

DIFFERENCES IN COLLAGEN GENE EXPRESSION IN MALE AND FEMALE ANTERIOR CRUCIATE LIGAMENT INJURED ATHLETES

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Abstract. Anterior Cruciate Ligament (ACL) injuries occur two to eight times more frequently in women than men. However, it remains unclear whether gender differences exist in ACL at the molecular level. Using reverse transcript polymerase chain reaction (RT-PCR) with histological analysis, the gene expressions of collagen types I and III of fibroblasts from ACLs of 17 male and 17 female athletes with acute ACL tears were studied. Female athletes were found to have a significantly lower gene expression of collagen I. No significant difference was found in type III collagen gene expression between male and female athletes. This finding may help us to explain the higher incidence of ACL injury in female athletes from the molecular perspective.

(Biol.Sport 26:255-261, 2009)

Key words: Anterior Cruciate Ligament (ACL) - Collagen - Gene Expression - RT-PCR

Introduction

Anterior cruciate ligament (ACL) injuries occur two to eight times more frequently in women than men [2,3,10]. Intrinsic differences between males' and females' ACLs have been considered to be the major factor contributing to the disparate incidence of ACL injury between males and females [5]. Many studies have evaluated gender differences in ACL at the gross anatomical level. Difference in the alignment of ACL between males and females has been reported [5].

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Cadaveric studies have shown that the width of the ACL and the ratio of the width of the ACL to the width of the femoral intercondylar notch in females were significantly smaller than those in males [9]. However, it remains unclear whether gender differences exist in the ACL at the molecular level. This information may be critical not only because the properties of ACL are determined by its molecular components, but also because ACL cell functions are regulated at this level.

Collagens are major components in the extracellular matrix of the ACL. Type I collagen is the most abundant component in ligament. It comprises approximately 90% of the collagen in this tissue. Type III collagen is the second most abundant collagen in ligament. It accounts for approximately 10% of the collagen in this tissue [1]. The gene expression level of these two components in ACL will profoundly affect the components and properties of this tissue. The goal of this study is to determine and compare the gene expressions of type I and III collagens in male and female ACL fibroblasts using reverse transcript-polymerase chain reaction (RT-PCR). The hypothesis is that there exists a gender difference in the ACL fibroblast collagen gene expression.

Materials and Methods

ACL samples were harvested from 17 male and 17 female athletes with acute ACL tears (33.5 ± 13.3 days for female and 16.4 ± 7.8 days for males from injury to operation) undergoing ACL reconstruction. The age for the female patients was 24.2 ± 12.7 years and 18.0 ± 5.3 years for the males. In order to preserve their pre-injury properties, all samples were taken from an area of the ACL far from the rupture ends during surgery. Informed consent and our Institutional Review Board ethics committee approval were obtained for this study.

Each sample also underwent histological analysis in order to ensure that no post-injury effects involved the tissues to be used for further RT-PCR study. ACL samples were fixed in 10% buffered formalin and embedded in paraffin. Five micron-thick sections were cut, and stained using hematoxylin and eosin (H & E) staining. Ten micron-thick serial sections were cut for RNA extraction. The target areas with well-organized collagen fibers and fibroblasts, but no inflammatory cells or blood vessels, were cut macroscopically with a fine needle referring to the microscopic observation of the morphology of the H & E stained sections [4].

The isolated tissues were digested with proteinase K overnight and total RNA from the fibroblasts was extracted with TRIzol reagent according to the manufacturer's instruction. One-step RT-PCR analyses were performed using primers for collagen I ($\alpha 1$), collagen III, and β -actin (as an internal control).



Sequences of primers and PCR product fragment sizes are summarized in Table 1. PCR cycle numbers were carefully selected within the linear zone of each gene. PCR products were electrophoresed in 4% agarose gels and densitometric analyses were performed using the image analysis software AlphaEase™ (Alpha Innotech Corp. San Leandro, CA). The resultant data were expressed as a ratio to the internal control value [7].

