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Role of Cell-Cell Interactions on the Regeneration of Soft Tissue-to-Bone Interface

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Abstract—**Soft tissues such as the anterior cruciate ligament (ACL) connect to bone tissue through a characteristic fibrocartilagenous interface. This interface is essential for load transfer between soft and hard tissues, and its absence is the primary cause of graft failure post ACL reconstruction surgeries. Currently, the mechanism of interface regeneration is not known. Based on *in vivo* observations that a fibrocartilage-like tissue forms when the graft is in direct contact with bone, we propose here the original hypothesis that fibroblast-osteoblast interactions may lead to the recruitment and differentiation of mesenchymal stem cells or progenitor cells for interface regeneration. To test this hypothesis, a tri-culture model of fibroblasts-osteoblasts and interface-relevant cells was designed. This model mimics the graft-to-bone interface, supports direct cell-to-cell contact, as well as controlled homotypic and heterotypic cell-to-cell interactions. We used this model to determine the effects of fibroblast-osteoblast interaction on the response of interface-relevant cells such as bone marrow stromal cells (BMSCs), chondrocytes and fibroblasts. The response of osteoblasts and fibroblasts in triculture were also assessed. It was found that tri-culture with chondrocytes led to significant changes in cell proliferation and reduced osteoblast-mediated mineralization, accompanied by increased fibroblast mineralization. Interestingly, BMSCs in tri-culture measured higher ALP activity compared to controls. Positive glycosaminoglycan (GAG) production was detected in the chondrocyte tri-culture group. Moreover, expressions of interface-relevant markers such as type II collagen and GAG were detected in tri-culture with BMSCs and fibroblasts. Our results collectively demonstrate that osteoblast-fibroblast interactions modulate cell phenotypes, promote chondrocyte matrix elaboration and may initiate the differentiation of BMSCs into interface relevant phenotype. The findings of this study provide new insight into the mechanisms governing the regeneration of soft tissue-to-bone interfaces.**

I. Introduction

The anterior cruciate ligament (ACL) is a band of regularly oriented, dense connective tissue that joins the femur to the tibia. The ACL inserts into subchondral bone through a fibrocartilage interface region, and it is the primary knee joint stabilizer. The ACL is the most frequently injured knee ligament, with approximately 100,000 reconstruction procedures performed each year in the United States[1]. Injuries to the ACL do not heal due to the inherently poor healing potential and limited vascularization of the ligament[2], surgical reconstruction is necessary to restore normal joint function. There are currently two common reconstruction options, bone-patellar tendon-bone and hamstring tendon-based autografts. Autologous hamstring tendon-based grafts for ACL reconstruction are increasingly utilized due to donor site morbidity associated with bone-patellar tendon-bone grafts[3]. While these grafts may restore the physiological range of motion and joint function through mechanical fixation, biological fixation is not achieved as disorganized scar tissue forms within the bone tunnels, and there is no tissue transition between tendon and subchondral bone[4,5]. Therefore, the graft-bone junction has limited mechanical stability, and the lack of integration is the primary cause of graft failure[4,6].

It is well established that the ACL-to-bone interface consists of three distinct tissue regions: ligament, fibrocartilage, and bone[10]. The fibrocartilage region is further divided into the non-mineralized and mineralized fibrocartilage zones. The ligament proper is composed of fibroblasts embedded in a type I and type III collagen matrix. The non-mineralized fibrocartilage matrix consists of ovoid chondrocytes, and types I and II collagen are detectable within the proteoglycan-rich matrix. The next region is the mineralized fibrocartilage zone, with hypertrophic chondrocytes surrounded by a mineralized matrix[9]. Type X collagen, a marker for hypertrophic chondrocytes, is detected only within this region[8]. The last zone is the subchondral bone, within which osteoblasts, osteocytes and osteoclasts are embedded in a type I collagen matrix. The specific organization and controlled heterogeneity are believed to be important for minimizing stress concentrations and facilitating the transfer of complex loads between soft and hard tissues[7,11].

