

LIGAMENT CELL ADHESIVENESS AND REPAIRING *IN VITRO* UNDER INFLAMMATORY CONDITIONS

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Abstract

The increase in anterior cruciate ligament (ACL) and medial collateral ligament (MCL) fibroblast adhesiveness under inflammatory conditions [lipopolysaccharide (LPS), tumor necrosis factor- α (TNF- α), and C5a] may explain the delay in the wound healing process seen in the *in vitro* wound-healing assay. With the addition of inflammatory agents, the ligament cell wound-healing was observed to be delayed 3 to 10 times compared to control. This reduction in wound recovery rate can be correlated to the increase in ACL and MCL cell adhesiveness through the interaction between integrin and extracellular matrix (ECM) (e.g., fibronectin, FN) but not through changes in proliferation under the treatment of inflammatory agents. One mechanistic factor inducing the increase in ACL cell adhesiveness is the elevation of intracellular free calcium concentration ($[Ca^{2+}]_i$) by inflammatory agent treatments which facilitates cytoskeletal assembly. The increase in MCL cell adhesiveness under LPS treatment may be a result of both the elevation of $[Ca^{2+}]_i$ and the upregulation of FN expression. However, under TNF- α and C5a treatments, the changes in MCL cell adhesiveness may correlate to the upregulation of FN gene expression, but not to $[Ca^{2+}]_i$. Any factor which facilitates stress fiber assembly (e.g., elevation of $[Ca^{2+}]_i$) and/or ECM gene expression can enhance cell adhesiveness.

Key Words: Ligament fibroblast, adhesion, migration, proliferation, inflammation.

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Introduction

The topic of ligament healing has been an area of enormous orthopaedic research effort for decades. Particular investigations have studied the relative inability of the adult anterior cruciate ligament (ACL) of the knee to regenerate itself upon injury, unlike the medial collateral ligament (MCL) which elicits a functional healing response [10, 11, 15, 29]. There are approximately one and a half million ACL injuries per year in United States, many due to common sports injuries. The adhesive interactions of cells with other cells and the extracellular matrix (ECM) proteins play a fundamental role in the healing process and the tissue structure of differentiated organs [1, 4, 5, 28, 31]. There is increasing evidence that inflammatory factors can exert significant influence on ligaments during their healing process and modulate their biochemical and biomechanical performance [13, 31]. The early inflammatory phase of ligament healing has been found to have both positive and negative effects on ligament healing rate [7, 12]. This inflammation phase includes strong cellular adhesion to the matrix tissue and also increased cell migration towards chemotactic factors. These two cellular events, namely adhesion and migration, must be delicately balanced for tissue healing to occur [9, 16, 32]. Adhesion is necessary for the normal movement of ligament fibroblasts into the ruptured sites of the ligament. However, too much adhesion would impede motility and migration [9]. From the mechanical point of view, there are also two important factors in tissue healing due to the immobilizing nature of many ligament injuries, namely the decrease in ligament activity and cellular mechanical stresses by which inflammation may be accompanied [13]. Therefore, the objective of this study was to investigate quantitatively changes in cell adhesiveness to correlate with our migration studies involving the *in vitro* migration-healing assay.

This study focused on the adhesion characteristics of fibroblasts from the ACL and MCL to fibronectin (FN) by measuring single cell attachment forces with the use of a micropipette micromanipulation system [22]. Cell adhesiveness was examined and correlated with intracellular calcium concentration fluctuation and FN gene expression

under the effects of inflammatory agents, e.g., lipopolysaccharide (LPS), tumor necrosis factor- α (TNF- α), and complement C5a. This study will help to elucidate the mechanisms of cell adhesion behavior under inflammatory conditions *in vitro*.

Materials and Methods

Human ligament fibroblasts were obtained from culture of ACL and MCL explants harvested freshly at autopsy within 6 to 24 hours after death of the subjects (3 subjects ages from 22 to 55) [14]. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), non-essential amino acids {0.10 mM, L-glutamine (4 mM)}, penicillin (100 U/ml), streptomycin (100 μ g/ml), and fungizone (0.25 μ g/ml) (Biowhittaker, Walkersville, MD) [24]. Cultures were maintained at 37°C and 5% CO₂. At confluency, the ACL and MCL fibroblasts were trypsinized for 3 minutes using a trypsin-Versene (Biowhittaker) mixture (1:250 trypsin, 200 μ g/ml Versene ethylenediaminetetraacetic acid, EDTA), washed twice with DMEM, resuspended and gently agitated in complete medium (DMEM with supplements outlined above) for 2 to 4 hours prior to being seeded in 6-well plates (wound recovery assay) or in micropipette chambers (cell adhesion assay) [19, 22, 24].

Wound recovery assay *in vitro*

When a confluent monolayer was reached, *in vitro* wounds were simulated in the 6-well plates by streaking the cells, creating a cell free area [14, 17, 30]. An inoculating wire was used to generate a consistent wound width of 400 \pm 10 μ m. After streaking all the wells in the plate, the plate was gently rocked back and forth and the old medium was then removed and replaced with fresh culture medium. This was to assure that the cells scraped away were suspended in the medium so they would be removed when the medium was changed. Before streaking, the loop was wiped with alcohol, flamed until red, and then allowed to cool for at least 20 seconds [30].

The following inflammatory factors were used: LPS (1.0 μ g/ml), TNF- α (20 ng/ml) and complement C5a (100 ng/ml). One inflammatory factor was added to the medium of the cultured cells (one factor per plate) after the wound was simulated and continually added with the 30% FBS medium at each medium change. In each plate, three wells contained ACL cells and three wells contained MCL cells. One ACL well and one MCL well were used as controls and did not have any inflammatory factors added after wound simulation.

The rate of recovery of cells into the wound was then observed and recorded on a video tape at approximately 12-hour intervals [30]. The data were analyzed using

Bioscan Optimas imaging software program (version 2.03, BioScan Inc., Edmonds, WA) after the areas were drawn on transparencies with different colored markers. Each color represented a different time interval and, at most, five time intervals were drawn on each transparency. The computer was then used to find the cell free area at each time. The length of the wound area was chosen as 1250 μ m in all experiments. The healing, called % Recovery, was calculated by subtracting the area at each time interval from the initial cell free area, and then normalizing the difference by the initial area:

$$\% \text{ Recovery} = 1 - [(\text{cell free area})_i / (\text{cell free area})_0]$$

From the results of the experiments (the controls), plots were made of % Recovery versus time for each initial wound width. Because the wound width varied slightly along the length of the artificial wound, the initial wound width was calculated by dividing the initial wound area by the wound length of 1250 μ m. From these plots, the time it took to reach 50% and 80% Recovery were extracted. These times were then plotted against the initial wound width to observe the differences.

Cell proliferation under inflammatory conditions

Ligaments cells (ACL/MCL) were seeded in 96-well flat bottom plates at 37°C and 5% CO₂ in DMEM with 10% FBS. Fifty microliters of 1 X 10⁵ cell/ml solution (~5000 cells) were added to each well. The cells were allowed to incubate for 1 hour. Inflammatory agents were then added to the appropriate wells at the following concentrations: LPS 1 μ g/ml, TNF- α 20 ng/ml, and C5a 100 ng/ml. Six wells were kept inflammatory agent-free and used as control, and another 6 wells were kept cell-free for background effect calculation. The cells were incubated for 12, 24, 48, and 72 hours. One hour before the end of incubation, 10 microliters of Cell Titer 96 Proliferation Kit (Promega Corp., Madison, WI) [8] solution was added into each well including the background wells (culture medium only). The assay is based on the cellular conversion of the tetrazolium salt, MTS, into a formazan that is soluble in tissue culture medium. The culture plate was then placed on the orbital shaker and shaken for 1 hour. At the end of the incubation period, 100 μ l of the supernatant from each well was transferred into a new well of another 96-well plate. Any bubbles created as a result of the transfer were eliminated using a heated sterilized 32 gauge needle. The colored formazan product whose presence can be detected with a spectrophotometer [3]. This assay measures the number of living cells by measuring the amount of basal metabolism that take place within each culture well. The amount of metabolism is proportional to the number of living cells. Hence, the number of living cells present can be rapidly and accurately

determined. The absorbance of the content of each well was measured at wavelength of 520 nm. The 3 readings from each well were subtracted from the blank reading and averaged. This averaged reading was converted into an absolute cell number based on the standard curve generated from the assay of known number of cells.

