harvested from a patient with lateral epicondylitis during surgery. Then biopsy samples were taken from the extensor carpi radialis brevis tendon corresponding to structural changes observed at the time of surgery. All of the harvested tendons appeared to be dull, gray, friable, and edematous, which made them visually distinct from the healthy ones. Tenocytes were cultured and used for experiments. Meanwhile, half of the harvested tissues were analyzed histologically, and it was shown that they shared the same key features of tendinopathy (angiofibroblastic dysplasia or hyperplasia). A secondary analysis by immunocytochemistry confirmed that the cultured cells were tenocytes with the majority of the cells having positive stains for mohawk and tenomodulin proteins. The qualities of the degenerative nature of tenocytes were then determined by comparing the cells with the healthy control using a proliferation assay or qRT-PCR. The degenerative tenocyte displayed a higher proliferation rate and similar gene expression patterns of tendinopathy that matched previous reports. Overall, this new protocol might provide a useful tool for future studies of tendinopathy.

INTRODUCTION:

Tendinopathy is a chronic degenerative musculoskeletal condition that develops in various parts of the body. Recently, the number of cases of tendinopathy has increased greatly in the developed world due to growing participation in recreational sports and increased life expectancy[1,2]. The cause of tendinopathy is considered to be multifactorial and these causes include ischemia, oxygen free radical injuries, an imbalance between vasoconstrictor and vasodilator innervations, internal micro-tears, and changes to neuro-regulation[3-8]. Most treatments for tendinopathy only relieve its symptoms. Moreover, treatments without tissue regeneration require a long time for rehabilitation and achieve a limited response from injured tendons, which imposes a clinical challenge for physicians[9].

The incompetence of current treatment options along with the lack of the degenerative tendon's ability to self-heal has lead researchers to take interest in exploring alternative treatment strategies. Recently, new studies reported many promising results for enhancing the healing efficacy of the tendinopathy tendons using growth factors, stem cell based therapy, and gene therapy[10-12].

Through a literature review, we found that the involved studies may be divided into two categories based on their analysis materials: animal models such as a rat, a mouse, or a rabbit; and human models. With regard to the animal model, currently there are two popular techniques to generate tendinopathy: chemical induction of injury or mechanical overloading

88   the model. However, each animal model was limited in reproducing the complex human
89   tendinopathy pathology[13,14].
90
91   Most papers using human samples were analyzed histologically or performed the in vitro
92   experiment based on a healthy human tenocyte instead of a degenerative tenocyte[15-21]. Only a
93   few papers reported that they used a human degenerative tenocyte, but they did not describe
94   in detail the protocol used to get the degenerative tenocyte from the human[22,23]. In this context,
95   it should be noted that successful results from either the animal model or the healthy
96   tissue/tenocyte may not necessarily predict human efficacy or efficacious dosing because
97   tendon degeneration is a complicated process and the pathogenesis is still not fully understood.
98
99   Collectively, it is necessary to describe the standard protocol for obtaining the degenerative
100   tenocyte from human tissue without causing adverse effects to the donor. This article describes
101   a protocol on how to acquire the human degenerative tenocyte. To validate the protocol, the
102   harvested tissues were analyzed histologically. Then, the cultured cell was confirmed as the
103   degenerative tenocyte by using immunocytochemistry (ICC), a quantitative real time-
104   polymerase chain reaction (qRT-PCR), and a viability assay.
105
106   **PROTOCOL:**
107
108   The protocol was conducted in accordance with the Declaration of Helsinki and the protocol
109   was approved by the Institutional Review Board at CHA Bundang Medical Center.
110
111   **1.   Degenerative tendon tissue harvest from the patient**
112
113   1.1.   Diagnose lateral epicondylitis by taking a medical history and using physical exam
114   findings: tenderness over the tendinous origin close to the lateral epicondyle; pain evoked by
115   resisted extension of the wrist joint.
116
117   1.2.   Use the following inclusion criteria: over 18 years of age; a history of lateral epicondylitis
118   for a minimum of six months; recalcitrant for non-operative treatment; at least three months
119   have passed since injection of corticosteroid, platelet rich plasma, or botulinum toxin
120
121   1.3.   Exclude the following: pregnant women; patients with a history of cervical spine disease,
122   elbow arthritis, rheumatologic disease, surgery around elbow, and nerve entrapment
123   syndrome; patients refusing to donate tissue.
124
125   1.4.   <mark>Surgical technique</mark>
126
127   NOTE: See **Figure 1**[24].
128
129   1.4.1.   After general anesthesia by isoflurane and endotracheal intubation, put the arm on the
130   table and apply a tourniquet. Then apply betadine agent on the whole arm and drape with an
131   aseptic surgical sheet.

