ORIGINAL ARTICLE

MORPHOLOGIC CHANGES IN THE INJURED ANTERIOR CRUCIATE LIGAMENT (ACL) CORRELATED WITH THE EXPRESSIONS OF PROTEIN S100 AND NFAP: MAJOR THERAPEUTIC IMPLICATIONS OR A WRONG GATEWAY TOWARDS IMPROVING ACL RECONSTRUCTION ?

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ABSTRACT

Objectives: We examined the histoarchitecture of the anterior cruciate ligament [ACL] stumps in different stages of evolution after injury and we analyzed the correlation with the expressions of protein S100 and neurofilament associated protein [NFAP]. **Materials and Methods:** A total number of 102 cases of ruptured ACL was included in our study. Immunohistochemistry was performed for protein S100 [56 cases] and NFAP [46 cases] in order to evaluate the nervous structures located in the ligament and in the synovial tissue. **Results:** Protein S100 and NFAP were identified in the quasi-normal ligament, in the disrupted ligament and in the synovial tissue. Protein S100 was positive in the nervous structures of the synovial tissue. The nervous fibers from the ligament also reacted to protein S100. The synovial tissue reacted to NFAP at the level of the small nervous fibers and free nerve endings. In the ligament, NFAP expression was predominantly found in the small nervous fibers and in the free nerve endings. The quasi-normal ligament showed a greater number of NFAP positive free nerve endings. **Conclusions:** We found significant differences between the quasi-normal and the disrupted ligament regarding the expressions of protein S100 and NFAP. The low number of nervous structures identified using the two markers may suggest a time- and injury-dependent loss of nervous fibers following post-rupture ligament remodeling. The persistence of nervous fibers in the remnant stumps of the ruptured ACL is not 'absolute' and may not always ensure a successful recovery of the patient after ACL reconstruction.
Key Words: Anterior cruciate ligament [ACL], protein S100, neurofilament associated protein [NFAP], remnant stumps, nervous fibers, free nerve endings.

INTRODUCTION

Few data from the literature considers the histopathologic changes that occur in partial and complete rupture of ligamentous structures. At the time being, few information is available regarding different bone lesions or inflammatory processes that may affect the adjacent ligamentous tissue. Many studies have focused on the diagnosis and management of ACL injuries, resulting in data of great interest for the clinical practice [1, 2]. Despite these aspects, ACL has little been subject to research studies, although some experimental models on rodents are currently available [3]. ACL injuries affect the surrounding structures of the knee and are associated with a decrease in the patients' life quality. It has been demonstrated that ACL traumas affect the meniscus, induce cartilage damage and determine the occurrence of osteoarthritis [4, 5]. Also, ACL ruptures are difficult to treat and are in need of close medical follow up after surgical intervention hence the works that have focused on improving the treatment of ACL injuries and the patients' long term outcome [6, 7, 8, 9]. ACL ruptures are common lesions diagnosed in physically active individuals but may also be age-related and result in motion instability even after reconstruction [10]. Usually encountered in young individuals, ACL injuries seem to be more frequent in women [11, 12, 13]. It has also been evidenced that ACL ruptures are associated with disruption in the strength of the quadriceps muscle following ACL reconstruction [14]. It is well known that ligaments are active structures that possess the ability to undergo modifications in different pathologic conditions. Tissue destruction may be positively correlated with the remodeling response after injury. Matrix metalloproteinases appear to stimulate angiogenesis and inflammatory reactions in the synovium of patients with rheumatoid arthritis [15]. These metalloproteinases are not expressed in the synovial tissue in normal conditions [15]. Also, various metalloproteinases play a critical role in the process of ligamentization [16]. In case of ligament reconstruction, they seem to facilitate cell migration, proliferation and angiogenesis in the synovium [16]. Other lesions, such as osteoarthritis, are accompanied by tissue remodeling due to inflammation and angiogenesis in the synovium [17, 18] and inhibition of angiogenesis seems to promote cartilage repair in the affected joint [18].