Table 1

Sequences of primers and product fragment sizes used for RT-PCR

Target gene	Forward primer	Reverse Primer	Size (bp)
Collagen I	GGCTCCTGCTCCTCTTAGCG	CATGGTACCTGAGGCCGTTTC	132
Collagen III	CCAGTACAAGTGACCAACTA	TAGCACCATTGAGACATT	182
β-Actin	GGTGTCCAGAGGCGCTCTT	TCCACGTCGCACTTCATGAT	97

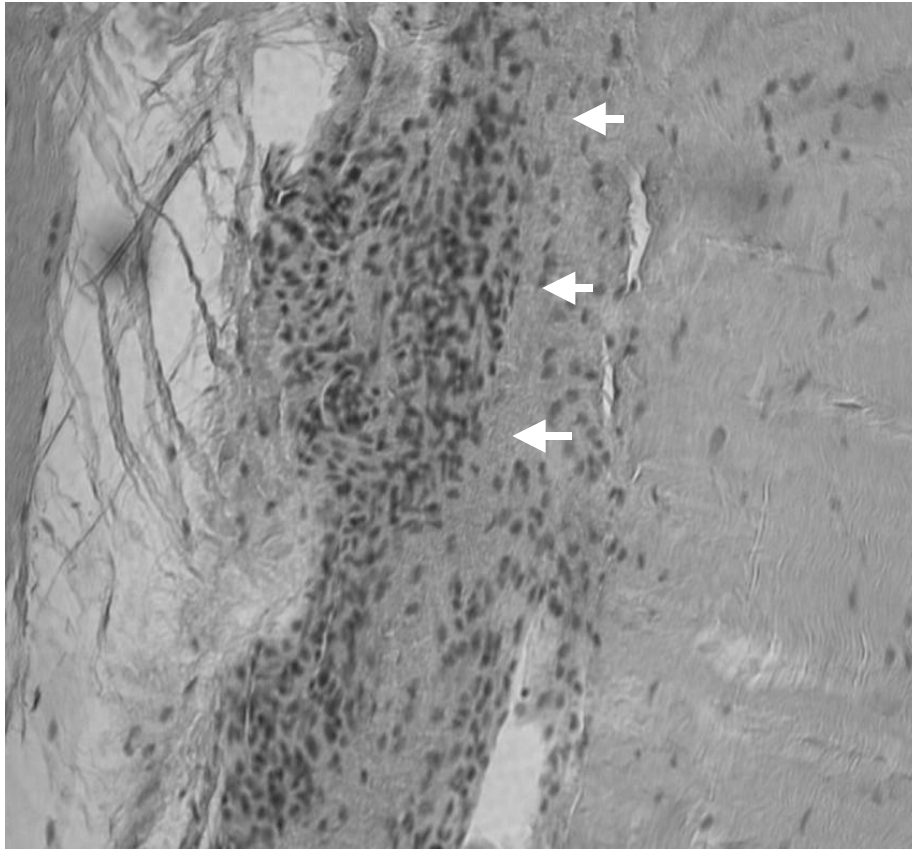
Student T-test was employed for statistical analysis of the relative mRNA amount of collagen types I and III of groups of male and female athletes. All differences were considered to be significant at a probability greater than 95% ($P < 0.05$).

Results

Although our samples were selected from non-injury sites of torn ACLs using visual observation, histology studies showed focal inflammatory infiltrates in some samples. However, the filtrations were not uniform and well-organized collagen fibers and fibroblasts were still preserved (Fig. 1). In every sample, only the areas with well-organized collagen fibers and fibroblasts were used for further RT-PCR study.

The relative expression of collagen I (normalized to internal control) was 1.57 ± 0.58 in males and 1.14 ± 0.75 in females. The relative expression of collagen III was 0.21 ± 0.18 in males and 0.34 ± 0.26 in females. There was a significant difference between males and females in collagen I ($P < 0.05$), but not in collagen III ($P > 0.05$) (Fig. 2).



**Fig. 1**

H & E staining of a typical injured ACL sample. Inflammatory infiltrates, mainly lymphocytes and plasma cells were found in some areas (arrows). However, organized collagen fibers and fibroblasts were also preserved (right portion)