1.4.2. Expose the lateral elbow through a 3-5 cm incision with No. 15 surgical scalpel blade just anteromedial to the lateral epicondyle and proximal to the level of the joint.

1.4.3. Visually identify the extensor carpi radialis longus (ECRL) and extensor aponeurosis interface and make a splitting incision 2-3 mm in depth between the ECRL and extensor aponeurosis with a No. 15 surgical scalpel blade at the identified interface.

1.4.4. Extract the ECRL anteriorly, which will bring the pathologic extensor carpi radialis longus brevis (ECRB) origin below into direct view.

1.4.5. Visually identify the pathologic degenerative tissue by its characteristic dull grayish color and usually edematous and friable tissue.

1.4.6. After identification, carefully resect all degenerative tissue sharply using No. 15 surgical scalpel and mini-blades.

1.4.7. Embed the resected tissue (about 1 cm^3) into phosphate buffered saline (PBS) immediately and remove all the surrounding tissue except tendon tissue carefully into about 10 mL of PBS as soon as possible.

1.4.8. In cases with bony exostosis at the lateral epicondyle, remove exostosis using a rongeur and smoothen it with a rasp. Make multiple drill holes (diameter 1.6 mm, about 6-8 holes) in the anterolateral condyle to enhance vascular supply. This is optional.

1.4.9. Close the wound layer by layer using non-absorbable suture material.

1.5.   Healthy control tenocyte

1.5.1. Obtain healthy tenocytes from the remaining tendon of the auto-hamstring or auto-patella tendon during anterior cruciate ligament reconstruction, and use it as the control for the following experiment[25].

NOTE: The number of samples used in this study were three in each group.

2.   Histology

2.1.   Store about half of the harvested tissue in neutral buffered 10% formalin in a chemical fume hood or equivalent.

2.2.   Embed the tissue in a paraffin block (4 – 5 mL) and section it in 4 – 5 μm sections coronally.

2.3.   Stain sections from control and degenerative tendon with Hematoxylin and Eosin stain (H&E) and Alcian blue stain at a pH of 2.5. Perform H&E staining in an automated stain machine as follows.

2.3.1.   Deparaffinize using xylene (3 times, 5 minutes, respectively).

2.3.2.   Hydrate from graded alcohols to water (100% alcohol, 4 min; 100% alcohol, 3 min; 95% alcohol, 2 min; 70% alcohol, 1 min; water, 2 min).

2.3.3.   Stain in Hematoxylin for 5 min. Wash well in water for 2 min.

2.3.4.   Differentiate in 1% acid alcohol (1% HCl in 70% alcohol) for 3 s. Wash well in water for 3 min.

2.3.5.   Neutralize HCl by dipping in an 0.5% ammonia for 10 s, followed by a 3 min water wash.

2.3.6.   Stain in Eosion for 5 min.

2.3.7.   Dehydrate through alcohols (80% alcohol, 30 s; 95% alcohol, 1 min; 100% alcohol 1 min, 100% alcohol 2 min).

2.3.8.   Clear in xylene (3 times, 2 min, 2 min, and 3 min, respectively).

2.3.9.   Mount with a cover slide.

2.4.   Perform immunohistochemitry (IHC) using the Bond Polymer Refine Detection kit according to the manufacturer's instructions with minor modifications.

2.4.1.   After deparaffinization using xylene, perform an antigen retrieval procedure using Bond Epitope Retrieval Solution for 30 min at 100 °C. The Bond Epitope Retrieval Solution contains a citrate based buffer and surfactant (1 liter, pH 5.9-6.1).

2.4.2.   Quench endogenous peroxidases by incubating the tissue with hydrogen peroxide for 5 min.

2.4.3.   Incubate the sections for 15 min at an ambient temperature (21-22 °C) with primary antibodies against vascular endothelial growth factor (VEGF) (1:200), tenomodulin (1:200), or mohawk (1:200).

2.4.4.   Perform secondary antibody tagging with a biotin-free polymeric horseradish peroxidase-linker antibody conjugate system.