Cell adhesion assay

Micropipette chamber / Loading of inflammatory agents: All cover glasses within micropipette chambers were pre-coated with 5 µg/ml of FN which is the same concentration used in our previous studies on fibroblasts [20, 23, 24] and is close to or less than the physiological concentration. The detailed coating procedure of FN on cover glasses within micropipette chambers has been previously described [20, 21, 24]. After having filled the chamber with medium containing all necessary supplements and the desired amount of the inflammatory agent of interest, LPS (1.0 µg/ml), TNF- α (20 ng/ml), and C5a (100 ng/ml). The chamber was loaded with suspended ACL and MCL cells which were then allowed to interact with the inflammatory factors and the FN-coated chamber for a 15-minute cell seeding time at room temperature (20-22°C) before beginning the cell adhesiveness measurements [20, 21, 23, 24].

Adhesion force measurements: The micropipette-micromanipulation system used for the measurement of adhesion force is similar to that previously described [22, 24]. Glass micropipettes with an internal tip radius of 1.5 to 3.0 µm were prepared by using a micropipette puller (Model P-87, Sutter Instrument Co., Novato, CA). They were then filled with complete culture medium and mounted on a hydraulic micromanipulator with the wide end of the pipette connected to a pressure regulating system. The adhesion characteristics were measured under direct microscopic observation in conjunction with a video recording system.

Intracellular calcium measurement

Video microscopy and a quantitative fluorescence system was used to measure fluctuations in intracellular free calcium concentration ($[Ca^{2+}]_i$) of ACL and MCL fibroblasts seeded onto a monolayer of FN (5 µg/ml) for 60 minutes [23]. The calcium ion selective fluorescent probe, Fura-2 (Molecular Probes, Eugene, OR), was used together with Image-1/FL software (Universal Imaging Corp., West Chester, PA), a ultra-violet (UV)-light Nikon (Tokyo, Japan) Diaphot-TMD microscope with quartz optics, and a video camera with a silicon intensifier target tube (Hamamatsu Photonics, Hamamatsu City, Japan) connected to a 486 CPU-based personal computer (Gateway 2000, North Sioux City, SD) [23]. The Fura-2 probe exhibits a shift in wavelength maximum and intensity level upon binding with calcium. Without calcium binding, the maximum excitation is at a wavelength of 380 nm, and 340 nm with calcium binding.

The wavelength ratio of 340/380 excitation is used to determine $[Ca^{2+}]_i$ [25]. Fura-2 was loaded into cells using its acetoxymethyl (AM) ester, Fura-2 AM, according to the following protocol. Stock Fura-2 AM at 50 µg was transferred into a 50 µl non-ionic detergent (mixture of 3 to 1 ratio by weight DMSO and low toxicity dispersion agent Pluronic F-127, Molecular Probes) to make a 1 mM stock solution. The stock Fura-2 AM solution was then added to the cell suspension (at a working concentration of 2-10 µM, diluted from the stock concentration by media solution) and incubated at 37°C (5% CO₂) for 15-60 minutes prior to seeding the micropipette chamber. The analysis includes a standard order of procedures, including spatial calibration, image acquisition, thresholding, and profile extraction [26, 27]. A standard calibration curve showing the relationship between calcium concentration and wavelength ratio was produced using a Calcium Calibration Buffer Kit II (Molecular Probes) [25]. After the cells have settled and adhered to FN for 30 minutes, the baseline measurements were made immediately. Fluorescence ratio images were obtained using 32 pseudocolor hues to represent the range of fluorescence ratio [23]. Fluctuations were observed for the baseline measurements, therefore, we averaged the data to generate a straight line. At the same time the baseline measurements were made, the treatment cells were also measured and a straight line was generated by taking the mean value of the fluctuated curve. The two lines were then superimposed with each other on the same graph since the system cannot generate two curves simultaneously. The results, therefore, are presented as a computer generated curves based on the mean of individual measurements.

Fibronectin gene expression measurement

The total cellular RNA was extracted from ACL and MCL cells, and the RNA was analyzed by Northern blotting with probes specific for the expression of genes for FN. Cells were lysed in a 4 M guanidium isothiocyanate solution. For the inflammatory treatment group, cells were lysed after incubating with the appropriate inflammatory agent added in culture medium for 4 hours. A phenol-chloroform extraction was performed and the aqueous phase was transferred to a new tube after centrifugation at 10,000 g at 4°C for 20 minutes. Total RNA was precipitated through two consecutive ethanol precipitations separated by an additional phenol-chloroform extraction step [18]. The final RNA yield was quantified by UV spectrophotometry at 260 nm. For Northern blot analysis, 5 to 20 µg of total RNA was electrophoresed on 1% agarose gels and transferred to nylon membranes, which were cross linked for 30 seconds by UV cross-linker. The membranes were then hybridized for 24 hours at 42°C using ³²P-radiolabelled probes prepared by random priming. After hybridization, membranes were washed and exposed to radiographic film (Kodak X-Omat

XAR film at -70°C , Kodak, Rochester, NY). To correct for loading differences, the membranes were dehybridized and subsequently rehybridized with a 780 base pairs (bp) cDNA fragment for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or a 29-bp DNA oligomer to 28S ribosomal RNA. All data were expressed as the ratio of mRNA/28S or mRNA/18S signals obtained from quantitative densitometry [18]. Probes for the FN were provided by the Adhesion Receptor Core Facility of the Institute for Biomedical Engineering, Univ. California San Diego.

Statistical analysis

For each set of data, the standard error of mean (SEM) was calculated from standard deviation (SD):

$$\text{SEM} = \text{SD} / (n)^{0.5},$$

where

$$\text{SD} = \sqrt{\sum (Y - Y_{\text{ave}})^2 / (n - 1)}$$

and n = number of samples. Two factor ANOVA F tests ($\alpha = 0.05$) were performed between controls and treated groups. Comparisons between controls and treated groups were performed using also the unpaired Student's t test (mean) to determine if any significant differences ($p < 0.05$) exist.

Results

During the experiments, ACL and MCL were taken from the same subject. We have compared the experimental data obtained between individual subjects and found that the variation was below 20%. This variation is below that of the single cell adhesion study (20-40%), the migration study (25%), the intracellular Ca^{2+} study (30%), and the gene expression study (20%). For the results presented here, experimental data obtained using ligaments from all 3 subjects were combined to minimize the variation.

