The Presence of Peripheral Biomarkers of ACL Injury and Recovery in Cadets Attending the United States Military Academy at West Point

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Abstract

The Presence of Peripheral Biomarkers of ACL Injury and Recovery in Cadets Attending the United States Military Academy at West Point

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ACL injury in military members is estimated to be 10 times that of the civilian population. These injuries are associated with prematurely ending careers and decreasing mission-readiness. Biomarkers such as collagen precursors, metabolites, and inflammatory signals may be correlated to the development of Osteoarthritis. Cytokines are one biomarker secreted in response to joint tissue trauma and are secreted by senescent cells (SnCs), which are associated with pathologies relating to age and cell death. Circulating factors can cause a senescence associated secretory phenotype (SASP). This study successfully converted RNA samples from de-identified United States Military Academy cadets to cDNA following Standard Reverse Transcriptase (RT) protocol, and then performed Quantitative PCR using a 96-well plate in order to compare gene expression in the control and injured patients at similar time points. 15 genes, associated either inflammation, cell cycle and senescence, or the extracellular matrix were tested in this study. Gene expression was tested at time of injury and two years post ACL repair. At the time of injury, four genes were found to be upregulated in injured patients compared to the control. Two years post ACL injury, there was found to be an increase in IL-6 and PRG4 but a decrease in MMP9. This study was a pilot study. Using only samples from 10 cadets, it was done to see if it would be feasible to do on a larger scale in the future. The hypothesis was supported. It was shown that some gene expression was influenced in the ACL injured group.

Introduction

Anterior cruciate ligament (ACL) injury is one of the most common traumatic knee injuries sustained by relatively young and physically active individuals, with an estimated 250,000 ACL injuries and 175,000 ACL reconstructions occurring annually in the United States (Griffin et al, 2006). ACL injuries have been directly associated with the onset of osteoarthritis which is a leading cause of morbidity among various populations (Chu et al, 2012). Approximately 50% of people who have ACL repairs develop OA (Wasilko, 2016). ACL injury in military members (also referred to as tactical athletes) is estimated to be 10 times that of the civilian population (Aguero et al, 2022). These injuries are associated with prematurely ending careers and decreasing mission-readiness (Galvin et al, 2016).

Biomarkers such as collagen precursors, metabolites, and inflammatory signals may be correlated to the development of osteoarthritis (Carbone et al, 2016). It may be possible to develop accurate patient prognosis for recovery and risk of osteoarthritis based on the presence of biomarkers in blood before and after the time of injury (Carbone et al, 2016). Cytokines are one biomarker secreted in response to joint tissue trauma that impact the immune system (Jeon et al, 2018). They are secreted by senescent cells (SnCs), which are associated with pathologies relating to age and cell death (Jeon et al, 2018). Circulating factors can cause a senescence associated secretory phenotype (SASP) (2018).

This is a portion of a larger study that aimed to successfully convert PBMC derived fibrocytes (stem cells) to chondrocytes, and maintain them in a laboratory environment and to use said PBMC-derived chondrocytes to understand the pathology of joint injuries. Data will be shown for the SASP biomarker project. Our hypothesis of this project was that SASPs may impact peripheral blood mononuclear cells (PBMCs) to the extent that they could have a significant impact on the development of post-traumatic osteoarthritis. If SASPs affect these cells before they differentiate into chondrocytes and form cartilage tissue, it follows that after an injury, patients would struggle to heal in the appropriate amount of time. It is expected that biomarkers indicating the presence and activity of SASPs would be present after the time of injury. PBMCs collected from control subjects should be better suited for chondrocyte conversion than those derived from injured subjects due to the presence of a more pronounced SASP phenotype in the injured suspects.

Methodology

The RNA samples utilized for this study were pre-existing, de-identified samples. The original samples were collected prior to 2020 and consisted of blood samples collected at the time of injury and two years post ACL reconstruction. There was a control and injured group, which were matched by sex, weight, height, and age. Only ten RNA samples were used in this study, five from ACL injured subjects and five from that of matched controls. Prior to 2020, peripheral blood mononuclear cells (PBMCs) were purified from the blood samples, and RNA was subsequently extracted from said PBMC samples. This research used the purified total RNA samples and converted them to cDNA. Following this, quantitative polymerase chain reaction (PCR) was performed to compare gene expression in control and injured patients.

Reverse Transcriptase Reaction

The RNA samples were converted to cDNA following a standard Reverse Transcriptase (RT) protocol. A 10X Taqman RT buffer, 25mM Magnesium Chloride, Random Hexamers for priming the RNA, an RNase inhibitor, a Multiscribe RT and RNase-free water was added to each sample of RNA. The samples were then centrifuged and added to a thermal cycling block. The tubes were heated to allow the RT to function. After heating was completed, the cDNA was stored at -80 degrees Celsius. A 100ul assay was typically used for the RT reaction.