Chang et al [19] have demonstrated that ACL rupture leads to post-traumatic osteoarthritis within decades after the injury. The mechanisms that lead to this disease imply gene expression changes that need to be further investigated [19]. ACL possesses a great healing potential through the formation of new blood vessels that occur in both the synovium and in the ligament itself in case the remnants are preserved [20]. The vessels found in the synovium present CD34+ endothelial cells while the remnants contained CD34+ cells which were identified amongst the collagen fibers and were characterized by hyperplastic stellate and stromal spindle shape cells [20]. Besides the formation of new blood vessels, the balance of soluble factors found in the synovial fluid is disrupted in traumatic conditions. Bigoni et al [21] have demonstrated an increase in IL-6, IL-10, IL-8 and TNF-alpha levels while the levels of IL-1ra and IL-1beta were strongly decreased. Despite the inflammatory changes encountered in the chronic phase of meniscal trauma, it seems that the increased level of pro-inflammatory cytokines does not influence cartilage degeneration [21].

It is well known that ACL injuries lead to post-traumatic osteoarthritis and cartilage degeneration [22, 23]. Additionally, different mechanic abnormalities have been encountered in ACL reconstructs and early biomarkers of cartilage degeneration have been evidenced [22]. Experimental models have shown that ACL rupture leads to an increase in the joint laxity along with motion reduction [3]. Also, it appears that a greater anterior knee laxity may represent a risk factor for ACL traumas depending on different particularities of ACL and bone geometry [24]. Re-stabilization after rupture seems to be ensured by the formation of condrophytes and osteophytes that will eventually lead to osteoarthritis [3]. Research studies in this field have shown that ACL is characterized by a poor repair capacity [25, 26]. This aspect may be supported by the differential levels of lysyl oxidases and matrix metalloproteinases in ACL compared to other ligaments [26]. Partial or complete rupture of the ligament determines changes in its morphological features. In response to stress stimuli, the histoarchitecture is modified through the presence of spindle shape and fibroblastic type cells [25]. Also, mechanoreceptors are detectable in the tibial remnant only they are less frequent [27] along with proprioceptive neuroreceptors that may ensure re-inervation [27]. Cabuk et al [28] have shown that mechanoreceptors from ligaments are usually located in the immediate vicinity of the bone insertion area and express protein S100. These mechanoreceptors play different roles in the regulation of knee motion and seem to be more numerous in cruciate ligaments compared to other ligamentous structures [28]. Moreover, the remnants of the human anterior cruciate ligament seem to hold healing capacities due to the blood vessels present in the synovium [29]. Unlike other ligaments, the human anterior cruciate ligament is characterized by the retraction of the remnants [29, 30]. Retraction after rupture is partially caused by the presence of myofibroblast-like cells, although their number is decreased in the early phases of response to injury [30]. ACL rupture is also accompanied by changes in the intercondylar notch which leads to stenosis [31]. Increase in fibroblast density, the expression of alpha smooth muscle actin and angiogenesis are common healing characteristics for the anterior cruciate ligament and for dense connective tissues in general [30]. The particularities found in the anterior cruciate ligament after rupture refer to the formation of a synovial cell layer on the surface of the rupture ends, the lack of tissue between the disrupted edges, and the presence of an epiligamentous repair phase [30]. ACL constructs tend to reproduce the native ligamentous tissue but with certain histologic and molecular differences [32]. Native ligamentous tissue is more abundant in fibrocartilaginous proteins while constructs contain a greater quantity of cellular-associated proteins and less collagen fibers [32].

The anterior cruciate ligament that was subject to trauma undergoes four reparative phases with different morphological features identified by Murray et al [30] in the article entitled "Histological changes in the human anterior cruciate ligament after rupture", namely: the inflammation phase, the epiligamentous regeneration phase, the proliferation phase and the remodeling and maturation phase.

The aim of our study is to evaluate the morphologic changes in complete and partial ACL ruptures correlated with the expression of protein S100 and NFAP that may be useful as predictors of the patients' outcome. An immunohistochemical profile of the injured ACL compared to normal specimens may help understand the molecular mechanisms that lead to the occurrence of posttraumatic changes in the affected ligamentous tissue. Markers that have a predictive role in ACL ruptures may be useful in order to improve the clinical and surgical approach of these cases and to ensure a better management of the patients.

