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Human anterior cruciate ligament fibroblasts from immature patients have a stronger *in vitro* response to platelet concentrates than those from mature individuals

Elise M. Magarian, Patrick Vavken, and Martha M. Murray

Department of Orthopaedic Surgery, Children's Hospital of Boston, Harvard Medical School, Boston, MA 02115

Abstract

A number of recently published studies have established a substantial age dependence of the response of ACL fibroblasts to stimulation by platelet-rich plasma (PRP). Further in-depth research of this age dependence revealed negative effects on both histological and biomechanical results in a large animal model. However, while it has been postulated that this association could affect potential human applications negatively too it remains to be proven that the same effects occur in human cells. Thus it was the objective of this study to search for age dependence in human fibroblasts before further human experiments are done. Human fibroblasts were obtained from 10 immature and adolescent patients, based on a-priori power calculations, and culture in a collagen-PRP composite. Three parameters that are pivotal for defect remodeling and wound healing – cell migration, cell proliferation, and scaffold contraction – were chosen as endpoints. Both migration and proliferation were significantly higher in immature cells, but no differences were seen in wound contraction. The former findings suggest that immature patients respond more favorably to treatment with PRP, which consequently might translate into better results in ACL tissue engineering.

INTRODUCTION

Injury to the Anterior Cruciate Ligament (ACL) is an important clinical problem leading with two main consequences: immediate joint instability and early onset of osteoarthritis, both considerably painful and disabling ¹. A growing body of evidence has recently been accumulated that consistently shows that while current treatment options alleviate instability almost entirely, the risk for premature osteoarthritis remains high even in those patients receiving the best currently available treatment, ACL reconstruction ^{2–4}. Due to these poor long-term results even with the current gold standard of treatment, new methods for ACL repair are being sought ^{3; 5}.

Ongoing research has had an increased focus on repairing ACL ruptures using tissue engineering in the form of a suture repair enhanced with implanted provisional scaffold materials (namely collagen-platelet composites) to promote regeneration and healing ^{3; 6–9}. With the use of the provisional scaffolds as structural support and a platelet concentrate as a

Corresponding Author: Martha Meaney Murray, Department of Orthopaedic Surgery, Children's Hospital of Boston, 300 Longwood Ave, Boston, MA 02115, Phone: 617 355 7132, Fax: 617 730 0459, Martha.murray@childrens.harvard.edu.

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source of growth factors, a marked improvement in ligament healing can be stimulated^{3; 6–12}. A body of literature has been published on the development and testing of such an approach in large animal models^{3; 5–9; 12}. This research has produced a consistent and comprehensive picture of the *in vitro* and *in vivo* mechanisms, from the cell to the tissue and organ level, of enhanced primary ACL repair, but its human application still awaits confirmation.

One of the probably most intriguing findings in this model was clear evidence for age dependence of ACL fibroblast behavior, particularly in response to stimulation by a platelet-concentrate. This difference was mostly seen when comparing skeletally immature (open physes) with either adolescent (closing physes) or adult (closed physes) individuals^{13–15}. While this seems biologically plausible and is consistent with conventional orthopaedic wisdom, suggesting that fractures in skeletally immature patients will heal faster than those in adolescents or adults^{16; 17}, the influence of age on the healing of ligaments has not been sufficiently studied or determined before. Moreover, considering that there is still a lack of clinically convincing treatment options in skeletally immature patients with ACL tears and that immature individuals have disconcertingly high failure rates for ACL reconstruction, this population could profit most from a tissue engineering repair procedure⁵. Such an interpretation is supported by recent findings showing that age-dependence does not only exist on the cellular level, but translates on to the tissue level as well, thus being reflected in biomechanical outcome after tissue engineering enhanced primary repairs¹⁵.