This complex, multi-tissue insertion site is lost post-surgery as existing methods focus on mechanically anchoring the grafts to bone with no attempt to re-establish the interface. We propose that biological fixation of soft tissues may be achieved by regenerating the fibrocartilage interface between soft tissue and bone, and the presence of this transition zone will mediate load transfer and prevent premature failure of the soft tissue graft.

Currently, the mechanisms and parameters governing interface regeneration are not known. While tendon-to-bone healing following ACL reconstruction does not lead to the re-establishment of the native insertion, a fibrous tissue is formed within the bone tunnel[5,12]. This fibrous layer matures and reorganizes into a fibrocartilage-like tissue during the healing process. While the location of this

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neo-fibrocartilage tissue is non-physiologic, these findings demonstrate that a fibrocartilage-like tissue can be regenerated *in vivo*. Moreover, the fact that a fibrocartilage-like layer forms only at regions where the tendon graft is in direct contact with bone tissue is highly significant. This observation has led us to form the novel hypothesis that the interaction between cells derived from tendon (*e.g.,* fibroblasts) and bone tissue (*e.g.,* osteoblasts) plays a role in interface or fibrocartilage regeneration.

In a previous study, we examined osteoblast-fibroblast interactions in a 2-D co-culture model and found that cell trans-differentiation toward chondrogenic lineage is possible[16]. Furthermore, we hypothesized that the interface may be developed under 3-D culturing conditions and the interactions between osteoblasts and fibroblasts may result in the recruitment of mesenchymal stem cells and progenitor cells to the interface. These cells have the potentials to differentiate into chondrocytes[17] and their ability to regenerate the interface is likely modulated by cell-cell interactions. To test this hypothesis, the first objective of this study is to establish a reliable, 3-D tri-culture system of relevant cell types found at the interface, namely osteoblasts, chondrocytes, and ligament fibroblasts. After the development of the tri-culture model, the effects of osteoblast-fibroblast interaction on interface-relevant cell growth and differentiation will be determined. The three types of cells studied include bone marrow stromal cells, fibroblasts, and chondrocytes. The morphology, viability, growth and phenotypic response of each cell type during tri-culture will be evaluated. It is anticipated that findings of this study will aid in our efforts to elucidate the mechanism underlying the regeneration of the fibrocartilage interface between soft tissue and bone.

II. Materials and Methods

All chemicals were purchased from Sigma Chemical (St. Louis, MO) unless otherwise noted.

A. Cells and Cell Culture

Primary bovine fibroblasts (Fb) and osteoblasts (Ob) were obtained respectively from explant cultures of bovine ACL and trabecular bone fragments[16]. The tissue was harvested from freshly slaughtered steer obtained from a local abattoir (Fresh Farm, Vermont). Bovine articular chondrocytes (Ch) were isolated from the carpometacarpal joints of calves following an enzymatic digestion[13]. Bone marrow was extracted from the femoral and tibial cavities, and elongated, spindle-shaped cells that attached to the culture dish were collected as bone marrow stromal cells (St). The cells were incubated in fully supplemented Dulbecco's Modified Essential Medium (DMEM) containing 10% fetal bovine serum, 1% non-essential amino acids and 1% antibiotics (all purchased from Mediatech, Herdon, VA) under humidified conditions at 37°C and 5% CO_2.

B. Osteoblast-Fibroblast Tri-Culture Model

The tri-culture model was established by seeding fibroblasts and osteoblasts on cell culture treated plastic coverslips (Fisher, d=15mm) at $5x10^4$cells/section. Interface-relevant cells (Fb, Ch, St) were suspended in 2% agarose (Type VII) at $10x10^6$ cells/ml and cut into strips (120 µl). Interface strips and coverslips with osteoblasts and fibroblasts were secured in a culture well (Fig. 1). The well surface was pre-coated with agarose to minimize cell migration and adhesion. Cells were cultured at 37°C and 5% CO_2 in supplemented DMEM.