Component	Volume/Tube (µL)		Final Concentration
	10-µL Reaction	100-µL Reaction	
RNase-free water	see below[a]	see below[a]	–
10X TaqMan RT Buffer	1.0	10.0	1X
25 mM Magnesium Chloride	2.2	22.0	5.5 mM
deoxyNTPs Mixture	2.0	20.0	500 µM of each dNTP
Random Hexamers[b]	0.5	5.0	2.5 µM
RNase Inhibitor	0.2	2.0	0.4 U/µL
MultiScribe Reverse Transcriptase (50 U/µL)	0.25	2.5	1.25 U/µL
Total	6.15	61.5	—

Figure 1: "TaqMan Gold RT-PCR Kit." *Applied Biosystems*. 2006.

The protocol for the RT reaction was as follows:

25 degrees C, 10 minutes

37 degrees C, 30 minutes

95 degrees C, 5 minutes

4 degrees C, HOLD

Quantitative Polymerase Chain Reaction (PCR)

qPCR was conducted using a 96-well plate in order to compare the control and injured patients at similar time points. Each well received Master Mix (2x) , RNase free water, a gene primer and the cDNA of the respective sample. A 20ul assay was used. In each sample, 23 genes and one -RT control were analyzed. Each primer had two trials. The 96-well plate was sealed with a plastic covering and placed in the 7500 Fast Real-Time PCR system. The plate underwent 40 cycles. The system was held at 50 degrees Celsius for two minutes. In TaqMan PCR, the temperature is initially raised in order to denature the double-stranded cDNA. The signal from the fluorescent dye on the 5' end of the TaqMan probe gets quenched by the non fluorescent quencher on the 3' end. After this happens, the temperature is lowered and the primers and probe combine with their targets. New strands are formed by the Taq DNA Polymerase. When the Taq DNA Polymerase comes in contact with the Taqman Probe, it separates the dye from the quencher. More dye molecules are released during every cycle of PCR, and this increases the fluorescence proportional to what was amplified.

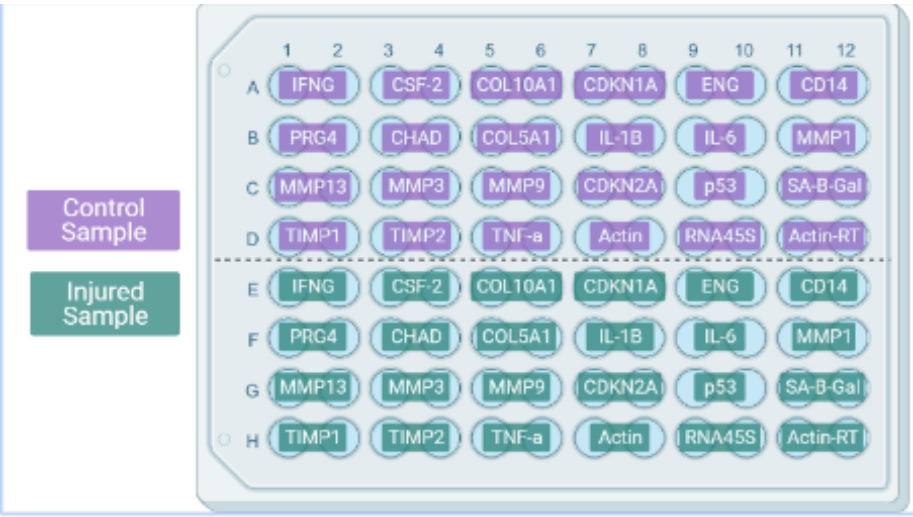

Figure 2: 96 well plate setup with the injured and controlled sample genes

qPCR Quantitation

The ΔΔCt method was used to analyze the fluorescence. This is comparative quantification. The ΔΔCt method compares results from experimental samples with both a calibrator and a normalizer. With this method, Ct values for the gene of interest in both the test sample (control and injured) and calibrator sample are adjusted in relation to a normalizer gene. CT stands for the Cycle Threshold of the sample. It is the cycle number in which the fluorescence generated by the PCR machine becomes detectable. ΔΔCt is the difference between the ΔCt values of the injured sample and the control sample. The lower the Ct value, the higher expression of the gene.

Tested Genes

The genes tested in this study include inflammation associated genes (Il-6, TNF-a, IL-1B, and IFNG), Cell cycle and senescence associated genes (CDKN2A andCDKN1A, p53, and SA-B-Gal), and genes associated with the extracellular matrix (COL5A1, ENG, TIMP1, PRG4, TIMP2, CHAD, and MMP9). Below is a chart detailing the functions of each gene.