Wound recovery assay *in vitro*

Effects of LPS on ligament wound recovery: The migration time is the time it would take for a wound of an initial width of $400 \pm 10 \mu\text{m}$ to reach 50% and 80% recovery for ACL and MCL cells under the influence of inflammatory agents. Comparing the results at 50% and 80% recovery, the times required for ACL cells under LPS treatment increased 1.2- and 2.1-fold, respectively, compared to control. Same trends were observed in MCL cells under the influence of LPS, for the MCL cells took 1.5 and 2.2 times longer compared to control to reach 50% and 80% recovery, respectively (Fig. 1). In addition, it was observed that the MCL control group reached 100% recovery in 48 hours, whereas the MCL LPS treatment group did not reach 100% recovery until after 132 hours (5.5 days). Also, the ACL

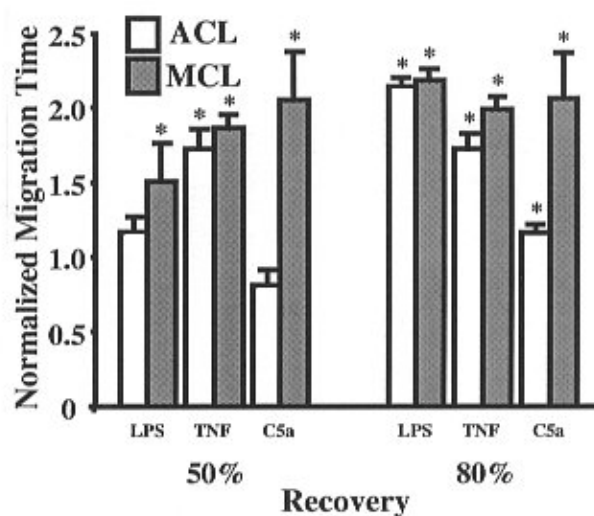


Figure 1. Effects of inflammatory agents on the wound recovery of anterior cruciate ligament (ACL) and medial collateral ligament (MCL) fibroblasts. Lipopolysaccharide (LPS), tumor necrosis factor- α (TNF- α), and complement C5a (respective concentrations of $1.0 \mu\text{g/ml}$, 20 ng/ml , and 100 ng/ml) were used as inflammatory agents. The amount of time (migration time) for the initial wound simulated by streaking the cells with an inoculating loop ($400 \pm 10 \mu\text{m}$ in width) to reach 50% and 80% recovery with the presence of inflammatory agents were normalized to that of the control group (without inflammatory agents) and plotted. For the ACL control groups, the times required for 50% and 80% recovery were 31.7 ± 3.2 hours and 45.3 ± 1.4 hours, respectively. For the MCL control groups, the times required for 50% and 80% recovery were 24.7 ± 2.6 hours and 36.4 ± 2.1 hours, respectively. Experimental groups with normalized migration times greater than 1 experience wound recovery delays with respect to control. * denotes a significant difference with respect to control ($p < 0.05$).

control group reached 100% recovery in approximately 70 hours, whereas the ACL LPS treatment group never fully reached 100% recovery even after 3 weeks (results not shown).

Effects of TNF- α on ligament wound recovery:

Under the treatment of TNF- α , ACL and MCL both exhibited a 1.7-1.8 and a 1.7-2.0 fold increase in the amount of time required to reach 50% and 80% recovery compared to control, respectively (Fig. 1).

Effects of C5a on ligament wound recovery: In the case of C5a treatment, no significant difference was observed between the control and treatment group for ACL cells at both 50% and 80% recovery (Fig. 1). However, the ACL control group and the C5a treatment group required

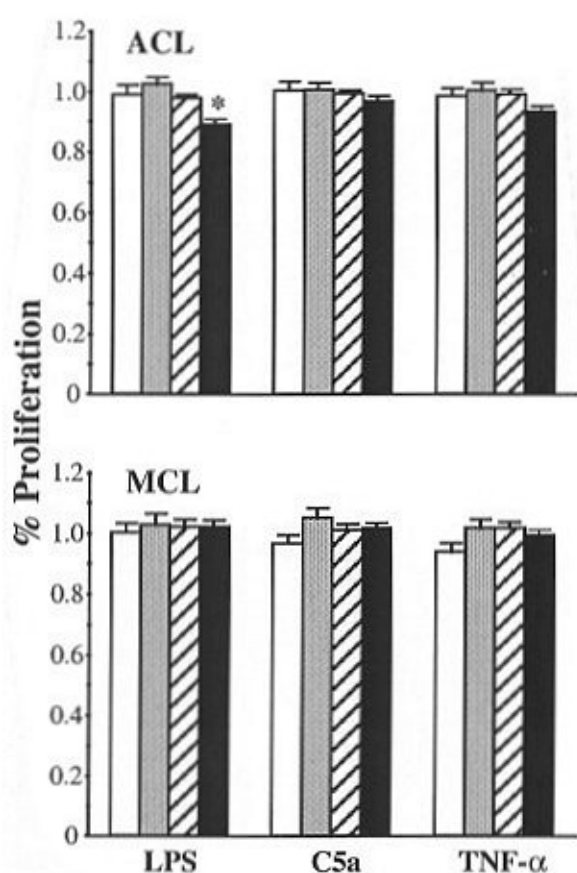


Figure 2. Effects of inflammatory agents on the cell proliferation of ACL and MCL fibroblasts. LPS, TNF- α , and C5a (respective concentrations of 1.0 $\mu\text{g/ml}$, 20 ng/ml, and 100 ng/ml) were used as inflammatory agents. Cells were seeded into wells in a 96-well plate and incubated for 12 (blank), 24 (gray), 48 (hatched), and 72 (solid) hours under the three separate inflammatory conditions. The number of cells in each well at the end of each incubation period was measured by the Cell Titer 96 Proliferation Kit (Promega). Data from experimental groups were normalized using control (no inflammatory treatment). * denotes a significant difference with respect to control ($p < 0.05$).

48 hours and 110 hours, respectively, to reach 98% recovery (results not shown). For MCL cells, the addition of C5a approximately doubled the time required to reach both 50% and 80% recovery compared to control.

Cell proliferation under inflammatory conditions

Approximately 5000 ACL/MCL cells were seeded in 96-well flat bottom plates at 37°C and 5% CO₂ in DMEM with 10% FBS. Upon adhering to bottom of the wells, the cells were incubated for 12, 24, 48, and 72 hours under three separate inflammatory conditions: LPS 1 $\mu\text{g/ml}$, TNF- α 20

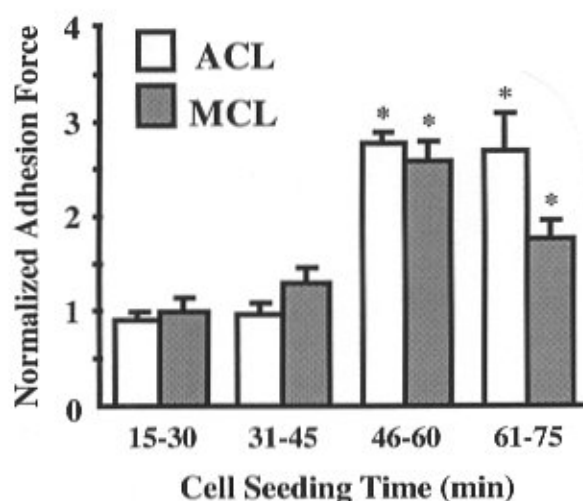


Figure 3. Effects of the inflammatory agent LPS (1.0 $\mu\text{g/ml}$) on the adhesion of ACL and MCL fibroblasts to 5 $\mu\text{g/ml}$ fibronectin. The adhesion strengths of the fibroblasts under the influence of LPS were normalized to that of the cells in the control group and plotted. For the control groups, the adhesion force at the 15-30, 30-45, 45-60, 60-75 minutes time periods were 7.33 ± 0.70 , 9.01 ± 0.77 , 11.9 ± 1.2 , and 12.4 ± 1.2 mdyne, respectively for ACL fibroblasts, and 6.23 ± 0.43 , 8.66 ± 0.57 , 9.88 ± 0.77 , and 8.17 ± 0.74 mdyne, respectively for MCL fibroblasts. Experimental groups with normalized adhesion forces greater than 1 experience adhesion force increase with respect to control, and *vice versa*. * denotes a significant difference with respect to control ($p < 0.05$).

ng/ml, and C5a 100 ng/ml. The number of cells in each well at the end of each incubation period was measured by the Cell Titer 96 Proliferation Kit (Promega Corp., Madison, WI). The results from experimental groups treated with inflammatory factors were then normalized by control. We found in our cell proliferation assay under inflammatory conditions that cell proliferation is not affected by inflammatory treatments except for ACL cells under LPS treatment for 72 hours (Fig. 2).

