MECHANICAL AND CHEMICAL FACTORS TO ENHANCE LIGAMENT TISSUE ENGINEERING USING RESORBABLE FIBERS

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INTRODUCTION: The anterior cruciate ligament (ACL) and the medial collateral ligament (MCL) of the human knee play pivotal roles in stabilizing the knee joint. Unfortunately, the ACL heals poorly following injury and the current methods used to replace the ACL are not ideal. At present, an autograph such as the patellar and gracilis tendon is the ACL replacement of choice. Donor ligaments and tendons (allographs) can be used in lieu of autographs. However, these biological graphs do have their shortcomings. Autographs require the harvesting of tissue from the patient, which can result in some loss of function and mechanical instability, while allographs from donors can carry the risk of disease transmission. If viable ligaments can be engineered, these problems can be eliminated. There are certain key factors to developing a reliable process for generating viable ligaments for implantation. First, a framework or scaffolding for the seeding of ligament cells must be found. The scaffolding must be biodegradable and be load-bearing. Second, an appropriate media must be synthesized that provides the right amount of growth factors, enzymatic cofactors, energy source in the correct concentrations, and certain amounts of inflammatory agents for optimal tissue formation and remodeling. Lastly, a method for vascularizing the engineered tissue must be found or else cells in the interior of the newly formed tissue will not be able to receive nutrients and remove wastes, and thus will not survive. Our study is directed at discovering what type of scaffolding will provide the best foundation for cell seeding and proliferation and also which biological and mechanical conditions promote the most tissue growth.

MATERIALS AND METHODS: Cells and Scaffolding Human fibroblasts from ACL and MCL were seeded dynamically by using an orbital shaker for twenty-four hours onto cylindrical fiber scaffolds (1 cm long x 0.2 cm diameter). The scaffolds were made by separating the individual fibers of Vicryl (polyglactin 910; Ethicon) and Dexon II (polyglycolic acid; Davis & Geck) and securing each fiber bundle at both ends with nonbiodegradable silk sutures.

Cell Proliferation Assay In order to determine the proliferation of ligament cells seeded on the scaffolds, the seeded scaffolds were allowed to incubate for one hour in a solution containing a tetrazolium compound (Cell Proliferation Assay, Promega, Madison, WI). Absorbances were taken on a 96 well plate reader at 520nm, converted to cell numbers, and then normalized to the initial cell seeding count.

Media Modulators Inflammatory agents lipopolysaccharide (LPS, 1μg/ml), complement C5a (100ng/ml), and tumor necrosis factor α (TNF-α, 20 ng/ml) were combined separately with Dulbecco's Modified Eagle's Medium (1000 mg/ml Glucose) containing 10% fetal bovine serum, 2% L-glutamine, 1% non-essential amino acids, and 1% penicillin/streptomycin/fungizone. The inflammatory agents were used separately as well as combined together. Growth factors used included: transforming growth factor β_1 (TGF-β_1, 0.5ng/ml) and epidermal growth factor (EGF, 5ng/ml). The growth factors were added in the proper concentration to Dulbecco's Modified Eagle's Medium (1000 mg/ml Glucose) containing 0.5% fetal bovine serum, 2% L-glutamine, 1% non-essential amino acids, and 1% penicillin/-streptomycin/fungizone. The growth factors were used separately or combined together.

Mechanical Modulator Different frequencies and durations of fluid shear stresses were applied to the scaffolds using an orbital shaker. Mechanical stimulus (using the orbital shaker) of the culture medium, Dulbecco's Modified Eagle's Medium, was used. The frequency of the shaker was 60 rpm with variable durations between 1 and 24 hours.

RESULTS: Synthetic bioresorbable polymer fiber scaffolds proved to be adequate scaffolds for the experiment. As early as one day after seeding cells flattened out on the fibers and began to proliferate rapidly and secrete extracellular matrix. Light microscopy show that these cells and their secreted matrix soon surrounded the scaffold fibers and even bridged the gaps between the fibers. In addition, the cells were found to be aligned along the length of the fiber. Beginning at two weeks, portions of the scaffolds were completely filled with tissue. By four weeks, the scaffold became a single bundle of tissue, with the scaffold fibers only faintly visible within the biological tissue. (Fig. 1) It was observed that the presence of TGF-β_1 and the combination of TGF-β_1 with EGF significantly increased cell proliferation for both MCL and ACL cells as compared to the control. EGF alone did not have a significant effect. (Fig. 2) The presence of inflammatory agents LPS and C5a showed a decrease cell proliferation for both cell types when compared to the control. The addition of TNF alone did not have a significant effect on either cell type. As for the combination of all three inflammatory factors, there was no significant decrease effect on the ACL and MCL cell proliferation. (Fig. 3) Mechanical stimulus in the form of fluid shaking increased the proliferation rate two fold for both MCL and ACL cells. (Fig. 4)

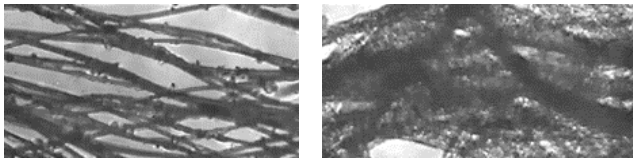
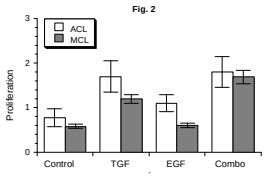


Figure 1. Light microscopic view of the MCL cell-seeded Vicryl fiber scaffold (**left**) with single cells appearing as spheres attached to the fibers immediately after seeding and the resulting tissue (**right**) after 4 weeks of culturing.



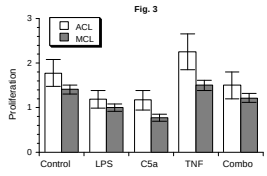


Figure 2. Four days of culturing with growth factors.
Figure 3. Four days of culturing with inflammatory factors

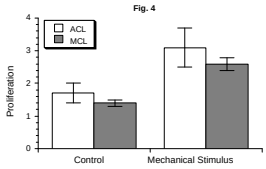


Figure 4. Four days of culturing with or without mechanical stimulus

DISCUSSION: In this study, we found that the use of biodegradable sutures serves as a promising framework for the seeding of knee ligament cells (ACL/MCL). Trysinized fibroblast (spherical shape) attach to the fiber scaffold and elongate to a normal fibroblast shape, indicating that the scaffold material is biocompatible with the ligament fibroblasts. Both cell proliferation and matrix synthesis occurred which is crucial to tissue engineering. The branching of the cellular matrix between adjacent fibers occurs quickly and is easily visible. Though the tissue formed is not ligament tissue with high tensile strength and arrays of collagen fibers, there is a potential for remodeling of the tissue once placed *in vivo.* The net decrease in cell numbers caused by the two inflammatory factors, C5a and LPS, was observed because it has been shown that the presence of some inflammatory agents serve to inhibit cell proliferation. The increase in cell proliferation due to TGF, TGF+EGF, and mechanical stimulus is a promising start. The possible combination of mechanical stimulus with growth factors may serve to provide an even better *in vitro* environment for the ligament cells to proliferate. These early experimental results serve to provide a foundation for more *in vitro* and *in vivo* studies.

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