2.4.4.1.   Use secondary rabbit anti mouse IgG in 10% (v/v) animal serum in Tris-buffered saline/0.09% 2-Methyl-4-isothiazolin-3-one solution to localize mouse antibodies.

219

220 2.4.4.2.   Use polymer anti-rabbit Poly-HRP-IgG (< 25 µg/mL) containing 10% (v/v) animal
221 serum in Tris-buffered saline/0.09% to localize rabbit antibodies.

222

223 2.4.4.3.   Use 3,3'-Diaminobenzidine tetrahydrochloride hydrate in a stabilizer solution to
224 visualize the complex via a brown precipitate.

225

226 2.4.4.4.   Counterstain with < 0.1% hematoxylin (blue) to visualize the cell nuclei.

227

228 2.5.   Observe the slides stained with H&E, Alcian blue stains, and IHC for VEGF, tenomodulin,
229 and mohawk under light microscope and obtain representative microscopic images of each
230 slide under one high power field (400X).

231

232 **3.   Tenocyte culture**

233

234 3.1.   Tissue preparation

235

236 3.1.1.   Prepare the tendon sample by removing all the surrounding tissue thoroughly with
237 micro-scissors and forceps.

238

239 3.1.2.   Wash with PBS and mince into small pieces (about 1 mm^3 or less).

240

241 3.1.3.   Digest tissue for 1 h in 30 mL of Dulbecco's Modified Eagle's Medium (DMEM)
242 supplemented with 30 mg/mL Collagenase II at 37 °C.

243

244 3.1.4.   Pass cells through a 100 µm cell strainer.

245

246 3.1.5.   Deactivate Collagenase II by adding 1 mL of fetal bovine serum (FBS).

247

248 3.1.6.   Centrifuge at 196 x g for 5 min.

249

250 3.2.   Cell culture

251

252 3.2.1.   Seed 3 x 10^5 cells into a 100 mm dish and culture with DMEM supplemented with 10%
253 FBS and 1% Antibiotic-Antimycotic solution at 37 °C in a 5% CO_2 incubator for 1 week.

254

255 3.2.2.   Change the culture medium every three days until 80% confluence occurs. Culture both
256 healthy control and degenerative cell samples until passage four and use for experiment.

257

258 **4.   Immunocytochemistry (ICC)**

259

260 4.1.   Transfer 200 µL of cultured cell (2 × 10^5 cells) to the eight wells of a chamber slide and
261 grow the cells to confluence with the addition of fresh media for 1 day.

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5.   Proliferation assay

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5.1.   Measure cell viabilities using water-soluble tetrazolium (WST)-1.

283
284
285

5.1.1.   Seed both healthy and degenerative cells into 96 well plates at a density of 2×10^3 cells per well for 24, 72, and 120 h.

286
287
288

5.1.2.   After incubation, add 10 µL of the 10% WST-1 solution to each well.

289
290

5.1.3.   Incubate the plates for 3 h at 37 °C in 5% CO_2.

291
292

5.1.4.   Measure the optical densities at 450 nm using a microplate reader. Take readings at least three times at all time points for each sample.

293
294
295

5.2.   Measure cellular DNA amount using the BrdU assay.

296
297

5.2.1.   Seed both healthy and degenerative cell into 96 well plates at a density of 2×10^3 cells per well for 24, 72, and 120 h.

298
299
300

5.2.2.   After incubation, add 10 µL of 10% BrdU solution to each well.

301
302

5.2.3.   Incubate the plates for 3 h at 37 °C in 5% CO_2.

303
304

5.2.4.   Add 100 µL/well of the Fixing/Denaturing Solution (60-70% ethanol, 0.1-0.5% sodium hydroxide) and keep the plate at room temperature for 30 min.