Cell adhesion assay

Effects of LPS on ligament fibroblasts adhesion:

Samples of 50-80 fibroblasts were used to test their adhesiveness to FN (5 $\mu\text{g/ml}$) for each time period (15-30, 31-45, 46-60, and 61-75 minutes). Their adhesion strength was observed to range from 0.5 to 18 mdynes for the control group and from 0.5 to 50 mdynes for the LPS group. The cell adhesion behavior from the ACL ($n = 260$) showed a seeding time dependence under the influence of LPS. The duration of LPS treatment had significant effects on ACL adhesion response. The adhesion strength started to

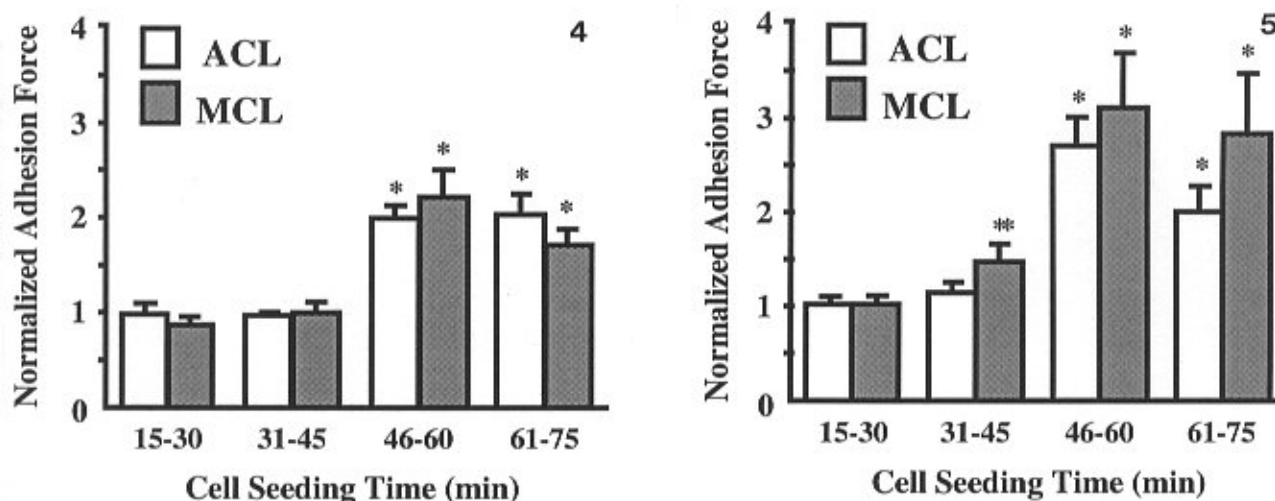


Figure 4 (at left). Effects of the inflammatory agent TNF- α (20 ng/ml) on the adhesion of ACL and MCL fibroblasts to 5 μ g/ml fibronectin. The adhesion strengths of the fibroblasts under the influence of TNF- α were normalized to that of the cells in the control group and plotted. For the control groups, the adhesion force at the 15-30, 30-45, 45-60, 60-75 minutes time periods were 11.6 ± 1.0 , 12.6 ± 0.7 , 13.2 ± 1.3 and 15.2 ± 1.4 mdyne, respectively for ACL fibroblasts, and 8.54 ± 0.63 , 9.28 ± 0.36 , 14.0 ± 0.7 , and 11.4 ± 0.8 mdyne, respectively for MCL fibroblasts. Experimental groups with normalized adhesion forces greater than 1 experience adhesion force increases with respect to control, and *vice versa*. * denotes a significant difference with respect to control ($p < 0.05$).

Figure 5 (at right). Effects of the inflammatory agent complement C5a (100 ng/ml) on the adhesion of ACL and MCL fibroblasts to 5 μ g/ml fibronectin. The adhesion strengths of the fibroblasts under the influence of C5a were normalized to that of the cells in the control group and plotted. For the control groups, the adhesion force at the 15-30, 30-45, 45-60, 60-75 minutes time periods were 8.28 ± 0.41 , 8.24 ± 0.42 , 9.90 ± 0.51 and 7.96 ± 1.23 mdyne, respectively for ACL fibroblasts, and 6.95 ± 0.52 , 8.68 ± 0.60 , 9.31 ± 0.69 , and 9.67 ± 0.78 mdyne, respectively for MCL fibroblasts. Experimental groups with normalized adhesion forces greater than 1 experience adhesion force increase with respect to control and, *vice versa*. * denotes a significant difference with respect to control ($p < 0.05$).

increase 45 minutes after the initial exposure of cells to LPS. Fibroblasts from MCL ($n = 286$) also showed a dependency on the duration of LPS treatment. Both ACL and MCL had similar response in their adhesion behavior when treated with LPS. However, MCL adhesion strength decreased during the 61-75 minute period compared to the 46-60 minute period. This decrease in adhesion strength indicated that the cells increased their motility during the 61-75 minute period. Fibroblasts from both ligaments showed the sharpest increase in adhesion strength beginning at 46 minutes after the initial exposure of cells to LPS. ACL cells had greater adhesion strength than cells from MCL during the 61-75 minute period after their initial exposure to LPS. The normalized adhesion forces of ACL and MCL are shown in Figure 3.

Effects of TNF- α on ligament fibroblasts adhesion:

Samples of 100-150 fibroblasts were used to test their adhesiveness to FN coated surface under the influence of TNF- α for each of the four different time periods (15-30,

31-45, 46-60, and 61-75 minutes). The results of the normalized adhesion forces exhibited by ACL and MCL fibroblasts under the influence of TNF- α were plotted as a function of the four time periods (Fig. 4). The effects of TNF- α on cell adhesiveness became evident during the 46-60 minute time period for both ACL and MCL. The increased adhesiveness of ACL and MCL fibroblasts was at approximately the same level during that 46-60 minute time period. During the 61-75 minute period, the adhesiveness of MCL fibroblasts showed a similar tendency (compared to LPS treatment) to decrease under TNF- α treatment.

Effects of complement C5a on ligament fibroblast adhesion:

Samples of 50-100 fibroblasts were used to test their adhesiveness to FN coated surface for each of the four different time periods (15-30, 31-45, 46-60, and 61-75 minutes) under the C5a complement treatment. The normalized adhesion force as a function of four different time periods was plotted in Figure 5. The adhesion of ACL fibroblasts

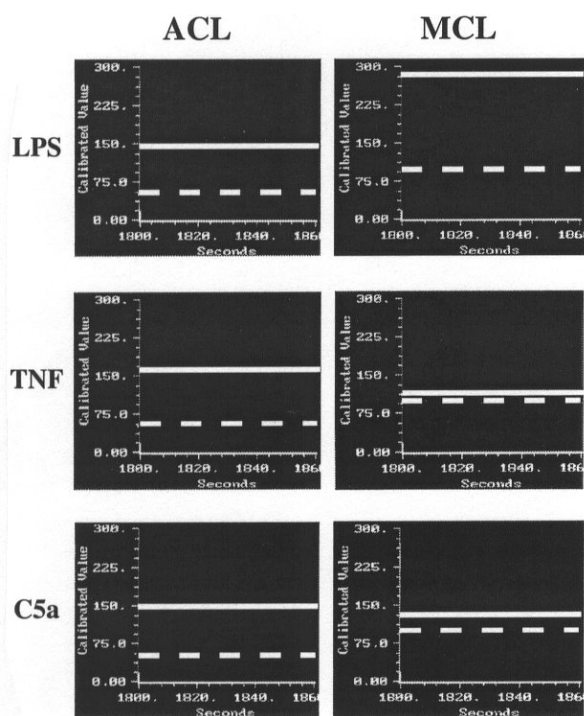


Figure 6. Effects of inflammatory agents on the intracellular free calcium level of ACL and MCL fibroblasts. Video microscopy and a quantitative fluorescence system was used to measure fluctuations in intracellular free calcium concentration ($[Ca^{2+}]_i$) of ACL and MCL fibroblasts seeded onto a monolayer of FN (5 $\mu\text{g}/\text{ml}$) for 60 minutes. This curve was generated by a computer system, and the baseline and the treatment group were grouped on one graph using the computer system. The dotted lines denote the basal $[Ca^{2+}]_i$ of control ACL and MCL fibroblasts with no external factors added, whereas the solid lines denote the $[Ca^{2+}]_i$ of ACL and MCL fibroblasts under the treatments of inflammatory agents LPS (1.0 $\mu\text{g}/\text{ml}$), TNF- α (20 ng/ml), and C5a (100 ng/ml) for 30 minutes.

was increased during the 46-60 minute period and slightly decreased during the 61-75 minute period. However, the MCL adhesiveness showed an early increase during the 31-45 minute time period while the adhesiveness of the ACL cells remained relatively the same as the control group (without C5a treatment). During the time periods of 46-60 and 61-75 minutes, the adhesiveness of the MCL fibroblasts increased with the same amplitude under the inflammatory condition induced by C5a.