IL-6	Proinflammatory cytokine, correlates with osteoarthritis severity
TNF-a	Inflammatory cytokine made by macrophages/monocytes during acute inflammation
IL-1B	Pyrogenic cytokine
IFNG	Cytokine involved in innate/adaptive immunity, primary activator of macrophages
CDKN2A	Tumor suppressor, cellular senescence marker
CDKN1A	Cyclin kinase inhibitor, cellular senescence marker
p53	Tumor suppressor, cell cycle arrest
SA-B-Gal	Indicator of senescent phenotype
COL5A1	Type V collagen, contributes to bone and interstitial matrix
ENG	TGF beta co-receptor, involved in chondrogenesis
TIMP1	Metalloproteinase (MMP9) inhibitor, degradation of extracellular matrix
PRG4	Chondrocyte proteoglycan
TIMP2	Metalloproteinase (MMP2) inhibitor, degradation of extracellular matrix
CHAD	Chondroadherin, cartilage matrix protein
MMP9	Matrix metalloproteinase, collagenase, wound repair

Figure 3: Genes analyzed in this study and their associated functions

Results

Four genes were found to be upregulated in injured patients compared to the control. At time of injury, these four genes were: IL-6, CDKN1A, CDKN2A, and COL5A1 (Figures 4 and 6). However, two years after injury and repair, injured patients showed a downregulation in MMP9 and an upregulation in PRG4 and IL-6 (Figures 5 and 7). At time of injury, there was a 4.11 fold change in IL-6 of the injured sample, a 3.94 fold change in the injured sample of CDKN2A, a 3.12 fold increase of CDKN1A in the injured sample, and a 3.70 fold increase in COLA51 in the injured sample (Figures 4 and 6). Two years post injury there was found to be a 2.01 fold increase in IL-6, a 3.47 fold increase in PRG4 and 0.44 decrease in MMP9 (Figures 5 and 7). Fold changes were relative to a control value of 1, and the fold changes in the other genes were not found to be statistically significant. A T-test was performed at time of injury and two years post ACL repair. A p-value of 0.03 was found at the time of injury for ENG1 and a p-value of 0.005 was found for MMP9 two years post repair. These were the only significant p-values, which makes sense due to the small sample size.

Fold Change			
Control	Injured	SEM	Gene
1	4.11	1.87	IL-6
1	1.00	0.50	TNF-a
1	0.99	0.50	IL-1B
1	0.98	0.49	IFNg
1	3.94	1.97	CDKN2A (p16)
1	3.12	1.56	CDKN1A (p21)
1	1.10	0.55	p53
1	0.92	0.46	SA-B-Gal
1	3.70	1.85	COL5A1
1	1.81	0.91	ENG
1	1.22	0.61	TIMP1
1	1.07	0.53	PRG4

Figure 4 : Time of injury

Fold Change			
Control	Injured	SEM	Gene
1	2.01	0.71	IL-6
1	1.22	0.39	TNF-a
1	0.88	0.19	IL-1B
1	1.85	1.18	IFNg
1	1.91	0.66	CDKN1A (p21)
1	1.93	0.76	CDKN2A (p16)
1	1.61	0.37	p53
1	1.30	0.29	SA-B-Gal
1	1.76	0.68	COL5A1
1	1.75	0.47	ENG
1	1.18	0.19	TIMP1
1	3.47	0.82	PRG4