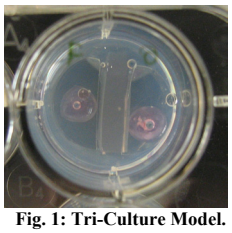


Fig. 1: Tri-Culture Model.

In the first part of the study, to determine model feasibility, tri-cultures of chondrocytes, fibroblasts and osteoblasts were established and their viability, growth and phenotypic responses were monitored over time. Control groups were each cell type alone (Fb, Ch, Ob), and the co-cultures among these three cell types (Fb+Ch, Ob+Ch, Fb+Ob).

In the second part of the study, osteoblasts and fibroblasts were tri-cultured with fibroblasts or bone marrow stromal cells encapsulated in hydrogel strips to examine the effects of fibroblasts and osteoblasts on the interfacial cells in 3-D environment. The positive control will be fibroblasts and osteoblasts tri-cultured with chondrocytes.

C. Effects of Tri-Culture on Growth and Diffferentiation

The effects of osteoblasts, fibroblasts and interface-relevant cells tri-culture on cell growth, alkaline phosphatase (ALP) activity, gene expression, and mineralization were determined at 1, 3, 7, 14, 21 days. At day 7, 10 µg/ml L-ascorbic acid and 1mM β-glycerolphosphate were added[16].

C.1 Cell Viability and Proliferation

Cell viability was determined using a Live (Calcein AM)-Dead (Ethidium homodimer-1) stain kit (Invitrogen, Carlsbad, CA). Green color indicates living cells. For proliferation, total DNA per sample (n=5) was measured using the PicoGreen® dsDNA assay (Invitrogen). The total number of cells per sample was converted by using the conversion factor of 8 pg of DNA per cell[14].

C.2 Alkaline Phosphatase (ALP) Activity & Mineralization

The ALP activity of the tri-cultured and control groups was assessed using both quantitative and qualitative assays[15]. Quantitative ALP activity (n=6) was measured using an enzymatic assay based on the hydrolysis of p-Nitrophenol phosphate (pNP-PO_4) to p-nitrophenyl (pNP). Qualitative ALP activity (n=3) was ascertained by Fast-Blue staining. To visualize mineral deposition, the Alizarin Red S (ALZ) assay staining was used. The samples (n=3) were fixed in 95% ethanol and incubated in 2% ALZ solution for 10 minutes.

C.3 Glycosaminoglycan (GAG) Production

Glycosaminoglycan (GAG) production was measured quantitatively using a Blyscan Kit (Biocolor Ltd, Ireland) (n=5), and visualized by Alcian Blue stain (n=2).

C.4 Gene Expression

Gene Expression (collagen types I, II, and X) was measured using the reverse transcription and polymerase chain reaction (RT-PCR). Glyceraldehyde 3-phosphate

dehydrogenase (GAPDH) was used as an inner gene control.

D. Statistical Analysis

Data are presented as mean±standard deviation. ANOVA and the Tukey-Kramer post-hoc test was used for all pair-wise comparisons and statistical significance was set at $p<0.05$. All statistical analyses were performed using the JMP statistical software package (SAS Institute, Cavy, NC).

III. Results

A. Ob-Fb and Interfacial Cell Tri-Culture Model

The tri-culture model used in this study was designed to emulate the graft-to-bone interface within the bone tunnel, with a layer of interface-like region situated in the middle. Fibroblasts and osteoblasts were seeded on coverslips so that the response of each type can be assesed at each time point. Interface-relevant cells were suspended in 3-D hydrogel so that they can communicate via soluble factors. In other words, when progressing from left to right in each tri-cultured well, three regions are found: fibroblasts only, interface, and osteoblasts only. The model is designed such that each region consists of the dominant cell population found in the graft, interface and bone respectively. It was observed that in time, the fibroblasts and osteoblasts formed confluent cultures. Live-dead staining revealed that osteoblasts, chondrocytes and fibroblasts remained viable during tri-culture, and maintained their phenotypic morphology (Fig 2).