Intracellular calcium measurement

Effects of inflammatory agents on ligament fibroblasts intracellular calcium: Intracellular free calcium concentration ($[Ca^{2+}]_i$) of 20 to 30 ligament fibroblasts were

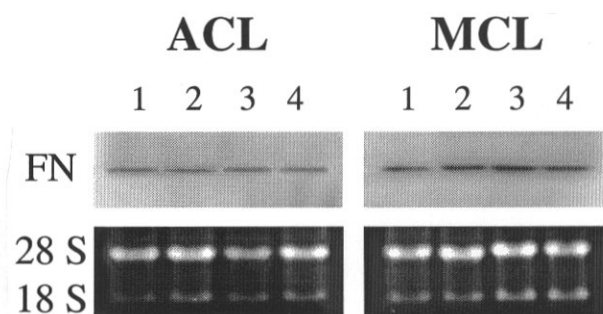


Figure 7. Effects of inflammatory agents on the FN gene expression of ACL and MCL fibroblasts. ACL and MCL fibroblasts were incubated with inflammatory agents LPS (1.0 $\mu\text{g}/\text{ml}$), TNF- α (20 ng/ml), and C5a (100 ng/ml) for 4 hours, and cellular RNA was then extracted and analyzed by Northern blotting with probes specific for the expression of genes for FN. Levels of gene expression were quantified using densitometry analysis. Lanes: 1 = control; 2 = LPS; 3 = TNF; and 4 = C5a.

measured for each cell line (ACL and MCL) and the mean $[Ca^{2+}]_i$ was calculated for each group (presented in Fig. 6). The results of ratio imaging measurement indicated that the $[Ca^{2+}]_i$ levels of ACL and MCL ligament fibroblasts were influenced by inflammatory agents. The ACL fibroblasts under LPS, TNF- α , and C5a treatments were found to possess significantly elevated $[Ca^{2+}]_i$ levels (Fig. 6, solid line) of approximately 3 times as high as the control group (Fig. 6, dotted line). The MCL fibroblasts exhibited different responses to different inflammatory agents. The inflammatory agent LPS were found to increase $[Ca^{2+}]_i$ level of MCL fibroblasts 3 folds over the control, which was a response similar to that seen in ACL fibroblasts. However, no significant increase in $[Ca^{2+}]_i$ level was seen in MCL cells under TNF- α and C5a treatments. It was also observed that ACL cells grown with LPS, TNF- α and C5a in DMEM had a lower basal line of $[Ca^{2+}]_i$ than the MCL cells grown in the same medium.

Gene expression assay

Effects of inflammatory agents on FN gene expression: The FN gene expressions of ACL and MCL cells under the influence of inflammatory agents (LPS, TNF- α , and C5a) for 4 hours were determined using the Northern blotting and expressed as the ratio of mRNA/28S or mRNA/18S signals obtained from quantitative densitometry. It was observed that while in MCL cells the inflammatory agents LPS, TNF- α , and C5a caused the FN gene expressions to increase 10-40% compared to control, in ACL cell the effects of the same set of inflammatory agents were minimal and

the FN gene expressions stayed relatively close to the control value (Fig. 7).

Discussion

The anterior cruciate ligament and medial collateral ligament are the major ligaments providing substantial stability and normal functioning of the knee joint. It is well documented that the adult human MCL has a functional healing response, whereas the ACL does not. The differential healing responses of the ACL and the MCL could be due to factors related to the different biological conditions (e.g., locations *in vivo*) [2, 4, 6, 13, 15, 39] and the different intrinsic properties of the constituent cells of these ligaments [13, 15]. At the cellular level, ligament wound healing involves cell attachment, detachment, migration, and proliferation. The goal of this study was to quantify the influence of inflammatory agents (LPS, TNF- α , and C5a) on the ACL and MCL ligament fibroblast adhesion behavior and to correlate the results to the intracellular free calcium fluctuation. This should provide important insights into the understanding of different healing responses of these two ligaments under various inflammatory conditions.

The major findings of this study are as follows: (1) The wound healing rate of both ACL and MCL fibroblasts decreased under inflammatory conditions. (2) For both ACL and MCL fibroblasts, the cell adhesion force increased 2-3 times under the incubation with inflammatory agents (LPS, TNF- α , and C5a). (3) Under the treatments of inflammatory agents, the earliest time when a change in cell adhesion could be observed was during the 31-45 minute period after the initial exposure of cells to the inflammatory agents. At the 61-75 minute time period, the cell adhesion force showed a decrease for the MCL cells in both LPS and TNF- α , and for ACL cells in C5a. Without normalizing the adhesion forces by the control (ACL control = 9.09 ± 2.00 mdyne; MCL control = 7.44 ± 1.38 mdyne), the ACL cells had significantly greater adhesion forces (20.63 ± 3.73 mdyne) than the MCL cells (15.69 ± 4.76 mdyne) under the influence of inflammatory agents. (4) Inflammatory agents (LPS, TNF- α , and C5a), caused ACL cells to increase their cytoplasmic free calcium concentrations approximately 3 folds with respect to control. However, MCL cells only responded to LPS treatment with also a 3-fold increase in their cytoplasmic free calcium concentrations with respect to control. (5) The FN gene expressions of ACL and MCL cells responded differently to inflammatory conditions.

The effect of intracellular free calcium concentration ($[Ca^{2+}]_i$) on fibroblast adhesion strength has been previously reported [23]. Fibroblasts from ACL showed a significant increase in adhesion with increased levels of $[Ca^{2+}]_i$ after incubation with inflammatory agents LPS, TNF- α , and

C5a. Also, fibroblasts from both ACL and MCL showed a significant increase in adhesion with increased calcium after incubation with A23187, a calcium ionophore [23]. Our unpublished results obtained using immunofluorescence staining and epifluorescence microscopy method [24] indicated that both ACL and MCL undergo fast assembly of their actin filaments into stress fibers under inflammatory agent treatments. Therefore, this $[Ca^{2+}]_i$ elevation by inflammatory agents acting on ACL cells may play a role in increasing ACL cell adhesiveness that in turn causes the recovery delay of ACL cells seen in our *in vitro* wound-recovery assays. In MCL cells, however, trends were more difficult to identify. Surprisingly, although the LPS induced $[Ca^{2+}]_i$ elevation appeared to increase the MCL cell adhesiveness through the same mechanism as that of ACL cells, same conclusion could not be reached about TNF- α and C5a treatments. Under TNF- α and C5a treatments, the $[Ca^{2+}]_i$ of the MCL cells did not show significant elevation, although it was nevertheless observed that MCL cells undergo fast stress fiber assembly during incubations with all three inflammatory agents (results not shown). In our early studies on the adhesion of ACL and MCL cells to fibronectin, it was found that cell adhesion involves not only changes in cell shape, but also the redistribution of cytoskeletal proteins by way of polymerization, phosphorylation, and assemblage of stress fibers [23]. All of these processes require the communication with the extracellular matrix which helps in directing cellular activities. These activities include the triggering of signal pathways that convert the integrin receptor and ECM binding event into a cascade of intracellular signals and final protein phosphorylation [1, 23]. Since it was indicated from the results presented here that inflammatory agents TNF- α and C5a induced the increase in MCL cell adhesiveness with a mechanism other than that observed in ACL cells, namely $[Ca^{2+}]_i$ increase, another approach based on the above findings was taken in the attempt to explain the MCL cell response under those particular inflammatory conditions. FN gene expression in MCL cells under the treatments of LPS, TNF- α , and C5a was found to increase in our recent studies [19], and this elevation in FN gene expression may account for the increase in MCL cell adhesiveness observed.