While research in animal models has demonstrated efficacy of this technique it is yet unknown if human cells will have similar responses. In this paper, we hypothesize that skeletally immature human ACL fibroblasts (from patients with open physes) in a collagenous biomaterial have a stronger response to a platelet concentrate than cells from older individuals. Assuming that enhanced repair will primarily target younger individuals, and given that evidence suggests on differences in the behavior of adolescent and adult cells^{13; 14}, we chose adolescent, rather than adult, patients as controls. We chose the three parameters of cellular migration, proliferation, and scaffold contraction as endpoints for modeling the extent of wound healing and scar maturation *in vitro*.

METHODS

Institutional Review Board approval was obtained prior to beginning the study. Consistent with earlier published data^{13–15}, a sample size was calculated to allow for testing for an effect size of at least 2 with 80% at an alpha of 5%. Thus, with parental consent, ACL tissue was obtained from the tibial stump from 5 skeletally immature (11.0 ± 0.9 years, male) and 5 adolescent patients (15.0 ± 0 years, 4 female 1 male) undergoing surgical treatment for complete, midsubstance ACL tears within 6 months after the initial injury. Age grouping was based on physeal status about the knee, again in consistency with both methods and findings from said earlier studies. All tissues were obtained from patients undergoing ACL reconstruction surgery Children's Hospital Boston, MA, and Children's Hospital, Waltham, MA.

Cell Culture

ACL tissue was harvested intra-operatively using sterile technique. After harvest, explants were washed three times in 1% Antibiotic-Antimycotic solution (AB/AM) (Mediatech Inc., Herndon, VA.) followed by three washes with sterile 1X Phosphate Buffer Saline (PBS) (EMD Chemicals, Gibbstown, NJ), cut to approx 1mm^3 and transferred onto six-well plates. The explants were allowed to adhere to the plates, and then media (DMEM (Mediatech, Herndon, VA.) 10% FBS (HyClone Inc., South Logan, UT), 10% Fetal Bovine Serum (FBS) and 1% AB/AM) was added. The explants were maintained in culture, and the media was

changed two times per week. When the primary outgrowth cells were 80% confluent, they were trypsinized using a standard trypsin EDTA protocol and either reseeded in T-75 culture flasks or frozen and stored at -80°C until all groups had been collected for the experiment. All cells used for this experiment were first or second passage.

Migration Assay

Cell migration was measured using a modified Boyden chamber assay (Chemicon International Inc. Kit: QCM 24-well colorimetric cell migration assay). The upper and lower chambers are separated by an $8\mu\text{m}$ polycarbonate membrane and their migration through this membrane from the upper to lower chamber is measured using a cell stain solution. For each time point (4 and 24 hours) eight wells were analyzed per patient per age group. In addition, four negative control wells without cells (empty controls) were analyzed at each time point to control and adjust for potential interaction between the used scaffold and the test reagents. The results of these negative controls were subtracted from the actual samples.

Serum-free quenching medium was added to each T-75 flask (Serum-free DMEM containing 10% FBS) and incubated for 24 hours prior to assay. After 24 hours, cells were washed with sterile Phosphate Buffer Saline (EMD Chemicals, Gibbstown, NJ). Five milliliters of harvesting buffer (0.05% trypsin in Hanks Balanced Salt Solution (HBSS) containing 25mM HEPES, Cellgro, Mediatech, Inc, Herndon, VA) was added to the cells and incubated at 37°C for 15 minutes. After 15 minutes, cells were gently pipetted off the dish and 15mL of quenching medium (Serum-free DMEM containing 10% FBS) was added. Cells were centrifuged for 5 minutes at 1200rpm until pellets were formed. Pellets were resuspended in 5mL of quenching medium [serum-free DMEM containing 5% Bovine Serum Albumin (BSA)]. 300 μL of cell suspension ($0.5\text{--}1.0 \times 10^6$ cells/mL in chemoattractant-free media (serum-free DMEM containing 5% BSA) and 500 μL of serum free media (serum-free DMEM) were added to each insert. Inserts were handled with forceps sterilized with 70% ethanol. Plates were covered and incubated for 4 to 24 hours (37° , 5% CO_2). At the designated time points, cells were removed from the top side of the insert by pipetting out remaining cell suspension and placed into a clean well containing 400 μL of cell stain. New wells were incubated for 20 minutes at room temperature. Inserts were dipped into a clean well with water to rinse. Promptly after rinsing, the cells were removed from the interior of the insert using cotton-tipped swabs. Inserts air dried for 20 minutes and then transferred into a clean well containing 200 μL of extraction buffer (part no. 90145) for 15 minutes at room temperature. Stain was extracted by tilting insert back and forth several times during incubation. Inserts were removed from the well mixture was transferred onto a 96-well microtiter plate from which the optical density was read at 562nm.