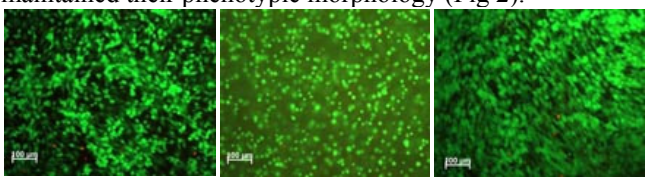


Fig. 2, live-dead stain of tri-culture, day 40, 10x. left to right: Fb, Ch, and Ob

B. Cell growth

In the first study, both fibroblasts and osteoblasts proliferated, while the number of chondrocytes remained relatively constant over time. The presence of other cell types delayed osteoblasts and fibroblast proliferation (Fig. 3).

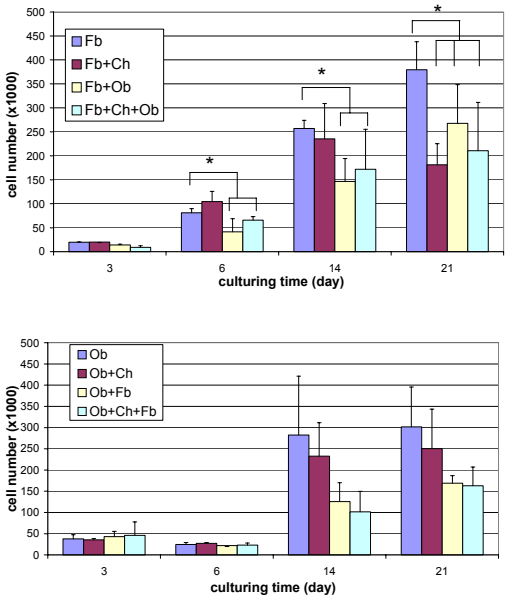
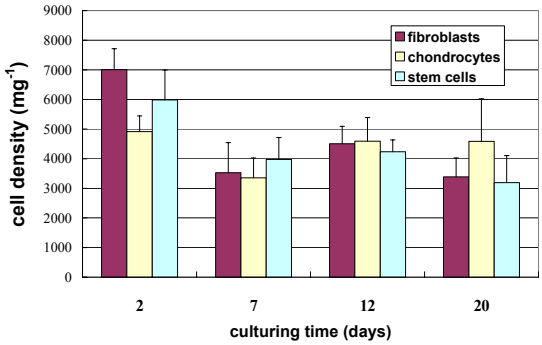


Fig. 3, cell proliferation over time. Top: fibroblast proliferation. Bottom: osteoblast proliferation.

In a separate study, interface-relevant cell proliferation was measured, and it was found that cells number remained the same over the culturing time (Fig. 4).

Fig. 4: Interface-Relevant Cell Proliferation



C. Alkaline Phosphatase Activity and Mineralization

Osteoblast ALP activity level decreased during co-culture and tri-culture (Fig. 5: A, B) in the first part of the study. In contrast, chondrocytes and fibroblasts measured negligible ALP activity. Interestingly, BMSCs showed high ALP activity during tri-culture over time (data not shown).

