Comparison of equine tendon-, muscle-, and bone marrow–derived cells cultured on tendon matrix

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Objective—To compare viability and biosynthetic capacities of cells isolated from equine tendon, muscle, and bone marrow grown on autogenous tendon matrix.

Sample Population—Cells from 4 young adult horses.

Procedures—Cells were isolated, expanded, and cultured on autogenous cell-free tendon matrix for 7 days. Samples were analyzed for cell viability, proteoglycan synthesis, collagen synthesis, and mRNA expression of collagen type I, collagen type III, and cartilage oligomeric matrix protein (COMP).

Results—Tendon- and muscle-derived cells required less time to reach confluence (approx 2 weeks) than did bone marrow–derived cells (approx 3 to 4 weeks); there were fewer bone marrow–derived cells at confluence than the other 2 cell types. More tendon- and muscle-derived cells were attached to matrices after 7 days than were bone marrow–derived cells. Collagen and proteoglycan synthesis by tendon- and muscle-derived cells was significantly greater than synthesis by bone marrow–derived cells. On a per-cell basis, tendon-derived cells had more collagen synthesis, although this was not significant. Collagen type I mRNA expression was similar among groups. Tendon-derived cells expressed the highest amounts of collagen type III and COMP mRNAs, although the difference for COMP was not significant.

Conclusions and Clinical Relevance—Tendon- and muscle-derived cells yielded greater cell culture numbers in shorter time and, on a per-cell basis, had comparable biosynthetic assays to bone marrow–derived cells. More in vitro experiments with higher numbers may determine whether tendon-derived cells are a useful resource for tendon healing. (*Am J Vet Res* 2009;70:750–757)

Tendinitis is a common debilitating injury in performance horses, comprising up to 43% of injuries in racing Thoroughbreds and resulting in retirement of up to 14% of horses.[1–3] Timely repair of injured tendon with restoration of normal tissue organization and biomechanical properties has not been consistently achieved.[4–6] Thus, reinjury, despite prolonged and costly rehabilitation, is common.

The inability of equine tendon to regenerate after injury, or to heal with mechanical properties comparable to the original tissue, is likely attributable to the low vascularity and cellularity of the tissue, low number of progenitor cells in the tissue, and healing under weight-bearing conditions.[2,3,7,8] Additionally, it is thought that tendinitis, which might more properly be termed tendinopathy, is caused by a chronic degenerative process rather than a single traumatic episode.[7,9] Strategies to improve tendon healing have focused on enhancing the metabolic response of the injured tenocytes, modulating the organization of newly synthesized extracellular matrix, or administering progenitor cells to enhance repair tissue.[5,10–13] The use of MSCs for tissue repair has been an extremely dynamic area of research during the past decade.[13] Most of those studies have focused on MSCs derived from bone marrow aspirates. The most prominent avenues of investigation with MSCs have been directed at repair of bone, cartilage, myocardium, and CNS tissues.[14–17] Isolation of a homogeneous population of progenitor cells from bone marrow is time-

Abbreviations

CPM	Counts per minute
COMP	Cartilage oligomeric matrix protein
DMEM	Dulbecco modified Eagle medium
DPM	Disintegrations per minute
FBS	Fetal bovine serum
MSC	Mesenchymal stem cells

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consuming, and there is much variation in cell numbers, cell viability, and growth rates among samples.[18,19] More recent studies[20,21] indicate that alternative sources of progenitor cells (eg, fat, synovium, cartilage, and satellite cells) might also be beneficial for specific therapeutic applications.

Tendon is composed primarily of type I collagen fibrils arranged into fibers aligned along the longitudinal axis of the tendon.[22] Collagen type III is also present and comprises approximately 14% of total collagen in normal tendon.[23] Cartilage oligomeric matrix protein is another important extracellular matrix protein produced by tenocytes.[24] Collagen type III and COMP participate in collagen type I fibrillogenesis,[24,25] and both are produced following tendon injury.[26,27] Collagen fibrils are surrounded by ground substance composed of proteoglycans and glycosaminoglycans, which help package the collagen fibrils. Proteoglycan and collagen synthesis are also increased in healing tendon.[28,29]

Bone marrow–derived MSCs have been used for tendon repair in horses, rabbits, and rats.[15,30,31] To date, most published studies, such as those by Awad et al[30] and Guest et al,[31] have used bone marrow–derived MSCs for tendon repair. Most studies in rabbits and rats have revealed an increase in the strength of tendon repair; however, 1 study[32] found bone formation within the tendon repair tissue. Progenitor cell–based treatments for tendon injuries have promise, but are not widely used or universally successful.