MATERIALS AND METHODS

A total number of 102 specimens of ruptured ACL were included in the study. Tissue specimens were fixed in 10% buffered formalin for 48 hours and paraffin embedded. Five micrometers thick sections were performed from each paraffin block and sections were mounted on silanized slides. Sections from each case were stained with routine hematoxylin and eosin method for histopathologic examination using Axiocam 506 color, Zeiss, Jena, Germany. The specimens were classified according to the four evolution phases evidenced by Murray et al. following ACL rupture [30].

Immunohistochemistry was performed for protein S100 [56 cases] and NFAP [46 cases] Immunohistochemical techniques included heat-induced epitope retrieval with Bond Epitope Retrieval Solution

1, a ready-to-use, pH 6.0 solution [Leica Biosystems, Newcastle Ltd, Newcastle UponTyne NE 12 8EW, UK] for 20 minutes, in case of the first antibody. The enzyme pre-treatment with Bond Enzyme 1 for 10 minutes was used for the second antibody. Endogenous peroxidase blocking was performed with 3% hydrogen peroxide for 5 minutes. Neurofilament, NF 200 KD [monoclonal, clone N52.1.7, ready to use, Leica Biosystems, Newcastle UponTyne, UK, 30 minutes incubation time] and S100 [polyclonal, ready to use, Leica Biosystems, Newcastle UponTyne, UK, 30 minutes incubation time] were used as primary antibody. The Bond Polymer Refine Detection System was used for visualisation. As chromogen 3, 3 diamino-benzidine dyhidrochloride was applied for 10 minutes and hematoxylin for 5 minutes, as counterstain. The entire immunohistochemical procedure was performed with Leica Bond- Max [Leica Biosystems, Newcastle UponTyne, UK] autostainer.

Immunoreactivity was estimated as positive in the cells that exhibited a cytoplasmic expression for both antibodies. A comparative analysis was performed between the areas that contained disrupted ligament, quasi-normal ligament and synovial tissue. The evaluation of positive elements was made at magnification ×400. Axiocam 506 color, Zeiss, Jena, Germany was applied for microscopic evaluation and image acquisition.

RESULTS

The majority of the examined specimens of ruptured ACL were classified in the remodeling/maturation phase according to the criteria described by Murray et al. [30], after routine hematoxylin and eosin examination.

Specimens containing injured ACL mimicked a dense irregular connective tissue morphology and were frequently associated with the presence of an adjacent quasi-normal ligamentous tissue.

The expressions of protein S100 and NFAP were identified in both the synovial tissue and in the ruptured ligamentous tissue. The extension of the expression for both markers was poorly represented. However, we noticed a more intense expression in case of protein S100 compared to NFAP in the examined specimens. Protein S100 was negative in 14 out of 56 examined cases and NFAP was negative in 17 out of 46 examined cases.

Protein S100 was detected in the large and small nervous fibers of the synovial tissue with a strong and mild to low intensity. The large nervous fibers exhibited a predominantly homogeneous expression pattern which was restricted to the cytoplasm of the Schwann cells [Fig. 1a] while the nervous fibers of small caliber presented a heterogeneous cytoplasmic distribution that ranged from mild to low. We also noticed a mild-low expression for protein S100 in the free nerve endings, occasionally located in the immediate vicinity of the small vessels from the synovial tissue [Fig. 1b].

The ligamentous tissue exhibited a positive reaction for protein S100 in the large and small nervous structures, although their number has proved to be quite low [approximately 2-3 nervous structures/microscopic field at 400x magnification in case of small nervous fibers and 1-2 nervous structures/microscopic field at 400x magnification in case of the large ones]. The expression of protein S100 was quantified as being intense in case of the large nervous fibers and mild to strong in case of the small ones.