We are not surprised that inflammatory agents were able to change the adhesiveness responses of both ACL and MCL cells to ECM through the modification of different signal pathways, for these results are quite similar to that seen in our previous studies where ACL and MCL cells were found to possess different signal pathways by which their cell adhesion is mediated [23], even without the presence of external factors such as inflammatory conditions. The different responses indicate that differences in intrinsic

behavior exist between ACL and MCL cells. Furthermore, the different adhesive behaviors of ACL and MCL cells under various inflammatory agents may account for their differential healing responses seen in the *in vitro* wound-healing assays, and it is our belief that inflammatory agents may play a significant role in ligament tissue healing *in vivo*. We found in our cell proliferation assay under inflammatory conditions that cell proliferation was not affected by inflammatory treatments except for ACL cells under LPS treatment for 72 hours. This delay in recovery has been demonstrated to be predominantly affected by LPS treatment, and the delay can be as much as 10 times slower than the corresponding MCL fibroblasts [30]. From this result, we conclude that the significant delay in ACL recovery under LPS treatment involves both a decrease in cell proliferation and an increase in cell adhesiveness. However, none of other treatments has a significant effect on ACL and MCL cell proliferation within the 72-hour treating time. Therefore, cell proliferation does not play a significant role, for the most part, in ACL and MCL cell migration and wound recovery under inflammatory conditions. Adhesion and migration are two of the fundamental steps involved in the wound recovery processes *in vitro*, and many other factors, e.g., cell necrosis and apoptosis (cell death), which could also be influenced by inflammatory agents will be examined in our future studies.

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Discussion with Reviewers

Reviewer I: What is the rationale for using an uncharacterized LPS preparation? Why do the authors think that the outer coatings of Gram negative bacteria would be important or relevant in this study? They should use pro-

inflammatory cytokines, not LPS.

K. Otsuka: The authors cite ACL injuries by sports, but I suppose these injuries are acute phase of inflammation. Can the authors explain why they choose LPS, TNF and C5a as inflammatory agents?

Authors: LPS is not easy to avoid, especially in open injury cases. We did look into other inflammatory factors (TNF- α and C5a). We agree with reviewer I on the use of pro-inflammatory cytokines (IL-1, 6, 8, 11) and we are currently working on them. However, one paper would be too much to address all the work. Also, work on TNF- α and C5a in ligament fibroblast has not been previously published.

Reviewer I: The presumptive fibroblast populations that are derived from the autopsy specimens are uncharacterized and may include various subpopulations and may not in fact be fibroblastic, a possible explanation for the unusual results and differences between the ACL and MCL populations. Did the authors consider assessing the expression of vimentin, collagens and various actin isoforms to assess whether the cells are indeed fibroblastic and whether are not there are various subpopulations?

Authors: The following procedures were used to ensure the purity of our ligament fibroblast culture. (1) Upon removal from the cadaver, the ligament tissue was dissected carefully to cut away the non-ligament tissues and only the middle portion of the ligament was preserved and used. (2) The ligament tissue was then cut in small pieces, washed 3 times with phosphate buffered saline (PBS) solution, and digested 3 consecutive times with collagenase (1/2 hour each time). (3) The cells released by the ligament tissue were then collected carefully and seeded onto a petri dish to observe their morphology. All cells observed bear the normal morphology of ACL and MCL fibroblasts. (4) The cells were also stained using rhodamine phalloidin to visualize their stress fiber formation [24]. We found that the stress fiber formation of our cells is typical of that of ligament fibroblasts. (5) Finally, the gene expression assay that we performed on these cells showed a high expression of collagen type I, which is typical of ligament fibroblasts. In addition, the cells were viewed under the microscope with a 5000X magnification during our adhesion force micropipette experiments, which made it easy for us to single out non-fibroblasts. Therefore, combining the results from the above tests, as well as our several published papers in major journals [20, 21, 23, 24], we are confident in the purity of our fibroblasts culture.

Reviewer I: What is the evidence that the cells that the authors have cultured are not endothelial (i.e., Factor VIII positive) or smooth muscle (desmin positive); what types of fibroblasts they have cultured (i.e. collagens, vimentin, actin isoforms)? Further, can they indicate why the ACL

and MCL populations should, *a priori* be different than one another.

Authors: The surrounding tissues removed were non-ligament tissue. A sample was prepared by dissecting the middle of the tissue and trypsinized twice with the supernatant discarded. The remaining tissue was then chopped and treated with a collagenase solution.

Under the Western Blot, the cells expressed collagen I positively and the medium used will not allow endothelium and smooth muscles to survive. Because of these two factors, we are certain the isolated cells are indeed ligament fibroblasts.

From the morphology of ACL and MCL, we cannot distinguish between the two cells, but their functional assay [15, 21, 22, 24, 25] indicates the two different behaviors.

Reviewer I: When fibroblasts are stimulated with TNF- α and possibly LPS, their degradative activity against matrix proteins is markedly increased. Did the authors consider that local degradation of the exogenous fibronectin may have altered the ability of the cells to migrate?

Authors: We thank the reviewer for pointing out and reminding us of this potentially confusing phenomenon.

Since the cell monolayers generally reached confluency within two days due to the high number of cells initially seeded onto the chamber, there is time for only a very small amount of fibronectin to be secreted on the bottom of the chamber. Therefore, since the chambers were not coated with any FN initially, we believe that there is negligible fibronectin coating at the cell free zone for both the control and experimental groups.

Also, since cells are capable of degrading FN on and around which they are bound, the increase in degradative activity of fibroblasts induced by inflammatory agent treatments influence both the exogenous FN directly bound to the fibroblasts as well as the FN around the fibroblasts. The degradation of FN to which the fibroblasts are directly bound increase fibroblast mobility and migration, but the degradation of FN around the fibroblasts should have an effect of decreasing their migration. Therefore, these two effects of the degradative activities of fibroblasts presumably result in a cancellation in their ability to influence cell migration.

Reviewer I: The authors speculation (in the previous answer) on possible cancellation of effect of TNF- α -induced protease expression and resultant effects on cell adhesiveness are not supported by any data when there are, in contrast, a large body of data to show that TNF- α strongly induces protease expression and degradation of matrix proteins produced by fibroblast *in vitro*. How can the authors account for this since it might be important in terms of their findings?

Authors: The reviewer has a good point about TNF- α inducing protease expression and degradation of matrix proteins. This could be the reason why TNF- α treated groups do not have a strong adhesiveness compared to LPS and C5a groups in later periods (46-75 minutes). We know with a short period adhesion assay, the matrix degradation is not so quick in the TNF- α group especially in the 46-75 minute period (please compare Fig. 4 with Figs. 3 and 5, LPS and C5a, respectively).

Reviewer I: One possible explanation for the behavior of the cells is that they simply were dead or dying, a view supported by the unusually high and prolonged $[Ca^{2+}]_i$. Did the authors consider the possibility of cell death and measure the incidence of dying cells?

