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Original Article

Claudin-5 (tight junction) expression level changes in achilles tendon healing

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Abstract

Aim: This study aimed to reveal the relationship between changes in Claudin-5 expression and the duration of healing in Achilles tendon injury.

Material and Methods: 18 Achilles tendons of Wistar-Albino rats were used in the study. Rats were divided into 3 groups as 6 rats in each group, group 1; sham group, group 2; tendon repair group (sacrificed after 3 weeks), group 3; tendon repair group (sacrificed after 6 weeks). Immunohistochemically, the tendons were stained with Claudin-5 and the degree of staining with light microscope was rated between 0 and 3. The obtained scores were compared with Kruskal Wallis test and Posthoc analysis.

Results: The scores were 0.5 ± 1 (0-1) in group 1.1 ± 1 (1-2) in group 2 and 1.5 ± 1 (1-2) in group 3. A statistically significant difference was found between the groups (p = 0.026). In the posthoc analyzes, there was a significant difference between group 1 and 3, but there was no significant difference between groups 1 and 2 and between groups 2 and 3.

Conclusion: The expression of claudins is regulated by many factors, including hormones, various cytokines, and epithelial-mesenchymal transition-related transcription factors. In this study, the increase in the expression of Claudin-5 was noticed in proportion to the progress of primary wound healing. This relationship may be a part of the repair mechanism. The role of claudin levels in intercellular passage is crucial for function as it is important for cell signaling. Achilles tendon healing can be attributed to a laboratory parameter such as claudin. This can help to understand the recovery rate and can help early return to work or sport. We believe that as a laboratory parameter Claudin-5 may be useful in the evaluation of tendon healing.

Keywords: Claudin-5; healing; tendon; achilles; rat.

INTRODUCTION

The junctions which extend adjacent to the cell membrane side surface's apical end are called Tight junctions. Barrier function and containment function are their two main functions: Regulation of ions's passage, water and macromolecules through paracellular spaces are the barrier function; and it also applies to cancer cells (1). Cell polarity are provided by surrounding function (1,2). Exchange and signaling are formed by Tight binding proteins which regulate proliferation, cell growth, differentiation and dedifferentiation (2).

There are a lot of different proteins in the tight junctions of the epithelium, endothelium and myelinated cells. Ocular and claudin are two main components of tight junction filaments. Claudin is a family of proteins with more than 20 members (1-4). Claudins are barrier forming proteins which make paracellular permeability arrangements. They can form especially small pores or provide water permeability (1-4). The claudins are thought to be the main determinants of the epithelial cells' permeability properties. Too many claudins identified in mammals and they are divided into eight subgroups; expression is made by a tissue-specific manner and are scattered throughout epithelium's all cell-cell contact regions. The function and tissue specificity of the claudins are well-known. At tight junctions multiple claudin isoforms are expressed concurrently (3-5). Loss of cell polarity is a function of epithelial-mesenchymal transition, a function that is clearly regulated by tightly linked proteins (6).

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In our work on rats, which are most similar to human beings biologically, differences in expression of Claudin-5 between tenosites resulting from physiological changes that took place over time were detected. Thus, it was aimed to reveal the changes of Claudin-5 expression in tendon injury and to relate it with the healing period.

MATERIAL and METHODS

During the experiment, rats kept at normal room temperature and humidity were fed with standard pellet feed and tap water for 10/14 hours in a light / dark cycle of light, 3 subjects per cage. Prior to the experiment, the weights of the animals were measured and group distributions were divided into 3 equal groups so that the weights of the animals were close to each other.

-Group 1 (Sham) (6 rats): the group that was sacrificed on the same day; the skin and subcutaneous tissues were passed through the incision of the right achilles tendon with 3 cm incision and the Achilles tendons were removed on the same day.

-Group 2 (6 rats): the group that was sacrificed after 3 weeks; After passing through the skin and subcutaneous tissues over the right achilles tendon with 3 cm incision, a full fold incision was made from 0.5 cm proximal of the calcaneus adesion site of the achilles tendon. Repair performed using the modified Kessler-type technique with 4.0 polypropylene suture (polypropylene, Doğsan, Istanbul, Turkey). Then the skin and subcutaneous tissues were closed. Achilles tendons were removed at the end of 3rd week.