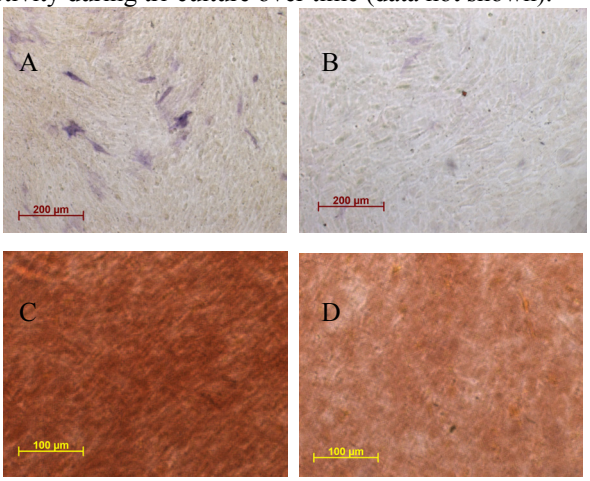


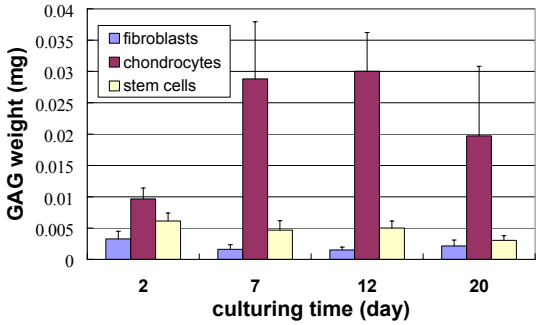
Fig. 5, A, B: ALP day 6, 10x. C, D: ALZ stain, day 14, 20x. A, C: Ob alone, B, D: Ob in tri-culture with chondrocytes.

In terms of mineralization, in the first part of the study, the surrounding fibroblast monolayer showed a higher level of mineralization in the presence of osteoblasts and interface-relevant cells. Lower level of osteoblast mineralization was observed in co-culture with fibroblasts and during tri-culture (Fig. 5: C, D). The presence of chondrocytes has no effect on mineralization by osteoblasts or fibroblasts.

D. Glycosaminoglycan (GAG) Production

It was found that chondrocytes produced a higher level of GAG when compared to other interface-relevant cells, and stem cells measured higher GAG production than fibroblasts (Fig. 6). Alcian Blue stain showed positive stain in the chondrocytes triculture group (data not shown).

Fig. 6: GAG Production



E. Development of Interface-Specific Markers

Fig. 7, RT-PCR analysis of the expression of GAPDH, type I and II collagens from fibroblasts in tri-culture, day 28.

It was observed that fibroblasts in tri-culture expressed type I and II collagens after 28 days (Fig. 7). The BMSCs expressed aggrecan indicating chondrogenesis tri-culture (data not shown).

IV. DISCUSSION

In order to determine the mechanisms of interface regeneration, this study evaluated the effects of osteoblast-fibroblast interactions on the response of interface-relevant cells such as chondrocytes, fibroblasts and stem cells using a tri-culture model. Tri-culture model with chondrocytes represents the native ligament-to-bone insertion site as fibroblasts, chondrocytes and osteoblasts are the major cell populations of the ACL, fibrocartilage interface, and bone respectively, therefore can serve as a positive control. Mesenchymal stem cells are located within the bone marrow that might be recruited during the soft tissue graft healing process. In addition, a fibrous tissue forms within the bone tunnel *in vivo*, and it later organizes into a fibrocartilage-like tissue[5]. We found that during tri-culture with chondrocytes, all cells remained viable and maintained their phenotypes. Osteoblasts and fibroblasts continued to express type I collagen, and the deposition of aggrecan was found only in chondrocyte cultures, which showed that this tri-culture model is able to support specific cell phenotypic responses. Tri-culture resulted in an increase in ALP activity in stem cells and aggrecan expression indicating a combined chondrogenic and osteogenic differentiation. Interactions between these cells led to the expressions of type II collagen in fibroblasts. The interactions of osteoblasts and fibroblasts also resulted in decrease of proliferation of osteoblasts and fibroblasts.

The findings of this study demonstrate that cell-to-cell communication modulates cellular responses in the three types interface-relevant cells studied. Both stem cells and fibroblasts are known to exhibit chondrogenic potential[17], and these cells may be responsible for forming the fibrocartilage interface. Future studies will focus on elucidating the mechanism of interface regeneration and the role of cell-to-cell interaction in this process.

V. CONCLUSION

The findings of this study provide preliminary validation of our hypothesis that cell-to-cell interactions play a significant role in the induction of interface-specific markers, and demonstrate the utility of *in vitro* tri-culture model system for investigating the mechanisms of interface regeneration.

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