Preparation of collagen-PRP provisional scaffold

Acid -soluble, Type I collagen slurry was made by solubilizing bovine patellar tendon tissue. In brief, bovine tissue was sterilely harvested and solubilized in an acid solution as previously described¹¹. Collagen content within the slurry was adjusted to greater than 5 mg/ml and neutralized with 0.1M HEPES (Cellgro, Mediatech, Inc, Herndon, VA), 5X PBS (HyClone Logan, Utah) to obtain a final pH of 7.4.

Platelet rich plasma (PRP) was prepared from human whole blood. Human platelets were prepared from six hundred milliliters of whole blood drawn from one hematologically normal individual. Blood was collected in a bag with 10% by volume acid-citrate dextrose. The blood was centrifuged for 6 minutes at 150g (GH 3.8 rotor, Beckman GS-6 Centrifuge, Fullerton, CA). The supernatant was aspirated and collected as platelet-rich plasma in a 50 ml tube. The platelet-rich plasma was then centrifuged again (GH 3.8 rotor, Beckman GS-6

Centrifuge, Fullerton, CA) and the platelet poor plasma supernatant was aspirated off. Complete blood counts (CBC's) were performed on the whole blood, platelet poor and platelet rich solutions. The resulting platelet pellet was resuspended in enough platelet poor plasma to bring the platelet concentration of the solution to approximately 3× that of the whole blood. A second CBC was performed on the platelet rich plasma to ensure the desired concentration was achieved.

The collagen-platelet composites were made by combining the cell-seeded platelet solution and cold, neutralized collagen solution. Each 0.5 ml CPC contained 0.19 ml cell-seeded PRP and 0.31 ml collagen-buffer solution and 7.5×10^5 cells per gel. The collagen solution was transferred into silicone semitubular molds with polyethylene mesh at each end to anchor the gels within six well plates using a repeat pipettor. This was done to ensure uniform volume across all scaffolds. The scaffolds were incubated at 37°C for one hour, to ensure complete gelation. Negative control scaffolds were created in the exact same fashion using PRP without cells. After gelation, six milliliters of complete media was added to each well to cover the composites. Samples were cultured in a 37°C CO₂ humidified incubator and the complete media was changed twice per week.

MTT Assay

Fibroblast number in the CPCs at day 2, 7, and day 14 was determined by using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay; MTT powder (USB Corporation, Cleveland, OH) was mixed with PBS at a ration of 1mg/ml for this assay. A total of eight cell-seeded scaffolds analyzed at each time point (2, 7, and 14 days) for each patient per age group. In addition, four cell-free, negative controls of collagen-PRP were analyzed at each time point and all samples were normalized using these empty controls.

At the designated time point, the media was aspirated from each well, and the scaffolds were removed from the silicone semitubular molds and placed into individual wells of twelve well plates. One milliliter of the 1 mg/ml MTT solution was added to each well. Each gel was fully immersed in the MTT solution. After the MTT was added, the plates were incubated for 3 hours at 37 C and 5% CO₂. Subsequently, the excess MTT solution was removed and 1 ml of sterile 1X PBS was added to each well. The plates were then placed on a horizontal agitator (Fisher Scientific Clinical Rotator, 100 rpm) and left to rinse at room temperature for 30 minutes. Rinses were repeated until absorbencies of the wash were less than 0.100 at 562 nm. All PBS was then removed and each gel transferred with a sterile spatula into a sterile 5.0 ml centrifuge tube. The gels were then placed in 1 ml of a detergent containing 20% aqueous Sodium dodecyl sulfate/formamide (1:1 volume ratio) in each tube and incubated overnight in at 37° C. Finally, the tubes were vortexed on high for 5 seconds and then centrifuged for 5 minutes at 1500rpm. Two hundred microliter aliquots of the supernatant from each tube were then transferred onto a sterile 96-well plate. The absorbencies were measured and 650nm, and the relative numbers of fibroblasts were determined.