Group 3 (6 rats): the group that was sacrificed after 6 weeks; After passing through the skin and subcutaneous tissues over the right achilles tendon with 3 cm incision, a full fold incision was made from 0.5 cm proximal of the calcaneus adesion site of the achilles tendon. Repair performed using the modified Kessler-type technique with 4.0 polypropylene suture (polypropylene, Doğsan, Istanbul, Turkey). Then the skin and subcutaneous tissues were closed. Achilles tendons were removed at the end of 6th week.

In the course of this study, the compliance with principles of Care and Use of the Laboratory Animals and the animal rights were provided. All procedures were carried out in accordance with ethical rules and the Ethical Committee of the Experimental Animals of the Faculty of Medicine approved this study (Date: 30.01.2018, Number: 82678388/04).

Surgical Procedure

Operations were performed under general anesthesia. Ketamine 90 mg/kg (Ketalar; Eczacıbaşı, Istanbul, Turkey) and xylazine hydrochloride 3 mg/kg (Rompun; Bayer, Leverkusen, Germany) are injected intraperitoneally. After appropriate anesthetic conditions, animals were shaved with care to avoid damaging the skin with a razor blade and disinfected with polyvinyl pyrrolidone-iodine (Batticon©, Adeka, Samsun, Turkey). The surgical field was covered with sterile compresses. Surgical procedure was applied sequentially (Figure 1). After the surgical procedure, all animals were regularly treated with wound dressing every day.

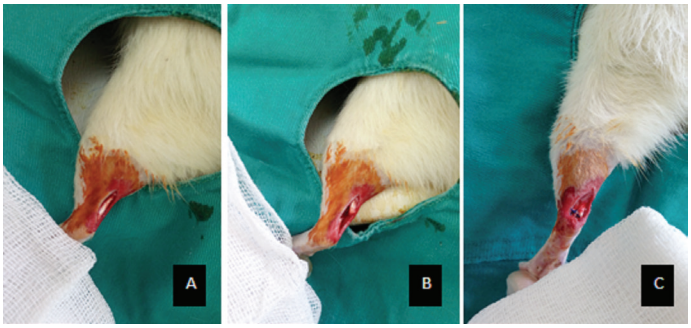

Figure 1. A. Exploration of Achilles tendon. B. Achilles tenotomy was made from 0.5 cm proximal of the calcaneus adesion site. C. The Achilles tendon was repaired with 4.0 polypropylene sutures using modified Kessler-type technique.

Histopathological Examination

Samples were taken from the tissues and then sections with a thickness of 5µ were taken on the poly-laminated slide. The sections for immunohistochemical study were stained with Leica Bond-Max IHK staining device (Vision Biosystems, Melbourne, Australia) using Claudin-5 (Genetex / GTX37465 Polyclonal / 1: 300) primer antibody.

Slayts were evaluated with light microscope. The ratio of cells with cytoplasmic membrane positivity is considered. And diveded four categories as; 0 staining, 1+ staining, 2+ staining, or 3+ staining and the categories were defined on the basis of no staining (none), 1% to 10% staining (light), 11% to 50% staining (moderate), and greater than 50% staining (severe), respectively (Table 1) (Figure 2-4).