Figure 5 : Two years post ACL repair

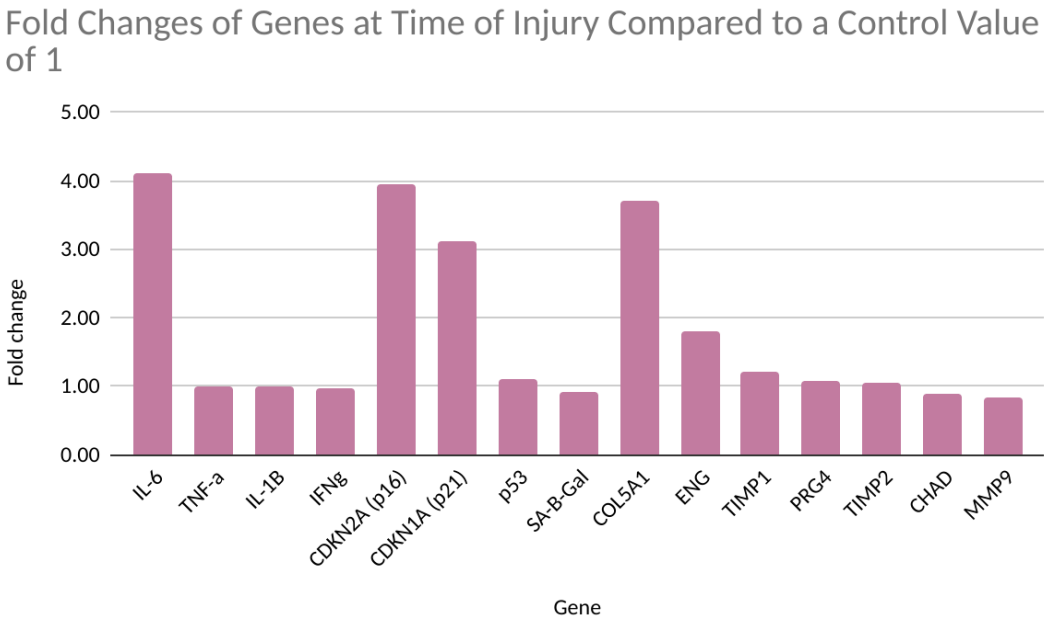

Figure 6: Chart depicting fold changes of gene expression in injured samples at time of injury

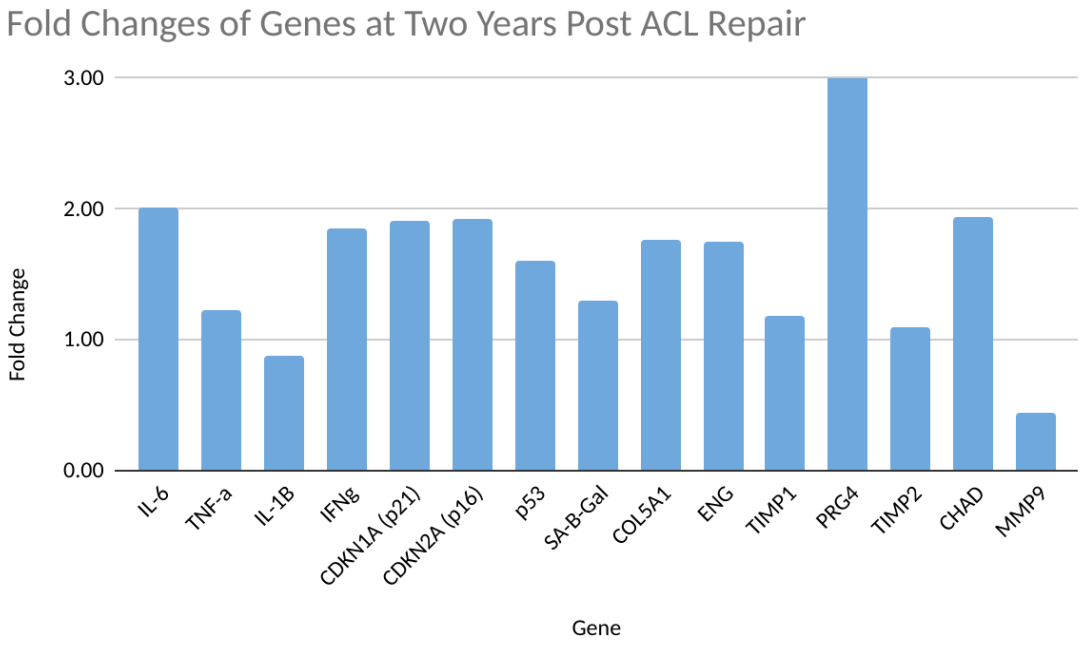

Figure 7: Chart depicting fold changes of gene expression in injured samples two years post ACL repair

Discussion

This study found that some investigated genes which were associated with inflammation were shown to be upregulated during initial ACL injury. This shows the chondrocytes did not maintain normal function during injury. Likewise, two years after injury and repair, other sample proteins were either up or downregulated which shows chondrocytes did not regain normal function even after repair. At the time of injury, only four pairs (injured and matched control) were analyzed. This was because for one of the matched injured and control samples, when the RNA was purified, there was a very low RNA yield and it gave no good data. Five pairs were analyzed two years post repair. This study was a pilot study. Using only a small sample size, it was done to see if it would be feasible and done on a larger scale in the future.

Conclusion

This study aimed to see how Senescence Associated Secretory Phenotypes may impact peripheral blood mononuclear cells (PBMCs) and what impact SASPs will have on the development of post-traumatic osteoarthritis. It was hypothesized SASPs will impact PBMCs and patient prognosis. The study was done to see if inflammatory biomarkers were changing after an ACL injury. The hypothesis was supported because it was shown that some genes had increased expression and some had decreased expression in the ACL injured group. Possible biomarkers of long or short term injury were discovered. This is a preliminary conclusion because not many samples were used in this study. A small sample size was a limitation to this study. With only ten people (20 samples), the results may not be a reliable indication of biomarkers in a larger population. In the future, more samples —at least 50 subjects—should be used. Samples can also be analyzed at the time of reconstruction, six months later, and one year after, in order to get a more accurate reading of when the biomarkers changed.

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