The objective of the study reported here was to compare cells isolated from 3 tissue types (tendon, muscle, and bone marrow) for ease of isolation, cell growth characteristics, extracellular matrix production, and expression of tendon matrix markers when grown on tendon extracellular matrix. Bone marrow–derived progenitor cells were used as the gold standard source for cell-based tendinitis therapy.[31] Skeletal muscle– and tendon-derived cells were isolated by use of a preplating technique used to isolate progenitor cells from muscle.[33,34] Our hypotheses were that tendon-derived cells would yield greater numbers in cell culture over a shorter time period, grow and adhere to the matrix better, and produce more tendon extracellular matrix than cells derived from muscle or bone marrow.

Materials and Methods

Collection of samples—Bone marrow, muscle, and tendon were collected aseptically from 4 young horses (1 to 3 years of age) euthanatized for reasons unrelated to musculoskeletal disease. All samples were obtained in accordance with guidelines reviewed and approved by the Institutional Animal Care and Use Committee of the University of Illinois. All horses were sedated with 0.5 to 1.0 mg of xylazine/kg administered IV prior to induction. Anesthesia was induced with 2.2 mg of ketamine/kg and 0.1 mg of diazepam/kg given IV. General anesthesia was maintained by IV infusion of 5% guaifenesin, 1 mg of ketamine/mL, and 1 mg of xylazine/mL. Following collection of the bone marrow aspirates, as described,[18,35] all horses were euthanatized with 104 mg of sodium pentobarbital/kg given IV. The tendon and muscle specimens were collected immediately following euthanasia.

Processing of bone marrow–derived cells—The right tuber coxae was clipped and aseptically prepared, and a bone marrow biopsy needle[a] was used to aspirate 30 mL of bone marrow into syringes containing 3,000 units of heparin diluted to a volume of 5 mL with PBS solution. The bone marrow aspirate was diluted with 15 mL of PBS solution and centrifuged at 300 × *g* for 15 minutes. The supernatant was removed, the pellet was resuspended in PBS solution, and centrifugation was repeated. Pelleted cells were resuspended in 12 mL of low-glucose DMEM[b] supplemented with 10% FBS,[c] 300 µg of L-glutamine[d]/mL, 100 U of sodium penicillin[e]/mL, 100 µg of streptomycin sulfate[e]/mL, and 1mM of sodium pyruvate[f]/mL. Resuspended cells were placed in a 75-cm^2 flask and incubated at 37°C in a 5% carbon dioxide atmosphere with 90% humidity. To obtain adequate cell numbers for experiments, the bone marrow–derived cells were passaged after they reached focal areas of confluence. Time to confluence and cell counts at trypsinization were recorded for all cell types.

Processing of muscle-derived cells—The skin over the right semitendinosus region was clipped and aseptically prepared prior to euthanasia. Immediately following euthanasia, a muscle sample (5-cm length by 2-cm width by 1.5-cm depth) was excised from the right semitendinosus muscle. Progenitor cells were collected from skeletal muscle by use of a published protocol.[33] Briefly, the muscle was diced into 0.5-cm^3 pieces, rinsed with PBS solution to remove RBCs, and dissociated by sequential digestion with 0.2% collagenase[g] for 30 minutes followed by 2.0% dispase[h] for 15 minutes at 37°C. All enzymatic digestions were carried out in high-glucose DMEM supplemented with 1% FBS, 37.5 µg of ascorbic acid[i]/mL, 100 U of sodium penicillin/mL, and 100 µg of streptomycin sulfate/mL. Following digestion, the suspensions were passed through 70- and 40-µm filters.[j] The isolated cells were collected by centrifugation at 300 × *g* for 5 minutes. The supernatant was removed, and the cell pellet was resuspended in media. Cell viability was determined by use of exclusion of trypan blue dye.[36,k]

Processing of tendon-derived cells—The superficial digital flexor tendons from both forelimbs were harvested aseptically from each horse. A 6-cm × 1-cm^2 sample of tendon was reserved for cell isolation. A 2-cm^3 sample was snap-frozen in liquid nitrogen for control RNA isolation, and the remainder was cryopreserved for cell-free tendon matrix production. The specimen for cell isolation was diced into 0.5-cm^3 pieces and digested for 16 hours at 37°C in 0.1% collagenase high-glucose DMEM supplemented with 1% FBS, 37.5 µg/mL of ascorbic acid, 100 U of sodium penicillin/mL, and 100 µg of streptomycin sulfate/mL. Following digestion, the isolated cells were processed as for muscle-derived cells.

