**Apoptotic Bodies from Tenocytes Enhance the Proliferation and Migration of Tenocytes and Bone Marrow Mesenchymal Stem Cells In Vitro Canine Model**

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**Disclosures:**  
C. Dong: None.  
A. Gingery: None.  
R. Reisdorf: None.  
K. An: None.  
P.C. Amadio: 4; Merck, J&J. 7; Elsevier, JBJS.  
C. Zhao: None.

**Introduction:** Despite advances in surgical repair techniques, healing of flexor tendons remains problematic because of poor vascularization and hypocellularity [1]. Intrinsic healing of injured flexor tendons is crucial because the extrinsic healing causes adhesion formation. Although it is clear that local tenocytes play a dominant role in flexor intrinsic healing, the mechanism that initiates cell proliferation and migration after tendon injury is not fully understood. Stem cell-based therapy, such as the use of bone marrow stromal cells (BMSCs) to enhance tendon intrinsic healing, is promising [2-5]. However, as with local tenocytes, the effect of the local environment on the transplanted cells is unknown. The immediate responses that occur after flexor tendon injuries involve cell death by necrosis and apoptosis [6, 7]. It is not clear how these dead cells and their related released cytokines affect the local live tenocytes or transplanted BMSCs. Therefore, the purpose of this study was to investigate the effects of tenocyte- derived apoptotic bodies-rich medium (T-ABRM) and tenocyte- derived apoptotic bodies-depleted medium (T-ABDM) on flexor digitorum profundus (FDP) tenocytes and BMSCs proliferation and migration in a canine tendon in-vitro culture model.

**Methods:** Tissues were harvested from two mixed-breed dogs which had been sacrificed for other Institutional Animal Care and Use Committee (IACUC) approved studies. Tenocytes were obtained from flexor tendons in zone II. Isolated tenocytes between passages 2 and 4 were incubated for 24 hours in basal medium without serum or growth factors in order to induce tenocytes apoptosis [8]. Medium from apoptotic tenocytes was collected and prepared according to published procedures [8]. Briefly, the medium was centrifuged at 800g for 10 minutes to remove dead cells and cell debris, and the supernatant, T-ABRM, was collected. The T-ABRM was then further centrifuged at 16,000g for 20 minutes to remove the apoptotic bodies. The supernatant of this second centrifugation, T-ABDM was also collected for cell stimulation.

Tenocytes and BMSCs were isolated from FDP tendons and tibia, respectively, using established procedures [9, 10]. Cells between passages 2-4 were used to test cell migration and proliferation under different culture conditions including conditioned medium with T-ABRM, T-ABDM, GDF-5, or basal medium with 2% fetal bovine serum (FBS) for 12, 24, 48 or 72 hours. Pilot data showed that T-ABRM resulted in increased cell proliferation, so we selected T-ABRM to test a dose-response effect on both tenocytes and BMSCs. T-ABRM (approximately 0.2mg/ml) was diluted with minimal essential medium (MEM) in different ratios (1:1, 1:3, 1:5) and cell proliferation was measured after 24 hours culture. The migration assay was performed using a QCMTM 24-Well Colorimetric Cell Migration assay Kit (Millipore, Darmstadt, Germany) according to user instructions. The proliferation assay was performed using Cell Counting Kit-8 (DOJINDO, Gaithersburg, Maryland, USA) following manufacturer’s instructions.
Data from the proliferation and migration assays were normalized by the basal medium with FCS (control) group. A Student-T test was used for statistical analysis. Any p value less than 0.05 was considered significant.

**Results:** Cell proliferation in the T-ABRM group was significantly increased in both cell types (tenocyte and BMSC) compared to T-ABDM, GDF-5, and control groups at 24, 48, and 72 hours. Cell proliferation increased in a time-dependent manner in all groups (Figure 1A&B). Cell proliferation in T-ABRM showed a dose response for both cell types, e.g. ratio 1:1 was significantly higher than 1:3, which was significantly higher than 1:5 (P<0.05) (Figure 1C). The cell migration rate in both cell types (tenocyte and BMSC) was significantly increased in T-ABRM compared with the T-ABDM, GDF-5, and control group (p<0.05). Cell migration was higher in T-ABDM as compared to the GDF-5 treatment group (p<0.05), and GDF-5 was higher than the control group (p<0.05) (Figure 2).

**Discussion:** In this study, we demonstrated that conditioned medium including apoptotic bodies, exosomes and growth factors promoted tenocyte and BMSC proliferation and migration. Conditioned media treatment with either apoptotic bodies present or depleted promoted migration and proliferation and this stimulation was significantly increased as compared to a single cytokine, GDF-5, or basal medium with 2% FBS. These results supported our hypothesis that apoptotic or necrotic tenocytes may provide a powerful stimulus promoting proliferation and migration for either residual tenocytes or transplanted BMSCs. This may explain why the residual tenocytes, as well as surrounding cells, proliferate and migrate to the injured site, where a large number of apoptotic and necrotic tenocytes exist following tendon injury. We also showed that the apoptotic bodies stimulate BMSCs migration and proliferation. Future studies are needed to identify factors, including growth factors and cytokines in the ABRM, which are responsible for the stimulation of cell migration and proliferation.

**Significance:** Apoptotic bodies from tenocytes have the ability to increase the proliferation and migration of tenocytes and BMSCs. Future in vivo study is needed to investigate the effect of apoptotic bodies on tendon healing and adhesion formation.
Figure 2. BMSCs Migration test under ABRM stimulated condition.
BMSCs were stimulated with different conditioned medium (T-ABRM, T-ABDM, GDF-5 and Control) for 24 hours. Colorimetric measurements were taken according to instructions of Elisa reader(A).
BMSCs were allowed to migrate towards different conditioned medium (T-ABRM, T-ABDM, GDF-5 and Control) for 24 hours. 200,000 cells were used in each assay. Migrated cells on the bottom side of the membrane were stained according to assay instruction with microscope(B). *P < 0.05, **P < 0.01