Authors: We always carefully check the cells using our regular high resolution microscope system (which can be easily switched back and forth with the calcium fluorescence setup) during the $[Ca^{2+}]_i$ measurements to make sure that the cells we were targeting were alive and undergoing shape changes, elongation, stress fiber formation, active extension of their filopodia, and granule movements. This ability to observe cell morphology in detail is one of the advantages of our high magnification microscope system (5000X). We usually discover dead cells by observing the leakage in cell membrane, the decrease of cytoplasmic viscosity, and the increase in the speed of granule movements. Also, if the cell death is through a necrosis pathway, we can observe an increase in cell surface roughness, and a tremendous change in cell viscoelastic properties, such as a 3-5 fold increase in cell rigidity compared to live cells (using the micropipette aspiration technique to determine single cell rheology; our group has 20 years of experience in this technique). Moreover, if cell death was induced by lysis (cell membrane is permeable to extracellular calcium), the increase in $[Ca^{2+}]_i$ would be close to 10-100 times as much as the normal $[Ca^{2+}]_i$. However, the $[Ca^{2+}]_i$ increase observed in our experimental system was only 3-4 fold. Therefore, the elevation in $[Ca^{2+}]_i$ should not be the result of cell death. From our observations, there was no cell death during the $[Ca^{2+}]_i$ measurements.

Reviewer I: The methods that the authors indicate that they have used are inadequate for assessing cell death and do not explain why there would be a persistent and greatly elevated intracellular calcium in their experiments. Further, the calcium traces that they show look very unphysiological and are not representative of real time calcium data which almost always shows some fluctuations due to camera noise, cell movement, and physiological variation. If they have used extensive data smoothing programs, why? Their current calcium data do not resemble anything remotely like living cells.

Authors: As we have clearly stated, this was a group of cells and the data are from a mean value of 20-30 fibroblasts for each line and the standard deviation was 15-25% of the mean. Thus, we would not see the calcium level fluctuation. We took this approach so that we could present our data in one picture with two curves (control and experimental). This mean value represents the real time in a duration of 60 seconds (Fig. 6). All cells were alive; if they were dead, we would not see the granules moving nor would the cells elongate into the normal shape of fibroblasts when the cells were seeded into the chamber.

Reviewer I: The calcium data that are shown presumably are representative of single cells or groups of cells. How much variation of calcium data was there between experiments? What were the means and the standard deviations? Why does one cell population respond very differently to the other?

Authors: The results of $[Ca^{2+}]_i$ presented in the manuscript was the mean $[Ca^{2+}]_i$ of one single fibroblast calculated using the data from 20-30 fibroblasts for each cell line (ACL and MCL). The standard deviation of the data associated with the mean was 15-25% of the mean value. We cannot give the reason for the difference observed in the $[Ca^{2+}]_i$ between ACL and MCL fibroblasts. However, we do know that the cytoskeletal protein formation as well as the signal transduction pathways in ACL and MCL fibroblasts are not the same due to their intrinsic differences [22, 23]. Therefore, this intrinsic difference might lead to the difference in $[Ca^{2+}]_i$ that we observed here.

Reviewer I: The authors discuss the idea that formation of stress fibers might be important in the increased adherence of the cells; this would be related to the increased calcium and actin assembly. Did the authors consider assessing actin to directly measure this possibility?

Authors: We are currently attempting to perform quantitative measurement of cell substrate focal contact using total internal reflection fluorescence technique (TIRF). This technique can enable us to directly measure focal adhesion plaque formation. From this measurement, we will be able to induce the actin assemblage rate that would be more relevant to the measurement of cell adhesion behavior.

Reviewer I: It is unclear how the authors could have measured the relative amount of RNA at a single wavelength (260 nm) and not the usual ratio measurement.

Authors: We used the 28S and 18S ratio to quantify the total mRNA. As stated in the manuscript, the 260 nm was used to check for purity.

Reviewer I: The relationship between the observed differences between the two groups of cells needs an con-

vincing explanation since it is not open to any straightforward explanation or hypothesis. Thus, the central findings are purely descriptive and not suggestive of an interesting mechanism of adhesion control.

Authors: In **Discussion**, we mentioned the differences in the mechanisms due to intracellular Ca^{++} changes or some correlation to FN gene activities to explain the cell adhesive behavior between ACL and MCL. From these limited results, we cannot exaggerate our findings to suggest unknown mechanisms. Furthermore, additional studies would be required in cytoskeleton assemblage, integrin conformation changes, and other gene activities under inflammatory condition to get a clearer picture of cellular activities.

Reviewer I: Have the authors examined actin staining in the cells? Can they address the notion that the presence of stress fibers, and the relationship between calcium and actin may be important in explaining their observed phenomena.

Authors: We agree with the reviewer that the presence of stress fibers will be correlated to cell adhesiveness [24, 25] between Ca^{++} and actin. Recently, we did a stress fiber formation of ACL and MCL under inflammatory condition and found that inflammatory factors can significantly enhance stress fiber formation.

Reviewer II: Can the authors provide a dose response graph of all three inflammatory factors used in the study. The authors chose to use a specific concentration for each factor but how did they determine the concentration used.

Authors: The concentrations of inflammatory agents that we were using in our experiments were obtained from literature related to this research [33, 35, 36, 37].

Reviewer II: It would be more specific if the authors used a factor that is known to be anti-inflammatory so they can see its effects and compare them with those obtained with the three inflammatory factors used in the study.

Authors: We agree and are currently planning on using anti-inflammatory agents in the next phase of our study (e.g., using anti-TNF- α antibodies to block the effects of TNF- α on the migration and proliferation of ligament fibroblasts).

K. Otsuka: The authors use 30% FBS for the assay without any coating the scratched area. It is not clear why they use 30% FBS for the assay, because they use fibronectin for determining the adhesion force assay.

Authors: From the results of our preliminary studies, we found that migration assays under the influence of inflammatory agents incubated in medium supplemented with only 10% FBS usually took months to complete. Therefore, medium supplemented with 30% FBS were used in our experiments instead to enable the completion of the assays in a reasonable amount of time.

K. Otsuka: The authors conclude that the wound recovery will depend on the correlation among cell adhesion force, intracellular calcium concentration and integrin expression. They showed the adhesion force was decreased over one hour with C5a treatment. It is necessary to explain why they choose 4 hour incubation to detect the integrin gene expression.

Authors: We chose the 4 hour incubation period based on the response of cells to inflammatory agents. After incubation for 4 hours, cells respond to inflammatory agents fully and their gene expressions remain active. We had also tried incubating the cells for 2 hours, but found that not all inflammatory agents had activated gene expression responses in the cells.

D.B. Jones: The paper deals with cultured cells. There is no evidence presented to determine how similar these cells are to the ones *in situ*. Are these cells actually tendon fibroblasts and do they produce the same matrix proteins and respond in the same way to the growth factors present *in situ*? There are usually a lot of similarities, but also some important differences. One of these major differences is the medium. Serum contains many factors not found in plasma, hence the growth rate, differentiation and response to other factors is quantitatively and qualitatively different in many cases. One usual objection to cell cultures, that the surface reactions and formation of focal adhesions in culture are artifacts, is perhaps, in this case, not such a large one as in fibromas or wound healing similar structures are also seen as in culture; but where are the comparisons to *in vivo*? Can we therefore extrapolate the findings of this paper to wound healing *in vivo*?

Authors: We never claimed that the cultured cells were identical to the ones *in situ*. We understand that it is incorrect to assume there were no differences between cultured cells and cells *in situ*. However, we still believe that cultured cells can be a good model for use since they allow the investigator to control experimental conditions and the factors and variables the investigator want to study. From our previous gene expression studies [19, 34], we are confident that the cultured ligament fibroblasts that we use do produce matrix proteins (e.g., collagen and fibronectin) just like the ligament fibroblasts *in situ*. Our next phase will involve the direct use of ligament tissue *in situ* to study the behavior of cells in ligament tissue under inflammatory conditions.

D.B. Jones: From the complexity of the study, several statistical methods should have been applied at different levels used. From the choice of 3 wells for experiment and 1 for control, I assume that they think that several measurements on one plate is enough. In any case, 1 control well will not be enough. Unless they can show that, from

each of their data sets, normal distributions are found, then, some form of non-parametric analysis should be used. This would imply at least 5 replicates for each observation. However, in dealing with measurements of wound healing per dish (%recovery) how did they determine the precision of their measurements? How many times did they measure, what criteria did they use as it applies to every methodology they have used and in the analysis of the results in general.