Muscle and tendon cell culture—The muscle- and tendon-derived cells were seeded at 1,300 cells/cm^2 in monolayer cultures in high-glucose DMEM supplemented with 20% FBS, 37.5 µg/mL of ascorbic acid, 300 µg of L-glutamine/mL, 100 U of sodium penicillin/mL, and 100 µg of streptomycin sulfate/mL. The rapidly adherent, fibroblast-like cells in these popula-

tions were excluded by a described preplating or differential attachment protocol whereby the culture medium and unattached cells were serially transferred to fresh culture flasks every 12 to 24 hours during the first 72 hours of culture.[33] The muscle- and tendon-derived cells that adhered after 72 hours of differential attachment were trypsinized at confluency and used in the experiments.

Tendon matrix culture model—Superficial digital flexor tendons were collected from donor horses and cut longitudinally with a dermatome to produce uniformly flat, 0.5-mm-thick tendon sheets. These sheets were cut into 1 × 1-cm square explants. The explants were subjected to 4 rounds of freeze-thaw cycles at –80°C and 4°C to kill the endogenous cells. These cell-free tendon explants (matrix only) were maintained in culture without the addition of cells to serve as negative controls or seeded with 250,000-cell aliquots of the expanded bone marrow–derived, tendon-derived or muscle-derived cells. This experimental design comprised 4 treatment groups (matrix only, matrix and bone marrow–derived cells, matrix and muscle-derived cells, and matrix and tendon-derived cells). Each treatment group had 20 replicates, with an overall total of 80 samples/horse.

The cell-seeded tendon matrices were supplemented with high-glucose DMEM supplemented with 10% FBS, 37.5 µg of ascorbic acid/mL, 300 µg of L-glutamine/mL, 100 U of sodium penicillin/mL, and 100 µg of streptomycin sulfate/mL. The media was changed every 2 days. All culture samples were collected on day 7.

Cell numbers on matrices—Three replicates of each treatment group were used to measure cell viability and number on day 7 by use of a cell viability assay,[l] as per manufacturer's instructions. In brief, 50 µL of the assay reagent was added to fresh ascorbate–free media in each well, and the cells were incubated at 37°C for 2.5 hours. One hundred microliters of media from each well was transferred to a 96-well plate, and absorbance was measured at 492nM in a microplate reader.[m] All samples were assayed in duplicate, and a mean value was calculated to provide a single data point. These optical density values were converted to cell number by reference to standard curves determined from plated cells from all 3 tissue sources from each horse.

Collagen synthesis—Collagen synthesis was determined via [^{3}H]proline[n] incorporation according to a published protocol.[37] On day 6, 3 wells of each treatment group were radiolabeled with 50 µCi of [^{3}H]proline/mL of media and incubated overnight. Following radiolabeling, the samples were stored at –80°C.

Radiolabeled samples were freeze-thawed 3 times, digested, and homogenized to disrupt cells and matrix prior to RNase treatment. The total protein was precipitated with tricholoroacetic acid and washed 3 times. The resulting pellets were dissolved, digested with purified collagenase,[o] and centrifuged. The supernatant and pellets were separated and added to scintillation liquid, and the radioactivity was counted via scintillation counter.[p]

Newly synthesized collagen was detected on the basis of radioactivity in the sample supernatants following collagenase-digestion.[37] Percentage collagen synthesis was expressed by relating collagen synthesis to total protein synthesis (sum of the activity in the collagenase-digested supernatant and the pellet) in each sample.

Proteoglycan synthesis—Proteoglycan synthesis was determined by measuring $^{35}SO_4$ incorporation into each sample. Three wells of each treatment group were radiolabeled with 10 µCi of ^{35}S methionine/mL[q] during the last 24 hours of the experiments. The samples were digested in 1 mL of 0.1% papain[r] at 65°C for 16 hours.[38] Twenty-five-microliter aliquots of ^{35}S-labeled, papain-digested tendon matrices were placed in multiwell punch plates,[s] precipitated with alcian blue dye, and counted via scintillation. All values were adjusted for radioisotope decay and for the total volume of papain digestion buffer added to the sample.

RNA isolation and gene expression—Nine samples from each treatment group were pooled, snap-frozen in liquid nitrogen, and stored at –80°C for RNA isolation. The RNA was isolated by use of a protocol adapted from a technique for cartilage RNA isolation.[39] Briefly, tissues were pulverized under liquid nitrogen, homogenized in guanidinium isothiocyanate lysis buffer, extracted with phenol-chloroform, precipitated with isopropanol, and purified by use of a commercially available column-based protocol.[t] This protocol included an on-column DNase treatment to exclude genomic template contamination. One microgram of RNA in each sample was converted to cDNA with a commercial reverse transcription kit[u] and oligo(dT) primers. Target cDNAs were amplified via real-time PCR by use of *Taq* DNA polymerase[v] and gene-specific primers designed from available published sequences in Genbank and by use of a multiple sequence alignment program.[w] Primer specificity was confirmed by cloning and sequencing the PCR products (**Appendix**). Real-time quantitative PCR assay was performed in duplicate for collagen I, collagen III, and COMP mRNAs and the reference gene, elongation factor-1α. A PCR detection system[x] was used to perform the assay. The RNA from freshly collected, snap-frozen tendon was used as a reference control for the gene expression analyses, to relate in vitro expression levels to in vivo expression. All reactions were run as singleplex, and the relative gene expression was quantified by use of the $2^{-\Delta\Delta CT}$ method.[40]