Table 1. Staining levels in groups. 0: none, 1: light, 2: moderate, 3: severe

Group 1	Group 2	Group 3
1	2	2
1	2	2
1	1	2
0	1	1
0	1	1
0	1	1

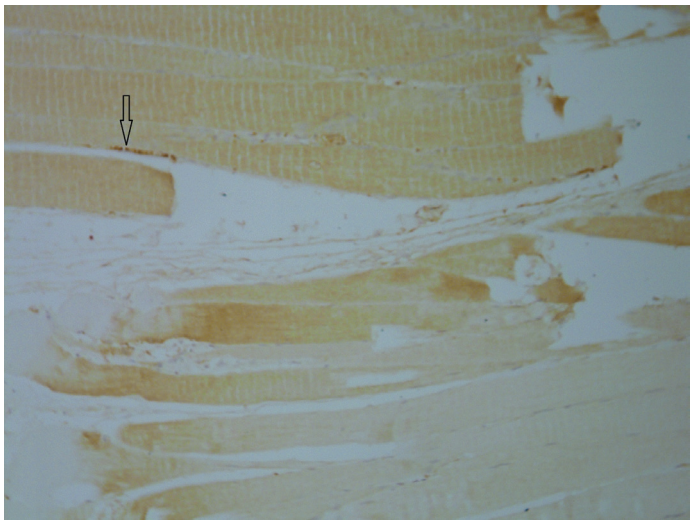

Figure 2. Linear appearance on cytoplasmic membrane with Claudin-5, 1 positive staining (Claudin-5 X 200)

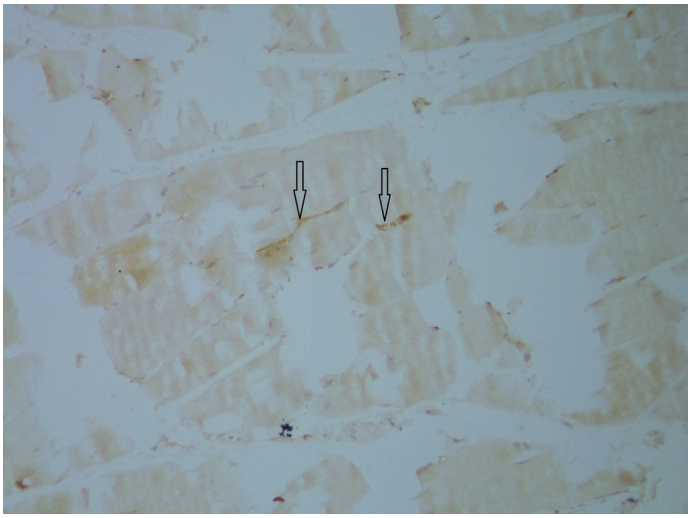

Figure 3. Linear appearance on cytoplasmic membrane with Claudin-5, 2 positive staining (Claudin-5 X 200)

Figure 4. Linear appearance on cytoplasmic membrane with Claudin-5, 3 positive staining (Claudin-5 X 200)

Statistical analysis

For statistical analysis SPSS (SPSS Inc., Chicago, Ill., USA) 13.0 was used. Descriptive statistics were given as median ± ICR (min-max). The staining difference between the three groups was compared with Kruskal Wallis test and Posthoc analysis. $p \leq 0.05$ is considered as significance level.

RESULTS

Main scores for groups were as: 0.5 in group 1; 1.33 in group 2; 1.5 in group 3 and according to group 1 the scores were significantly higher in the other groups. Between groups 2 and 3, there was no significant difference.

When the results are examined, the scores were 0.5 ± 1 (0-1) in group 1, 1 ± 1 (1-2) in group 2 and 1.5 ± 1 (1-2) in group 3. Difference between the groups were statistically significant ($p = 0.026$). According to the posthoc analyzes, there was only a significant difference between group 1 and 3, the other results were not significantly different between the groups.

DISCUSSION

Tight junctions (TJs) extend adjacent to the cell membrane side surface's apical end. Exchange and signaling are formed by Tight binding proteins which regulate proliferation, cell growth, differentiation and dedifferentiation (2). Paracellular transportation is regulated by Tight junctions serving as a barrier. These are barriers which are expressed in the body (7). There are a lot of different proteins in the tight junctions of the epithelium, endothelium and myelinated cells. Ocular and claudin are two main components of tight junction filaments. Most of (TJs) contain different types of claudins to form complexes (8). Claudin family consist of 27 members which first identified in TJs (9-11). Claudin-5 is a transmembrane protein known to form TJs between endothelial cells (10-12). Too many claudins identified in mammals and they are divided into eight subgroups; expression is made by a tissue-specific manner and are scattered throughout epithelium's all cell-cell contact regions. Claudins are differentially expressed in various tissues possing different properties and functions (13). Factors which are responsible for adjusting the expression of claudins are hormones, various cytokines, and epithelial-mesenchymal transition-related transcription factors (7). The function and tissue specificity of the claudins are well-known. At tight junctions multiple claudin isoforms are expressed concurrently (3-5). Loss of cell polarity is a function of epithelial-mesenchymal transition, a function that is clearly regulated by tightly linked proteins (6). Howewer, the function of Claudin-5 in the achilles tendon healing is unknown and in our knowledge there is no study about this perspective in the literature. Examining the molecular mechanism of achilles tendon healing can lead to early treatment goals and approaches.