Scaffold Contraction

Images of the gels were captured at Days 0, 2, 7 and 14 using a Canon, Rebel XT, EOS digital camera. The area of each scaffold was measured at each day using NIH Image J 1.37V. For each group, the scaffold areas were averaged and the mean and standard deviation reported. In addition, the percent of Day 0 size was calculated at each subsequent day. Again, 4 unseeded collagen-PRP scaffolds were used as negative controls.

Statistical analysis

Data analysis was done using intercooled Stata 10 (StataCorp, College Station, Tx). Repeated measure ANOVA or two-tailed t-tests were used; the Bonferroni method was employed to adjust p-values in case of multiple testing. All results are given as means $\pm$ SD. A p-value of less than 0.05 was considered significant.

RESULTS

Platelet Solution Preparation

Platelet (PLT) count, Red Blood Cell (RBC) count, and White Blood Cell (WBC) count of the whole blood, platelet rich plasma, and platelet poor plasma can be found in Table 1. The enrichment factor for the platelet rich plasma was 3.39 $\times$ of the whole blood platelet concentration.

Migration Assay

No significant differences were seen between age groups at 4 hours ($p=0.8605$). However, at 24h the immature cells had a 14% greater increase in migration than the adolescent cells ($327\%\pm 99\%$ vs $287\%\pm 117\%$, $p=0.0377$, Figure 1).

MTT Assay

At day 2 there were no significant differences between groups. At days 7 and 14, the immature cells had significantly higher MTT results ($p=0.0004$ at 7d, $p=0.004$ at 14d, Figure 2)

Scaffold Contraction

There was no difference in scaffold contraction rates between the immature and adolescent groups. At day 0 there were no significant differences in scaffold size between any age group or control scaffolds ($p > 0.019$ for all comparisons). At day 2, immature scaffolds were 46% smaller, adolescent 48%, than their day 0 size, differences that were significantly greater than the change in size of control scaffolds (61%, $p = 0.0062$). At day 7, adolescent scaffolds were 47% smaller, and immature scaffolds were 46% smaller than their day 0 size, differences that were significantly greater than the change in size of control scaffolds which had shrunk to 39% of their initial size and stay at this size until day 14 (62%, $p = 0.0041$ for adolescent scaffolds, and $p = 0.0038$ for immature scaffolds), but not significantly different across age groups. (Figure 3)

DISCUSSION

Tissue engineering enhanced ACL repair holds some promise as a new technique for the management of ACL tears. A body of literature that supports this claim has also consistently shown that the outcome of such a procedure depends on the age of the patient^{13; 14}. These findings are not only biologically and empirically plausible, but also clinically important since it is young and immature patients, who otherwise lack treatment options and thus often perform badly after ACL injury, that would probably benefit most from tissue engineering a repair of the ACL. However, this age-dependence has only been shown in large animal models so far, and while validity of these findings in human could be assumed, proof of their reproducibility in a human model was still missing. In this study we looked at age-dependence in three surrogate parameters of tissue healing and repair tissue maturation – cell proliferation, cell migration, and wound contraction and found a significant difference in cell proliferation and migration, but no difference in scaffold contraction, suggesting

some age-dependence in humans, but maybe to a lesser extent that seen in large animal studies.