Histologic examination—Two samples from each treatment group were fixed in 4% paraformaldehyde and paraffin embedded by use of routine protocols. Six micrometer-thick sections were stained with H&E for evaluation of the cell layers colonizing the matrix surfaces.

Statistical analysis—Mean ± SE for each statistic was calculated for each cell type from the 4 horses in the study. Background values detected in the matrix-only group were subtracted from the values for the other groups. Cell number, proteoglycan synthesis, and collagen synthesis data were log transformed to account for between-animal variability.[41] In addition, the mean logs of collagen synthesis and proteoglycan synthesis were normalized to the mean log of cell number. Collagen

type I, collagen type III, and COMP mRNA values were normalized to expression of the reference gene elongation factor-1α. The effect of cell type was evaluated by use of 1-way repeated-measures ANOVA to control for the differences among horses. Pairwise multiple comparisons were made by use of the Holm-Sidak method to evaluate differences in means. A commercial statistical program[y] was used to perform statistical analyses. Values of $P \leq 0.05$ were considered significant. Descriptive statistics (mean ± SE) were determined for days to confluence and cell number following monolayer expansion of bone marrow–, muscle-, and tendon-derived cells.

Results

Cell isolation and expansion—Following preplating and attachment, the muscle- and tendon-derived cells proliferated in uniform monolayer cultures and adopted a tightly packed, fusiform morphology (**Figure 1**). Bone marrow–derived cells grew in clonal expansion groups that had focal areas of tightly packed cells with fusiform morphology. Bone marrow–derived cells reached focal areas of confluence requiring trypsonization in 25.0 ± 3.4 days. Muscle- and tendon-derived cells achieved full confluence more rapidly (after 12.8 ± 2.2 days and 14.6 ± 1.9 days, respectively). Cell numbers at trypsonization were 2.3 ± 0.4 × 10^6 cells from bone marrow, 14.0 ± 2.9 × 10^6 cells from muscle, and 14.3 ± 4.8 × 10^6 cells from tendon.

Cell numbers on matrices—After 7 days in culture, the mean log numbers of cells adherent to the acellular tendon matrices were significantly ($P = 0.01$) increased in the tendon- and muscle-derived cell groups in comparison to bone marrow–derived cells (4.75 ± 0.21 bone marrow cells vs 5.14 ± 0.16 muscle cells and 5.09 ± 0.21 tendon cells). The tendon- and muscle-derived cell numbers adherent to matrices were not significantly different. Values for the cell-free matrix-only group revealed there were no viable cells, as expected. The power of the test was 0.85. Summary statistics for the transformed and untransformed data were determined (**Table 1**).

Collagen synthesis—Mean collagen log synthesis was significantly ($P = 0.004$) increased in the muscle- and tendon-derived cell groups in comparison to the bone marrow–derived cell group (bone marrow, 4.39 ± 0.19 DPM; muscle, 4.97 ± 0.40 DPM; tendon, 5.05 ± 0.33 DPM). There was no difference between muscle- and tendon-derived cell collagen synthesis. The power of the test was 0.97. Summary statistics for the untransformed, transformed, and normalized data were determined (Table 1). Collagen synthesis was increased by tendon-derived cells, when expressed as a percentage of total protein synthesis, compared with bone marrow–derived cells (bone marrow, 6.46 ± 2.64%; muscle, 9.28 ± 2.44%; tendon, 10.25 ± 1.66%); however, this difference did not reach significance ($P = 0.10$). The power of the test was 0.32. To determine whether this difference