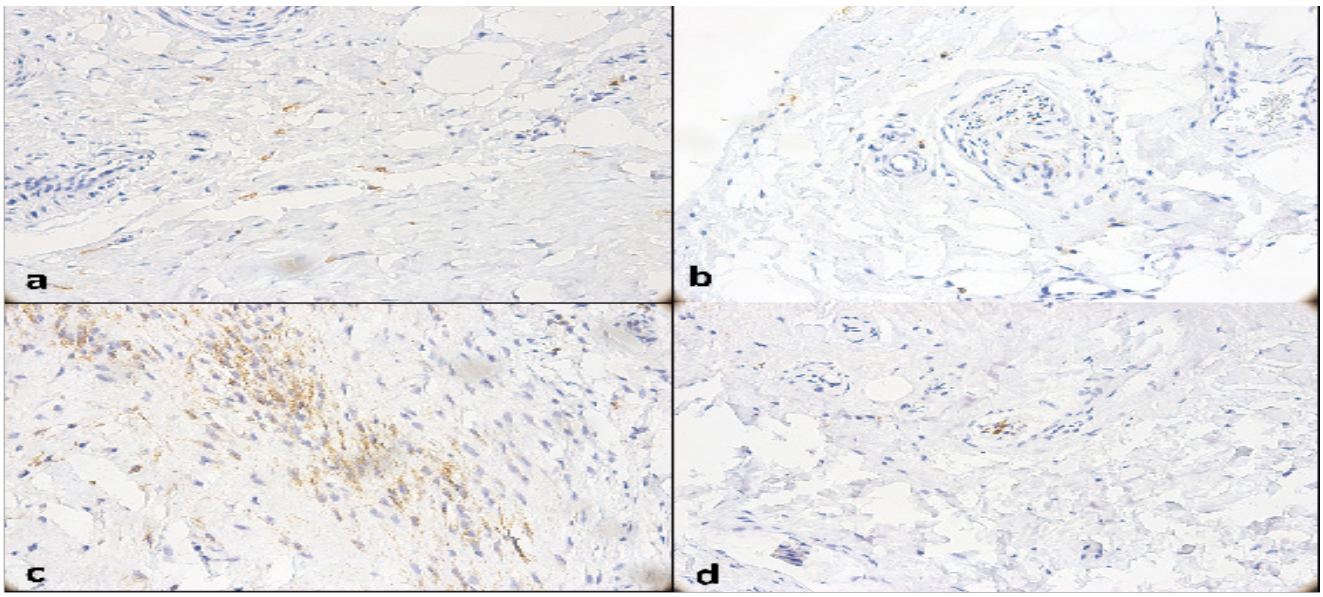

Figure 1. *The expression of protein S100 in the quasi-normal ligament, in the disrupted ligamentous tissue and in the synovial tissue. Note the intense cytoplasmic reaction in the Schwann cells in case of the large nervous fibers* **[a]** *and the presence of positive free nerve endings located in the immediate vicinity of the small vessels* **[b]**. *Note the presence of intensely positive large nervous fibers in the quasi-normal ligament* **[c]**. *The disrupted ligament is characterized by the presence of a lower number of nervous fibers exhibiting a mild-strong reaction for protein S100* **[d]**. *400× magnification.*

The pattern of expression was rather homogeneous and restricted to the cytoplasm of the Schwann cells, although a slight heterogeneity was evidenced in case of the small nervous structures. Both the intensity and distribution of protein S100 were quite similar in the disrupted ligament and in the adjacent quasi-normal one [Fig. 1c,d]. However, a more intense expression was noticed in the quasi-normal ligament [Fig. 1c], but no striking differences were evidenced in comparison to the disrupted areas.

NFAP expression was also noticed in the small nervous structures and free nerve endings of the synovial tissue. In comparison to the expression of protein S100, NFAP expression was poorly represented [Fig. 2a,b]. In the areas that contained ligamentous tissue we noticed a greater number of free nerve endings that were positive for NFAP [3-4 positive free nerve endings/microscopic field at 400x magnification or areas containing clusters of free nerve endings and small caliber nervous fibers] [Fig. 2c]. NFAP expression in the large nervous fibers was lower compared to protein S100, and was mostly restricted to the cytoplasm of a few Schwann cells with a mild-low intensity [Fig. 2d]. We noticed that the areas containing quasi-normal ligament were characterized by the presence of a greater number of positive nervous structures [free nerve endings and small nervous fibers] compared to the areas containing disrupted ligamentous tissue and synovial tissue.