305

306
307 5.2.5. Add 100 μL/well prepared 1x detection antibody solution and keep the plate at room
308 temperature for 1 h.
309
310 5.2.6. Remove solution and wash plate three times with 1x Wash Buffer.
311
312 5.2.7. Add 100 μL/well prepared 1x HRP-conjugated secondary antibody solution and keep the
313 plate at room temperature for 30 min.
314
315 5.2.8. Remove the solution and wash plate three times with 1x Wash Buffer.
316
317 5.2.9. Add 100 μL of 50% TMB Substrate.
318
319 5.2.10. Incubate for 30 min at room temperature.
320
321 5.2.11. Add 100 μL of STOP Solution.
322
323 5.2.12. Measure the optical densities at 450 nm using a microplate reader.
324
325 NOTE: Take readings at least three times at all time points for each sample.
326
327 **6.   Statistical analysis**
328
329 6.1.   Collect and analyze data using SPSS.
330
331 6.2.   Depict data by mean ± standard error of mean.
332
333 6.3.   Use Mann Whitney U test.
334
335 6.4.   Consider differences statistically significant when the p value is less than 0.05 ($p < 0.05$).
336
337 **REPRESENTATIVE RESULTS:**
338 Histological analyses revealed that the harvested tissue from lateral epicondylitis had the
339 characteristics of a tendinopathic tendon. H&E section of tendinopathy degenerative tendon
340 revealed a disorganized collagen bundle with a loss of polarity and fine straight, strongly packed
341 parallel fiber structures. Histological signs suggestive of degeneration such as higher cellularity
342 and enlarged nuclei without the typical spindle shape were common in samples. Additionally,
343 collagen bundles of the degenerated tendon were relatively weak eosin stained by H&E but
344 were positively stained by Alcian blue indicating increased proteoglycans and
345 glycosaminoglycans substance.
346
347 In all cases, blood vessels were increased in number and aggregates occupying spaces
348 embedded between fibers were compared to normal slit-like blood vessels. In the IHC staining,
349 cells of degenerated tendons showed cytoplasmic granular positive reaction to VEGF in

350 comparison to the negative reaction of control ones. Collectively, these findings confirmed that
351 the harvested tissues from lateral epicondylitis were degenerative tissues and suitable for the
352 degenerative tenocyte culture (see **Figure 2**).
353
354 The cultured cells were confirmed as tenocytes by ICC and the harvested tissues were
355 confirmed by IHC as tendon tissue. Most of the cultured cells and cells in tendon tissue showed
356 a positive stain for the representative tenocyte markers known as mohawk (green) proteins and
357 tenomodulin (red) proteins with an elongated appearance under microscopy (see **Figure 3**).
358 Also, the harvested tissues had the positive staining for mohawk proteins and tenomodulin
359 proteins immunohistochemically (see **Supplementary Figure 1**). These suggested that the
360 harvested tissues were composed by the tendon tissue.
361
362 Cultured tenocytes from the harvested tissue of lateral epicondylitis had the characteristic
363 features that correspond to tendinopathy. Both degenerative and healthy tenocytes were
364 cultured for 4 days under the same condition. At each time point, the proliferation rate was
365 measured by BrdU assay and WST-1 assay. The results showed that the proliferation rate of the
366 degenerative cells was significantly higher compared to the controls as previously reported ($p <$
367 0.05) (see **Figure 4A**).
368
369 Five genes which are known to be up-regulated in the degenerative tendon were analyzed by
370 qRT-PCR. Expressions of the genes, *COL3A1, ACTA2, TAC1 (SP), TAC receptor 1,* and *PTGS2*
371 *(COX2)* were increased more significantly in the cultured cells from the degenerative tendon
372 than in the cells from the healthy ones. The relative expressions for all genes are shown in
373 **Figure 4B** and Supplemental **Figure 2.**
374
375 **Figure and Table Legends:**
376 **Figure 1. Surgical photo of degenerative tendon harvest.**
377 **(A)** After skin incision and dissection, the pathologic degenerative tissue on the common
378 extensor origin was exposed, showing a characteristic dull-grayish color with edematous
379 change (red line). Conversely, the general appearance of normal tendon tissue was shiny and
380 firm with a slightly grayish tone (black line). (**B**) All identified degenerative tissues were
381 resected sharply and then the radial head bone was exposed. (**C**) All resected tissues were
382 embedded into the phosphate buffered saline immediately and the surrounding non-tendinous
383 tissue was carefully dissected as soon as possible. ECRB: extensor carpi radialis brevis. EDC:
384 extensor digitorum communis. ECU: extensor carpi ulnaris.
385
386 **Figure 2. Confirmation of degenerative tendon histologically.**
387 **(A)** In the H & E staining, degenerative samples from the lateral epicondylitis had
388 disorganized collagen bundles with loss of polarity and increased cell number compared to the
389 healthy control. (**B**). Alcian blue staining showed that the degenerative tendon had increased
390 ground substance consisting of proteoglycans and glycosaminoglycans with several mucoid
391 patches and vacuoles between fibers (Arrow). (**C**). Additionally, VEGF immunohistochemistry
392 demonstrated increased staining in vessel formation in degenerative tendon samples compared
393 to the control samples. VEGF: Vascular endothelial growth factor. Scale bar = 50 μm.