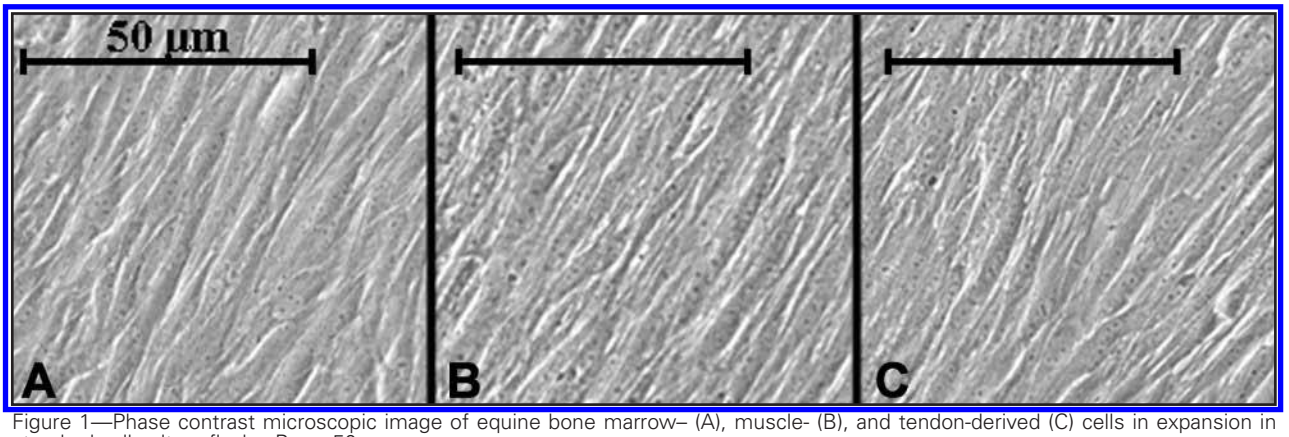

Figure 1—Phase contrast microscopic image of equine bone marrow– (A), muscle- (B), and tendon-derived (C) cells in expansion in standard cell culture flasks. Bar = 50 µm.

Table 1—Mean ± SE values for cell number, collagen synthesis, and proteoglycan synthesis of equine bone marrow–, muscle-, and tendon-derived cells after 7 days of culture on a tendon matrix.

Variable	Bone marrow	Muscle	Tendon
Cell number	73,227 ± 20,869	167,217 ± 59,380	164,672 ± 65,098
Log cell number	4.755 ± 0.213	5.135 ± 0.164*	5.087 ± 0.212*
Collagen synthesis	25,961 ± 4,365	134,971 ± 71,504	137,992 ± 50,271
Log collagen synthesis	4.389 ± 0.093	4.973 ± 0.202*	5.050 ± 0.163*
Log normalized collagen synthesis	0.930 ± 0.056	0.974 ± 0.067	1.001 ± 0.072
Percentage collagen synthesis	6.460 ± 2.639	9.282 ± 2.437	10.249 ± 1.663
Proteoglycan synthesis	2,470 ± 1,392	7,091 ± 3,388	5,784 ± 3,268
Log proteoglycan synthesis	3.220 ± 0.209	3.733 ± 0.170*	3.594 ± 0.203*
Log-normalized proteoglycan synthesis	0.681 ± 0.053	0.729 ± 0.037	0.709 ± 0.042

*Value differs significantly ($P \leq 0.05$) from bone marrow value.