DISCUSSIONS

The healing potential of ACL during reconstruction was studied using a panel of markers that include NFAP and protein S100 [33] although further research in the field is needed. Studies conducted on ACL injuries have evidenced the presence of morphologically normal mechanorecetors in the ruptured ligaments [34, 35]. It is well documented that the preservation of the remnant stumps following ACL rupture plays an important role in ACL reconstruction [34]. Bali et al have shown a positive expression for protein S100 in the free nerve endings in the residual stumps in specimens of ruptured ACL [34]. Unlike protein S100, NFAP expression is present in the persistent residual proprioceptive fibers of ruptured ACL [35].

The majority of the specimens of ruptured ACL used in our study were positive for protein S100 and NFAP but the number of identified nervous structures has proved to be quite low. Most of the specimens were positive for protein S100 in comparison to NFAP. NFAP positive expression was evidenced in a lower number of cases and was mostly restricted to the small nervous structures of the ligament and of the synovial tissue. It is well documented that nervous structures ensure the proprioceptive function of ligaments [36, 37] and their preservation post-rupture comes as a necessity in order to ensure the patients' life quality after surgical intervention.

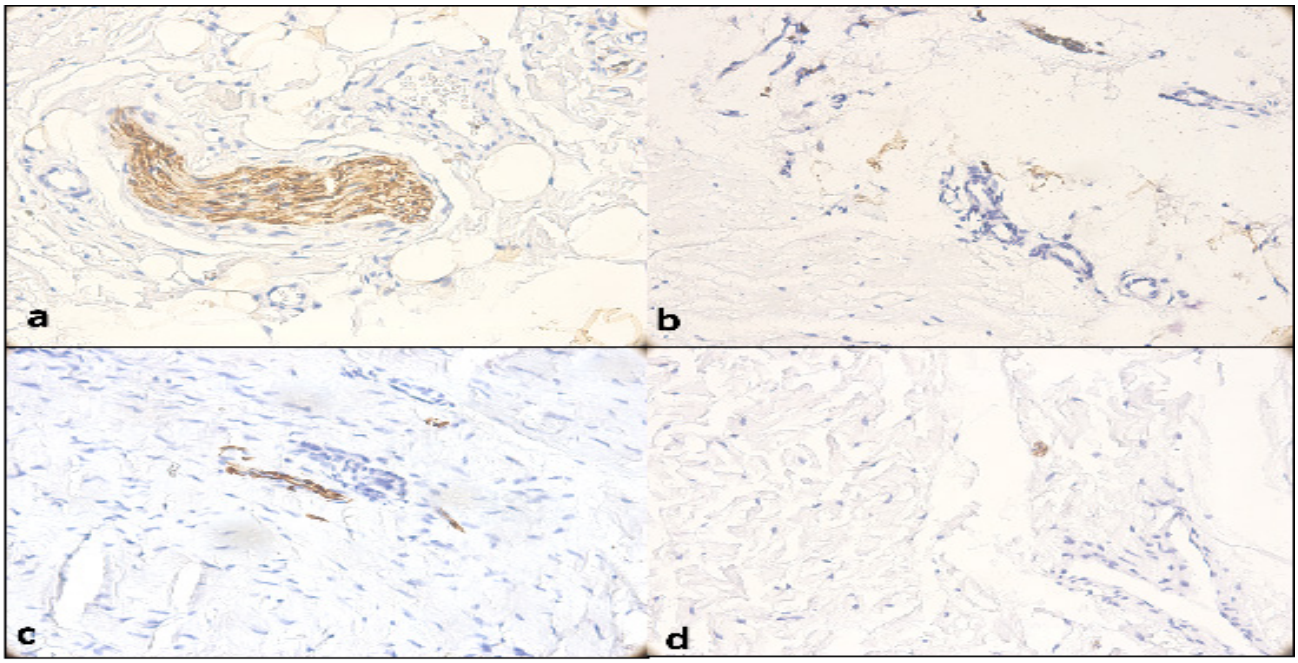

Figure 2. *The expression of NFAP in the quasi-normal ligament, in the disrupted ligamentous tissue and in the synovial tissue. Note the presence of positive small nervous structures* **[a]** *and the presence of large nervous fibers that exhibit a focal, mid-low reaction which is restricted to the cytoplasm of a few Schwann cells* **[b]**. *The quasi-normal ligament was characterized by the presence of a greater number of small nervous fibers and free nerve endings* **[c]**. *In the disrupted ligament, the number of small nervous structures was lower compared to the quasi-normal areas* **[d]**. *400x magnification.*

It may be possible that the remodeling process following rupture determines the loss of both small and large nervous structures. These findings may partially explain the difficulties that occur after ACL reconstruction and the patients' long term recovery. The quasi-normal ligament seems to possess a larger number of nervous structures compared to the disrupted one. These observations may suggest a gradual loss of nervous fibers as the remodeling process evolves. The differences evidenced in the number of nervous structures found in the quasi-normal and disrupted ligaments and also within the areas that contained the same type of ligamentous tissue may suggest that the loss of nervous fibers is both a time-dependent and an injury-dependent process. According to the previous data on ACL changes post-rupture, we support the implications of associated diseases such as the arthrosis of the knee that may lead to more severe ACL damages and may determine a further loss of nervous fibers along with the remodeling process.