The Achilles tendon is the largest and strongest tendon, and is also the most commonly ruptured tendon in the body. This is a frequent injury during leisure-time sporting activities, in the 30-50 years age group with a male predominance (14-16). Several factors increase the risk of rupture including male gender, use of steroids or fluoroquinolones, poor tendon vascularity, tendon degeneration and prior rupture on the contralateral side. The healing process is an active dynamic process that starts from the moment of injury and consists of three phases, namely inflammation, proliferation and maturation-remodeling, which are intertwined with each other and which are not able to draw successive boundaries with a complex set of effects (17-19). Recovery is taking place with developing histopathologic events during this process. Studies have shown a positive correlation between TJs and healing, and decreased TJs with oxidative stress (20). In this study, the increase in the expression of Claudin-5 was noticed in proportion to the progress of primary wound healing. This relationship may be a part of the repair mechanism. The role of claudin levels in intercellular passage is crucial for function as it is important for cell signaling.

Claudin-5 is especially expressed in endothelial TJs such as vascular endothelium of the blood-brain barrier to form TJs that inhibit the passage of macromolecules (8). Furthermore, although Claudin-5 is in morphologically normal blood vessels with Claudin-12, it functions as a small molecular sieve, as shown by Claudin-5 knockout mice showing impaired permeability to small molecules, especially smaller than 800 Da (21). In the murine kidney and pancreas, Claudin-5 was shown to be decreased in age21. In a study on the kidney, a new model of healing was introduced after exposure of the oxidative stress to the kidney epithelial cell line. In this study it is found that expression and localization of Okludin, Claudin-1 and Claudin-2 contributed to functional changes during recovery after oxidative stress exposure (20). It has been emphasized here that it is important to recapture the junction that occurs during healing, resulting in a modified tight junction, and producing long-term functional outcomes that potentially alter tissue physiology.

Decreased Claudin-5 levels have been demonstrated in the hearts of dystrophin/utrophin-deficient mouse model and humans with Duchenne muscular dystrophy and Becker muscle dystrophy. This occurs at a very early stage of disease progression with the reduction of the physiological and histological indication of heart failure (dystrophin) (7,22). In the cardiomyopathy, Claudin-5 promoter was one of only four genes, found to be hypermethylated in conjunction with reduced Claudin-5 gene expression (12). However, these studies show that changes in Claudin-5 regulation may be one of the most common alterations in human heart failure (23). Additionally, studies have highlighted the importance of future research on the role of TJs in damage and repair of other organ systems such as brain, gastrointestinal system and the lung following the conditions of oxidative stress (20,24).

In this study, we found that the level of Claudin-5 exspression increased during the healing process of the Achilles tendon, which indicates that tissue integrity is important during healing. The evaluation of the healing stage in the Achilles tendon injury is very important in early return to work or sports. Evaluating the level of claudin as an important laboratory parameter can be guiding about healing, follow-up and treatment stage. These data suggest that Claudin-5 may represent a new therapeutic goal in different pathologies.

CONCLUSION

In conclusion, claudins have different properties and functions and are expressed in different ways in various tissues. We demonstrated that Claudin-5 is widely expressed in the healed tendon and that the role of Claudin-5 in tendon healing may be a subject that needs to be investigated in new clinical studies. Documentation of the expression of other claudin proteins will be a more detailed and complete understanding of how selectivity and expression are produced.

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