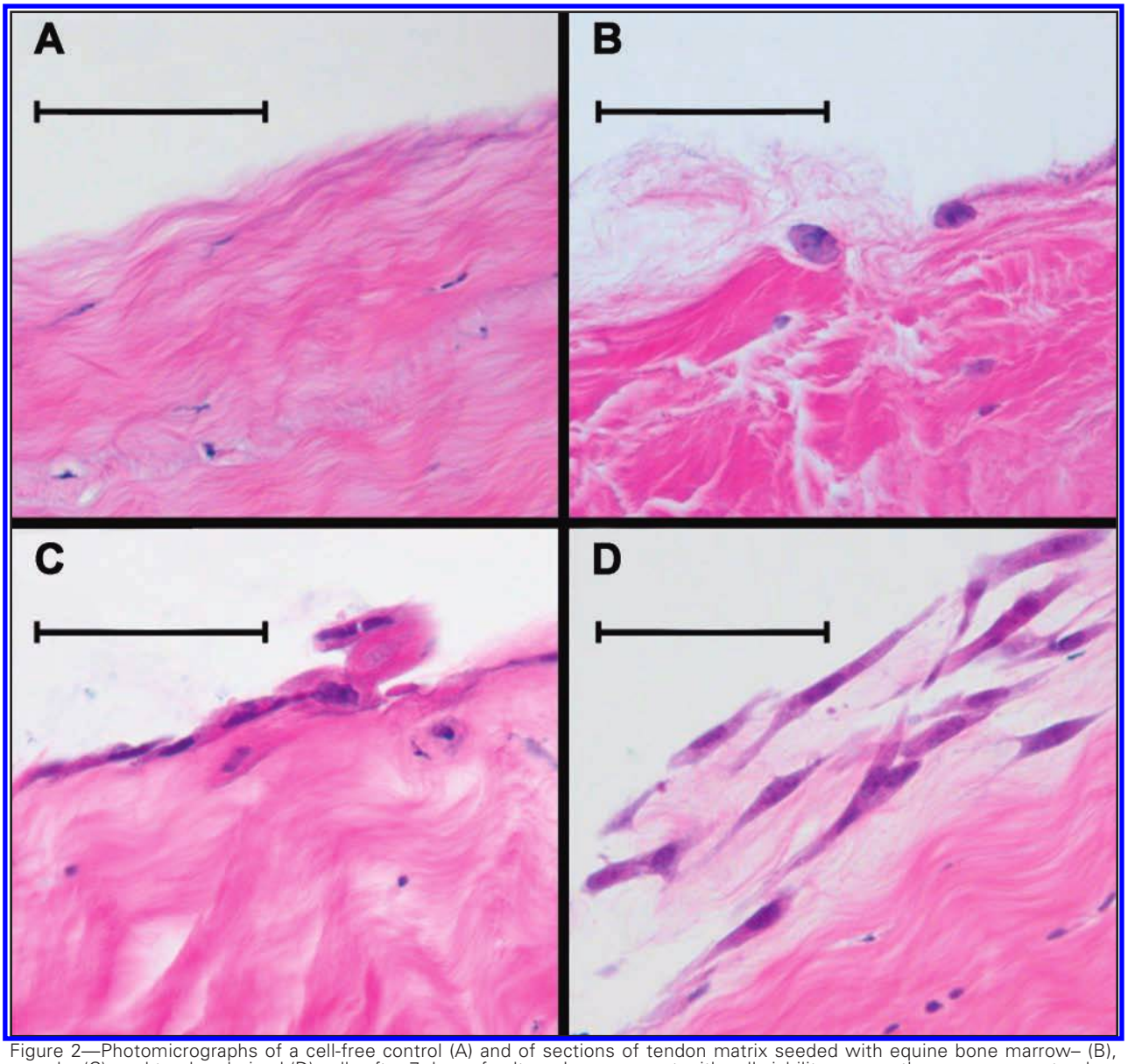

Figure 2—Photomicrographs of a cell-free control (A) and of sections of tendon matrix seeded with equine bone marrow– (B), muscle- (C), and tendon-derived (D) cells after 7 days of culture. In agreement with cell viability assays, there were more tendon- and muscle-derived cells on the surface of the matrix than bone marrow–derived cells. H&E stain; bar = 75 µm.

was significant, cells from an additional 24 horses would have been required.

When the mean log values of collagen synthesis were normalized to cell number, significant differences between the cell groups were not evident (bone marrow, 0.93 ± 0.06 DPM/cell; muscle, 0.97 ± 0.07 DPM/cell; tendon, 1.00 ± 0.07 DPM/cell; Table 1). The power for the test was 0.37.

Proteoglycan synthesis—Mean log proteoglycan synthesis was significantly (P = 0.001) increased in the muscle- and tendon-derived cell groups, in comparison to the bone marrow–derived cell group (bone marrow, 3.22 ± 0.21 CPM; muscle, 3.73 ± 0.17 CPM; tendon, 3.60 ± 0.20 CPM). There was no difference between muscle- and tendon-derived cell proteoglycan synthesis. The power of the test evaluating proteoglycan synthesis was 1.00. Summary statistics for the transformed and untransformed data were determined (Table 1). When mean log proteoglycan synthesis was adjusted for cell number, there were no differences (P = 0.19) between cell types (bone marrow, 0.681 ± 0.053 CPM/cell; muscle, 0.729 ± 0.037 CPM/cell; tendon, 0.709 ± 0.042 CPM/cell). The power for this test was 0.17.

Extracellular matrix mRNA expression—As expected, no mRNA was isolated from the acellular control tendon matrices that were subjected to 4 freeze-thaw cycles to kill resident cells. Quantitative PCR analyses of collagen type I mRNA expression in the cell-matrix groups revealed expression that was approximately 10% to 15% of in vivo expression. There were no significant (P = 0.64) differences in collagen type I mRNA expression among the different cell types (bone marrow, 0.14 ± 0.037; muscle, 0.12 ± 0.010; tendon, 0.11 ± 0.18). The power for this test was 0.05.

Collagen type III mRNA expression exceeded endogenous tenocyte expression in all 3 cell groups. Expression by tendon-derived cells was significantly (P = 0.04) increased in comparison to the bone marrow–derived cell group (bone marrow, 3.10 ± 1.05; muscle, 7.30 ± 1.92; tendon, 9.27 ± 2.56). There were no differences between muscle- and tendon-derived cells. The power of this test was 0.56.

The COMP expression in the cell-matrix samples was consistently lower than endogenous tenocyte expression. Tendon-derived cells had more COMP mRNA expression, compared with muscle-derived cells (bone marrow, 0.07 ± 0.04; muscle, 0.02 ± 0.006; tendon, 0.32 ± 0.14), but these differences were not significant (P = 0.10) because of large interanimal variation in expression. The power of this test was 0.31. To determine whether this difference was significant, cells from an additional 8 horses would have been required.