Our study has some limitations. First of all, while we calculated power a-priori, a point could be made that the lack of a significant difference in cell migration or contraction is due to a lack of power. However, while a large sample might have shown a statistically significant result, the absolute difference between groups would still have been too small to be clinically meaningful. Secondly, we based our categorization on biological age and skeletal maturity assessed by physal closure about the knee, which might have left some room for error given the natural variability of maturation. A recently published study on the role of growth-factor receptors in the age-dependence of ACL fibroblast behavior showed that this dependence is rather based on absolute age than age groups¹⁸. Thirdly, cells obtained from human individuals should be considered more heterogeneous than those obtained from research animals, since samples cannot be taken immediately after rupture/transection, but only during surgery, which is staged some time after the initial injury and not all time points are the same for all patients. However, these problems would also be encountered in a clinical scenario and might be appreciated as a shortcoming from a biological perspective, but an asset from a clinical one. Lastly, we were not able to obtain autologous PRP for all patients, because it is difficult to draw large volumes of blood from immature patients. Hence, we did not use autologous PRP, which limits the generalizability of our findings for future clinical application.

For healing to occur following ACL injury, population by ACL fibroblasts of the wound site and the biomaterial spanning it is pivotal, which happens initially by cell migration into the scaffold. These cells are critical to scar tissue formation in the joint, and have been found to migrate in the direction of the wound^{19; 20}. However, after 24 hours, there is a statistically significant and likely clinically meaningful, by absolute numbers, superior migration in immature cells. Previous *in vitro* studies of porcine and ovine ACL cells have supported this finding of greater migration potential in immature cells than adolescent or adult cells²¹. It is important to note, however, that these cells were taken from intact ACL tissue, whereas those used in this study of human cells were from patients undergoing surgery following a marked ACL tear, thus there is potential for even larger differences.

After repopulation of the wound site by cell migration, proliferation of these cells is the next crucial step in wound healing. Here, evidence for a significant difference between age groups was seen suggesting that immature cells grow faster and migrate faster than adolescent cells. These findings corroborate earlier findings in porcine and ovine cells^{13; 14}.

Finally, scaffold contraction should be seen as surrogate for wound contraction, which is a characteristic of wound maturation. The initial decrease in scaffold size, which occurred in both seeded slurries and negative controls, might be interpreted partially as shrinkage due to fluid flowing from the slurry to the osmotic denser cell culture medium and partially due to contraction by PRP clotting. From day 2 to day 7 and day 14 there are only marginal changes in scaffold size. This fact can be interpreted in two ways. First of all, this suggests that the cell-collagen construct is stable, which is an important finding for future clinical applications. Secondly, it suggests that our wound model is not fully mature yet, following the generally accepted fact that cell differentiation and contraction occur as final stages of wound healing subsequent to an earlier stage of cell migration and proliferation.

In conclusion, our findings show that cellular migration and proliferation are affected by age of human ACL fibroblasts, in a similar way as it has been described for large animal models. These findings indicate that immature individuals would benefit from tissue engineering

ACL repair greater than adolescent individuals, or adults. Future research focusing on the reasons for such differences might allow bridging this rift.

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Figure 1. Migration of Immature and Adolescent ACL Fibroblasts at 4 and 24 hours showed no differences initially, but a significantly higher rate of migration in immature cells after 24h. Error Bars represent standard deviation. (repeated measure ANOVA with post-hoc testing)

Cell proliferation (MTT) by age group

Figure 2.

Shows the results from the MTT assay representing cell proliferation. Error Bars represent standard deviation. Equal results at day 2 suggest equivalent seeding efficiency in both groups. There was a trend for a decrease in cell numbers in adolescent cells leading to significant differences between groups at 7 and 14 days. (repeated measure ANOVA with post-hoc testing)

Figure 3. Scaffold size as a percentage of Day 0 size at Day 2, 7, and 14. Both groups showed almost identical rates of scaffold contraction. That fact the rate of contraction was similar to the unseeded scaffolds in the control group suggest that reasons other than cellular activity caused scaffold shrinkage.

Table 1

shows the cell counts for whole blood and PRP.

	PLT ($10^9/L$)	RBC ($10^9/L$)	WBC ($10^9/L$)
Whole Blood	202	4.79	3.36
Platelet Rich Plasma	684	0.05	2.78
Platelet Poor Plasma	154	0.00	0.00