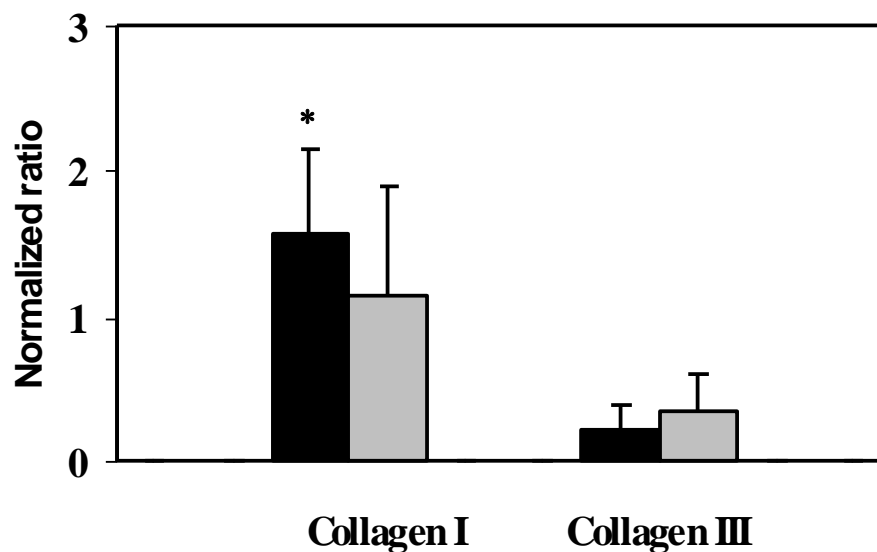


Fig. 2

Gene expression of collagens I and III. There is a significant difference between males (black) and females (grey) in gene expression of collagen I, but not of collagen III

Discussion

Using RT-PCR with histological analysis, the gene expression levels of collagen types I and III in ACLs from male and female ACL injured athletes were examined in this study. It was found the female athletes had significantly lower levels of type I collagen gene expression, as compared to those of the male athletes. However, no significant difference was found in type III collagen gene expression between male and female athletes.

Our finding that female athletes have lower gene expression of collagen I—the most abundant extracellular matrix molecule in ACL inferred that there may be a gender difference between the ACLs of males and females at the molecular level, which may be responsible for the higher incidence of ACL injury in female athletes. It is well known that type I collagen is the major component in ACL, which is considered to be responsible for the resistance to tensile mechanical

loadings. Therefore, a lower level of type I collagen in female ACL may cause weaker mechanical properties and higher incidence of injury to this tissue under mechanical loadings compared to those of males. The lower gene expression level of type I collagen in female ACL observed in this study may be a result of the effects of female sex hormones, unique to females. Though previous studies suggested that estrogen could increase type I gene expression in fibroblasts, a recent work has shown that under mechanical loadings simulating daily activities, the physiological level of estrogen can significantly reduce the gene expression of type I collagen in cultured ACL fibroblasts [7]. Their results were consistent with our findings in this study.

The purity of cells was critical for this study. Previous literature has shown that inflammatory cells are absent in the ruptured end of injured ACL, especially within three weeks after injury [8]. In our histology study, we confirmed the absence of infiltrated inflammatory cells in our samples taken from non-injury sites of torn ACL. Blood vessel endothelium cells are also common in ACL. Those non-fibroblast cells, though they produce little collagen, contribute significantly to β -actin and total RNA. Our preliminary study showed that the inclusion of those cells caused major errors in the RT-PCR results. However, the non-uniform distributions of inflammatory cells and blood vessels, as well as the local preservation of well-organized collagen fibers and fibroblasts shown in our histology study, made it possible to obtain pure fibroblasts using selective dissections in each sample of the H & E stained sections.

It was important to avoid the effects of ACL repair and remodeling after injury in this study. Previous studies showed that ruptured ends of torn ACL underwent repair and remodeling beginning at about eight weeks after rupture [8]. Animal studies also showed that ACLs of immobilized rabbits underwent degeneration beginning at about six weeks after immobilization [6]. In order to avoid the effects of degeneration or remodeling, several measures were adopted. First, we strictly chose patients with acute injury within two months. Second, the ACL samples were harvested far from the torn ends during surgeries. Third, histology analysis was done to assure that only the areas without obvious degeneration or remodeling were used.

In summary, the gene expressions of collagen types I and III of fibroblasts from ACLs of 17 male and 17 female athletes with acute ACL tears were studied. Female athletes were found to have a significantly lower gene expression of collagen I. This finding may help us to explain higher ACL injury incidence in female athletes, from the molecular perspective.



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Accepted for publication 1.03.2007

Acknowledgments

We thank Shelsy Utuph for her assistance in slides preparation and staining. We also would like to thank Carolyn Adams for her assistance in the preparation of this manuscript