Histologic examination—Small, condensed nuclear remnants of endogenous tenocytes were evident throughout the cross-sections of the freeze-thawed matrices. All 3 exogenous cell types were predominantly attached to the surface of the autogenous matrices **(Figure 2)**. More tendon- and muscle-derived cells were adherent to the matrix surface than bone marrow–derived cells, consistent with the cell count data.

Discussion

The goal of the present study was to determine whether cells isolated from tendon or skeletal muscle constitute an alternative to bone marrow–derived cells for cell-based therapies for tendon injuries. Results indicated that cells can be easily isolated from tendon and muscle tissue biopsy samples. These cells can be rapidly expanded in monolayer culture to produce clinically useful numbers within 2 weeks. In contrast, there are few proliferative cells in bone marrow aspirates (estimated to be approx 1 cell in 10,000).[42] Consequently, monolayer expansion of these populations is driven by a small number of the total cell isolates and requires a minimum of 3 to 4 weeks for adequate expansion.[18,35] In this respect, cell isolates from skeletal muscle or tendon biopsy samples have a clear advantage over bone marrow aspirates. These findings are supported by those of a recent study[43] that revealed that tendon-derived cells proliferated faster than bone marrow–derived MSCs from the same individuals. The differences in cell culture techniques also could have contributed to the higher cell numbers and shorter times to confluence seen with the tendon- and muscle-derived cells, compared with bone marrow–derived cells. Apart from the issues of isolation and expansion, tendon- and muscle-derived cell attachment and survival on tendon matrix were significantly higher than those of bone-marrow–derived cells after 7 days.

In this study, the muscle- and tendon-derived cell groups synthesized more collagen and proteoglycan than did bone marrow–derived cells. After normalizing matrix synthesis to cell number, the difference was not significant; much of the difference in matrix synthesis is attributable to the differences in cell attachment and survival over time. These results were similar to those of previous studies[44,45] of matrix synthesis by equine bone marrow–derived MSCs, in which increased synthesis reflected increases in cell numbers rather than per-cell changes. Nevertheless, this outcome is relevant to therapeutic applications because colonization of the tendon matrix and persistence at the site of administration would likely influence the clinical efficacy of these cells for treatment of horses with tendon injuries.

In the present study, expression of collagen type I and COMP were markedly lower than expression detected in normal tendon. There was no significant (P = 0.64) difference in collagen type I expression among the cell groups. Tendon-derived cells had increased COMP expression, compared with the other groups, but the difference was not significant (P = 0.10). Cartilage oligomeric matrix protein influences collagen type I fibril formation and is important for tendon matrix production and tendon repair.[24,26] The pentameric nature of COMP brings collagen molecules in close proximity to promote further collagen assembly. In 1 study,[24] COMP interacted with loose collagen type I to form fibrils that increased in diameter and in length.

Collagen type III mRNA expression was higher than endogenous expression in all 3 cell groups, and expression by tendon-derived cells was significantly higher than that of bone marrow–derived cells. Collagen type III expression is upregulated during embryonic tenogenesis and during tendon healing.[25,27] In some respects, the acellular matrix model used in this study represents the situation cells might encounter after injection or migration into injured tendon, and interaction with the acellular matrix might stimulate a reparative response in these cells. The repeated freeze-thaw protocol was designed to remove confounding biosynthetic contributions from endogenous tenocytes. Previous studies[46,47] reveal that freeze-thawing tendon does not alter the biomechanical properties of the tissue, suggesting that the collagenous matrix of tendon is not adversely affected by this procedure. However, it is possible that endogenous cellular debris might have influenced the activities of the exogenously administered cells.

These data were derived from cells collected from 4 young adult horses. There was considerable interanimal variation in the data, and as a consequence, many of the outcomes (mean percentage collagen synthesis-to-total protein ratio, mean log collagen synthesis-to-log of cell number ratio, and COMP mRNA expression) were greater in tendon-derived cells, but differences did not reach significance. All the analyses that yielded P values between 0.10 and 0.05 had low statistical powers (≤ 0.37) and should be interpreted with caution. On the basis of these data, a minimum of 12 horses would be necessary to provide sufficient power (0.8) for these analyses. However, in our experience, marked interdonor variability is a biological reality of mesenchymal cell isolation and in vitro activity[18,44,45] and is likely to be reflected in in vivo therapeutic efficacies.