394
395 **Figure 3. Identification of tenocyte by ICC.**
396 The cultured cells and cells in tendon tissue were confirmed as tenocyte by ICC. Most of the
397 cells showed positive stain for the representative tenocyte marker, including mohawk (green)
398 and tenomodulin (red) with elongated appearance under microscopy. Chondrocyte was used as
399 the negative control. ICC: Immunocytochemistry. Scale bar = 50 μm.
400
401 **Figure 4. Characteristics of degenerative tenocyte.**
402 (**A, B**) Degenerative tenocytes (red) had a higher proliferation rate with a notable increase in
403 cellularity. (**C**) Five genes that are known to be related in the development of tendinopathy
404 were significantly elevated in the degenerative tenocytes. Gene expression levels were
405 normalized against *GAPDH*. * and ** mean < 0.05 and < 0.01, respectively. SP: substance P.
406
407 **Supplementary Figure 1. Histological confirmation of the harvested tissue as the tendon**
408 **tissue.**
409 The harvested tissue was also analyzed by IHC for mohawk and tenomodulin to determine
410 whether the harvested tissue had the adequate characteristics of tendon tissue. IHC:
411 Immunohistochemistry. Scale bar = 50 μm.
412
413 **Supplementary Figure 2. Expression of tendinopathy related genes in tenocyte.**
414 The transcripts of five genes normalized by actin expression were significantly elevated in the
415 degenerative tenocytes and have similar trends to **Figure 4**. Gene expression levels were
416 normalized against *actin* with a mean < 0.05 and < 0.01, respectively. SP: substance P.
417
418 **DISCUSSION:**
419 A number of previous studies have reported how to create chronic tendinopathic animal
420 models using different procedures such as collagenase or kartogenin injection, treadmill
421 running, and more[26,27]. Although numerous studies showed promising therapeutic effects
422 based on these animal models, experiments using the human degenerative tenocyte would be
423 crucial in the field of tendinopathy in order to reproduce the efficacy of treatment. In this
424 article, we established a protocol to successfully harvest and culture human degenerative
425 tenocytes consistently. Both harvested tendon tissue and the cultured tenocyte showed the
426 common characteristics of the degenerative tendon and tenocyte[7,28].
427
428 In our experience, for the successful tissue harvest, it is essential to comprehend the
429 morphological changes frequently associated with degenerative tendon and to accurately
430 delineate that area during surgery. Degenerative tissue is typically grey or brown in color and
431 the tissue is thin, fragile, and disorganized with loose texture. The inclusion and exclusion
432 criteria of the protocol should be strictly kept with careful evaluation of previous treatments
433 before harvest.
434
435 Tissue size is also important for a successful cell culture. We recommend harvesting more than
436 1 cm^3. Usually, as long as all of the degenerative area is removed, the amount of collected
437 tissue would be enough for the cell culture. Lastly, we did not use tenocytes over passage six in

438 all experiments. Until passage six, we believe that the cultured cells still had the characteristics
439 of degeneration.

441 Our protocol has several advantages. These advantages include no harm to the patient since
442 the degenerative tissue was supposed to be removed during surgery, efficiency since the
443 amount of tissue required is enough for the experiment, highly ethical due to no violation of
444 healthy tissues, reproducibility in that pathological tissues from humans can be readily obtained
445 during surgery, and, lastly, ease of application once surgical techniques become familiar.

447 However, we acknowledge that our experiment has several limitations. First, we cannot
448 completely exclude the remnant effect of previous corticosteroid or platelet rich plasma
449 injection even though we included only patients who had passed at least three months from
450 receiving any previous treatment. In addition, in this protocol, we only harvested the ECRB
451 tendon and used it to culture degenerative tenocytes since the ECRB tendon is a common
452 tendinopathy site for surgery along with Achilles and rotator cuff tendons. Further studies
453 should be carried out with a larger sample size focusing on any differences between various
454 tendinopathy sites.

456 In conclusion, this validated protocol for acquiring human degenerative tenocytes will be a
457 useful tool for future investigations in tendon biology and for testing novel therapeutic
458 interventions.

465 **DISCLOSURES:**
466 The authors have nothing to disclose.

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