In a study conducted by Dhillon et al, it has been evidenced that the number of proprioceptive fibers found in the injured ACL was increased when ACL remnants were adherent to PCL [35]. Despite the frequent association of ACL and PCL ruptures, very few data is available regarding the immunohistochemical analysis of PCL specimens. In a similar manner to ACL, the preservation of mechanoreceptors and nervous fibers plays an important role in maintaining the proprioceptive function of PCL [36, 37]. Also, it seems that the mechanoreceptors found in PCL specimens belonging to ostheoarthritic patients are morphologically normal [38]. The immunohistochemical profile of PCL shows a positive reaction for NFAP in the axon while the periaxonal cells exhibit a positive reaction for S100 and Vimentin [39]. Both the nerve fibers and the free nerve endings were positive for S100 and NFAP [39].

The specimens used in our study did not include PCL samples, which is why further research studies are needed in the field of knee ligaments in order to fully understand the complex changes that characterize ACL changes after rupture. Could PCL undergo similar modifications regarding the loss of nervous structures in a similar manner to ACL? If this is true then surgical intervention applied for ligament reconstruction becomes even more difficult. The preservation of nervous fibers and free nerve ending is necessary but a possible loss of these structures should be taken into consideration in patients diagnosed with ACL rupture.

CONCLUSIONS

Our study partially confirms the previous data available in literature regarding the expressions of protein S100 and NFAP in the ruptured ACL. It appears that the number of large and small nervous fibers decreases in the disrupted ligament compared to the areas containing quasi-normal tissue. However, the large and small nervous fibers along with the free nerve endings were morphologically normal. It seems that, remodeling post-rupture does not lead to a disruption of the nervous structures, instead it determines their numerical loss. We conclude that the preservation of nervous structures in the remnant stumps of ruptured ACL is a possible but not a necessary fact, as the specimens included in our study did not present a spectacular distribution for the expressions of protein S100 and NFAP. These aspects may explain the difficulties that occur in ensuring a similar function of the ligament as it has possessed before rupture. The surgical applicability regarding the preservation of the nervous fibers in the remnant stumps of ruptured ACL should not be considered an 'absolute' status of the injured ligament and is in need of further research studies. At the time being, very few studies have focused on the expressions of protein S100 and NFAP in specimens of ruptured ACL. Considering the frequent ACL injuries that mostly occur in the active population we believe that a close study of the number and morphology of the persistent nervous structures present in the remnants may improve the surgical approach of ACL reconstruction.

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