In the present study, an in vitro model was developed to assess the ability of cells to adhere to, and survive on, tendon matrix. On the basis of results of this study, we propose that muscle- and tendon-derived cells may provide an alternative to bone marrow–de-

rived cells for cell-based strategies to promote tendon healing. This study evaluated autogenous cell-based strategies, and more research is necessary to determine whether allogenous cell-based strategies would yield similar results. For all autogenous cell-based strategies, the donor morbidity site will remain an important consideration, particularly for tendon-derived cells. In recent projects we have harvested tendon-derived cells from the lateral digital extensor tendon, muscle-derived cells from the semitendinosus muscle, and bone marrow–derived cells from the sternum of horses without any obvious donor site complications. However, donor site morbidity and in-vivo efficacy still need to be determined for these cell types.

The assays used in this study determined cell survival and matrix biosynthesis, rather than characterizing the phenotypes and lineages of these cells. Previous studies[43,48,z] reveal that MSCs with multilineage potential can be isolated from human and murine tendon as well as bone marrow and muscle. Other studies[aa–bb] indicate that cells expanded from equine tendon by use of techniques described in the present study have similar capacities. It remains to be seen whether these cells are effective in vivo, but efforts to optimize the cell isolation, expansion, and purification protocols could improve the clinical efficacy of muscle- and tendon-derived cells for treatment of performance horses with tendon and other musculoskeletal injuries.

a. Jamshidi bone marrow biopsy needle, Cardinal Health, Dublin, Ohio.
b. DMEM, Mediatech Inc, Herndon, Va.
c. Gemini Bioproducts, Woodland, Calif.
d. L-glutamine, 200 mM, Invitogen, Carlsbad, Calif.
e. Penicillin-streptomycin, BioWhittaker, Cambrex Bio Science, Walkersville, Md.
f. Sodium pyruvate, Sigma Chemical Co, St Louis, Mo.
g. Collagenase type II, Worthington Biochemical Corp, Lakewood, NJ.
h. Dispase, Roche, Indianapolis, Ind.
i. Ascorbic acid, WAKO, Richmond, Va.
j. Filters, BD Biosciences, Bedford, Mass.
k. Trypan blue, Sigma Chemical Co, St Louis, Mo.
l. Cell Titer 96 Aq_{ueous} One Solution Cell Proliferation Assay, Promega, Madison, Wis.
m. Microplate reader, FLUOstar Optima, BMG Laboratories, Durham, NC.
n. [^{3}H] proline, Sigma Chemical Co, St Louis, Mo.
o. Collagenase, purified, Worthington Biochemical Corp, Lakewood, NJ.
p. LS6500 multipurpose scintillation counter, Beckman Coulter Inc, Fullerton, Calif.
q. ^{35}S- labeled sodium sulphate, MP Biochemicals, Irvine, Calif.
r. Papain, Sigma Chemical Co, St Louis, Mo.
s. Multiwell punch plates, PDVF plate, Millipore, Bedford, Mass.
t. RNeasy Mini Kit, Qiagen, Valencia, Calif.
u. Superscript II, Invitrogen, Carlsbad, Calif.
v. iQ SYBR Green Supermix, Bio-Rad Laboratories, Hercules, Calif.
w. ClustalW, European Molecular Biology Laboratory European Bioinformatics Institute (EMBL-EMBI), Cambridge, England. Available at: www.ebi.ac.uk. Accessed September 3, 2004.
x. iCycler iQ real-time PCR detection system, Bio-Rad Laboratories, Hercules, Calif.
y. Sigma Stat, Systat Software Inc, San Jose, Calif.
z. de Mos M, Koevoet WJ, Jahr H, et al. Intrinsic differentiation potential of adolescent human tendon tissue: an in-vitro cell differentiation study (abstr). *BMC Musculoskelet Disord* 2007;8:16.
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bb. Barrett JG, Stewart AA, Yates AC, et al. Tendon-derived progenitor cells can differentiate along multiple lineages (abstr), in *Proceedings*. 34th Annu Conf Vet Orthop Soc 2007;31.

Appendix

Primers used for real-time PCR amplification of genes in a study of equine bone marrow–, muscle-, and tendon-derived cells.

Gene	Sequence	Amplicon (bp)
Eq Col I (S)	GAA AAC ATC CCA GCC AAG AA	231
Eq Col I (A)	GAT TGC CAG TCT CCT CAT CC	
Eq Col III (S)	AGG GGA CCT GGT TAC TGC TT	215
Eq Col III (A)	TCT CTG GGT TGG GAC AGT CT	
Eq COMP (S)	TCA TGT GGA AGC AGA TGG AG	223
Eq COMP (A)	TAG GAA CCA GCG GTA GGA TG	
Eq EF1-α (S)	CCC GGA CAC AGA GAC TTC AT	328
Eq EF1-α (A)	AGC ATG TTG TCA CCA TTC CA	

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