Authors: Regarding the control experiments of the adhesion assays, we did have 2 control wells for each experiment that we performed. We collected adhesion data from one control well at the beginning of the experiment, and data from another control well were collected at the end of the experiment. We then group the data from both control groups to generate the mean adhesion force that we used as the control value in normalizing the results of our experimental groups. In addition, we also had 2 wells for each experimental condition. Due to time consuming nature of our single cell assay on two different cell types (ACL and MCL), it was not possible to perform more than 2 wells per treatment condition in one single experiment. To compensate for that, we performed 3-5 repeat experiments, and then grouped all data together to calculate the average adhesion force, the standard error of mean, and performed the unpaired Student's *t* test to determine if significant differences exist between the control and the experimental groups.

To ensure the precision of our wound recovery assay, at the very beginning of the project, we carried out a test where one single wound recovery measurement was repeated 4 times by 4 different trained experimenters. We determined that an approximate 10% variation exist in their measurements. This 10% variation is insignificant since it is smaller than the standard error of mean of our experimental results.

D.B. Jones: What was the precision of the measurement and the spread of results in cell adhesion assay? What are the error bars; the errors are surely much too small for the methods used?

Authors: The error bars were the standard error of mean of the data. Since we performed the assay with a large number of cells, the error bars were relative small.

D.B. Jones: How many cells were analysed for cell calcium assay? What statistical methods were used?

Authors: For each inflammatory condition, we determined the intracellular free calcium level for 20-30 ACL and 20-30 MCL fibroblasts. Student's *t* test was carried out to determine statistical significance between the averaged intracellular calcium concentration of cells from each inflammatory conditions.

D.B. Jones: It is a matter of discussion whether a real calibrated value can be obtained. For instance, how were background values subtracted from the raw images. Slicing too much or too little will significantly change the ratio values. Each time a background is taken, it will vary slightly. The cameras are not linear across their imaging area and the shade correction will depend on the amount of intensifier gain and video gain. For this reason, many workers use ratio rather than attempt to apply a calibration. How were the calibration standards used applied: measured in solution, if so, these values will be all wrong and the “real” free calcium values much higher? How many images were taken, was any averaging carried out and how quickly were the ratios made?

Authors: We did obtain the wavelength ratio of the Fura-2 probe during our assay as well as our standard curve where a relationship between calcium concentration and wavelength ratio was produced using a Calcium Calibration Buffer Kit II (Molecular Probes). We believe the use of this wavelength ratio can minimize the effect of background distortion. In addition, each assay was performed with 2-3 cells and the calcium level analysis was carried out with the same cells continuously for the entire duration of that experiment. The experiment was then repeated many times and ACL and MCL fibroblasts (20-30 each) were assayed for each condition. The average value from those 20-30 cells was then calculated and presented in the graph.

D.B. Jones: It is assumed that because a Ca^{++} ionophore will raise adhesiveness, any agent that raises Ca^{++} will be directly linked to this effect. Well, an ionophore raises Ca^{++} to a very high level, is usually lethal and the calcium levels are long lasting. It is known that cells can react not only to the level of calcium, but also its frequency. Receptors to $\text{TNF-}\alpha$ also have other transducing pathways other than calcium. Many factors which raise intracellular free calcium also have other more significant transducing pathways. Also, there are no data in this paper on the other possible mechanisms (tyrosine phosphorylation?).

Authors: We know that the raise in intracellular calcium level by ionophore was not lethal in our system since the raise was usually only 3-4 fold and we constantly monitor the viability of the cells we assay during the experiments. However, it is true that we have not looked at the effect of the frequency of intracellular variation, although we still firmly believe that intracellular calcium level is one possible pathway by which cell adhesiveness is affected.

Reviewer VI: Have the calcium levels been calibrated, this is very difficult to do in cells if at all, hence ratios are better. Information about the type of ionophore used and its concentration do not seem to match any ionophore I know about: an increase of only 3-4 fold in intracellular free calcium

seems to be extremely low unless they are buffering extracellular calcium levels? Did they use a microchannel plate intensifier coupled to a CCD chip (what exactly is a silicone intensified camera)? The results they show indicate a static level of Ca^{++} over a very long time, these results do not seem real as there is no noise that one gets from these cameras! Real cells fluctuate as well. Is the graph in nM? There are no experiments to link the Ca^{++} results, if these are real, with their other data.

Authors: As mentioned, we used a calibration buffer kit from Molecular Probes. This kit contains a series of bottles of various Ca concentrations, thus, a calibration curve was generated with a wavelength ratio. No ionophore was involved in this particular study, but was used elsewhere [24].

We are not camera experts, but we have been told that the camera can pick up individual photons. The results given are a mean value of 60 seconds of 20-30 cells. Any fluctuations would be smoothed out by the mean. The units are in nM.

Reviewer VI: The authors make several claims. They first measure tendon fibroblast adhesiveness (no mention is made of the error of the methodology used or how the adhesiveness relates to other studies using similar or other methods) and report an increase in “stickiness” after using certain inflammatory agents. They then claim that this increase in stickiness is due to an increase in intracellular free calcium, which is suggested to cause this increase through increased cytoskeleton assembly in ACL cells but not in MCL cells, which perhaps increased adhesiveness through an increase in fibronectin gene expression. Due to uncertainties of methodology as presented and lack of certain pieces of evidence in the chain of reasoning, how can the authors substantiate these claims?

Authors: Our study is not on tendon fibroblasts, only on ligament fibroblasts. Our claim is that inflammatory agents produces multiple factors within a cell and thus lead to changes in ligament cell adhesion. In other publications we present a study of actin filaments assembly and bridge protein between actin and integrin [25] and a study on how calcium ions (BAPTA and A23187) affect cell adhesiveness [24]. Some unpublished results indicate inflammatory agents facilitates actin filaments to form stress fibers which linked to integrin molecules to promote the cell to form adhesion plaques (significant increase of adhesion plaques formation contributes to increase cell adhesiveness). In other words, an increase in intracellular calcium concentration may contribute to increases ligament fibroblast adhesion.

Reviewer VI: The authors use the *t* test for all their results. There is no evidence presented that this is an appropriate

test (the data for cell calcium will certainly not be normally distributed). There is no evidence presented that their other data are normally distributed, hence the Student's *t* test is inappropriate and there are not enough experiments done to analyze the data by other methods (preferably non-parametric such as Mann Whitney or Wilcoxon). Some groups like to compare F values through some sort of ANOVA. The authors appear to have assumed that the *t* test is universally applicable to all forms of data! In this sort of experiment, at least 5 replicates of control and experimental protocol should be carried out, experiments performed at least 3 times, or some sort of statistical reasoning should be used.

Authors: As stated in the manuscript, a 2 factor ANOVA F test ($\alpha = 0.05$) was performed.

Reviewer VI: Cells need attachment to move. Lack of attachment factors does not increase motility. This has been presented in a number of papers, also in those the authors cite.

Authors: We agree cells need attachment to move. But with strong attachment, the cell cannot move. We want to emphasize that this study is a short term adhesion study. Migration under inflammatory factors increases adhesiveness and decreases migration rate because cells are strongly attached [31].

Reviewer VI: It is not clear that an increase in FN, or an increase in cytoskeleton assembly are the factors leading to a drop in motility, they can be associated certainly, but as pointed out above other factors can also be expected to play a big role in this process. For instance, it can be assumed that cell growth is another parameter in addition to recruitment and chemotaxis in wound healing. Differentiation of stem cells also seems to be a strong factor in wound healing.

Authors: We agree that cell growth is another parameter in addition to recruitment and chemotaxis in wound healing; our Figure 2 from cell proliferation studies addresses this issue. Differentiation from stem cells is a powerful technique, but isolating stem cells is a difficult process and will require a new study.

Additional References

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