ORIGINAL ARTICLE ISSN: 2455-5274

Immunohistochemical Study of Matrix Metalloproteinase-9 in Medial Collateral Ligament Epiligament in Rat Knee after Grade III Injury

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Date of Submission: ***13-11-2016***
Date of Acceptance: ***19-12-2016***
Date of Publishing: ***04-07-2017***

ABSTRACT

Background: In the present study, we conduct an immunohistochemical analysis to evaluate the expression of matrix metalloproteinase-9 (MMP-9) in the epiligament (EL) after grade III injury of the medial collateral ligament (MCL) in an experimental model of a rat knee. **Methods:** Twelve 8-month old male Wistar rats were used in this study. Three animals were used as controls, while the remaining nine underwent grade III injury of the MCL. The MMP-9 immunoreactivity was evaluated on the 8th, 16th and 30th day after injury. **Results:** We observed an intensive expression of the enzyme in all periods after injury in contrast with the control group. We also discovered that the main source of matrix metalloproteinase-9 was localized in the epiligament tissue. Immunoreactivity was highest and homogeneously distributed on the 8th day and gradually diminished, concentrating on the EL-ligament border and the perivascular zones on the 30th day. **Conclusion:** We present the first immunohistochemical study of the expression and distribution of the enzyme MMP-9 in the EL of the MCL and track the changes in enzyme activity on the 8th, 16th and 30th day after damage.

Keywords: *Matrix metalloproteinase-9, medial collateral ligament epiligament, grade III injury, immunohistochemical study.*

INTRODUCTION

The epiligament is a thin layer of loose connective tissue which covers the ligaments and plays an important role as a supportive structure participating in their nutrition, regeneration and especially the process of healing after acute injury. According to Bray et al. the epiligament is a "surrounding adherent connective tissue removed simultaneously with the ligament but which was grossly distinguishable from ligament tissue proper".[1] The medial collateral ligament (MCL) of the knee joint is a commonly injured ligament used in order to compare normal healing and non-healing in ligaments.[2,3] Type I collagen is the main structural component in both normal and injured ligaments;[4,5] type III and V collagen have also been reported to participate in their structure.[6,7] Type I is primarily responsible for the ligament tensile strength and is apparently key for the long-term properties of the tissue matrix,[4,5] while type III is needed for ligament repair.[7] Healing of ligament injuries happens by the formation of scar tissue, similar to healing in other soft connective tissues, rather than regeneration.[8,9] MCL injuries can be classified intro several grades: grade I, which involve a few fibres of

the MCL with only localized tenderness (opening: 0–5 mm); grade II injuries include interruption of more fibres with generalized tenderness (valgus opening: 5–10 mm); grade III injuries are complete MCL tears with resultant medial joint laxity to valgus stress (>10 mm opening).[10]

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Matrix metalloproteinases (MMPs), also known as matrixins, are zinc-dependent endopeptidases known for their ability to cleave and rebuild extracellular matrix (ECM) constituents such as collagen, elastin, gelatin and casein,[11] as well as non-matrix proteins.[12] Normally, up-regulation of MMPs production by various cells such as fibroblasts, neutrophilic leukocytes is an indicator of abundant tissue remodelling during wound healing, bone resorption, morphogenesis, etc. On the contrary, excessive production and secretion of MMPs is a hallmark of many pathological processes, e.g. inflammation, metastasis, angiogenesis that involve connective tissue remodelling and degradation.[12] MMPs family members have been classified into different but closely related subgroups with fairly close characteristic features.[3] This classification recognises collagenases (MMP-1, 8, 13, 18), gelatinases (MMP-2 and 9), stromelysins (MMP-3, 10, 11), elastases (MMP-7, 12), and membrane type MMPs (MT-MMPs, MMP-14, 15, 16, 17) and a group of unnamed members.[13] Gelatinases play an important role in many extracellular mechanisms involved in tissue remodelling, repair and basement membrane degeneration and it has been proved that they are involved in the healing of acute tears, tumour invasion, neovascularisation and metastases. MMP-9 is also known as gelatinase B and primarily cleaves denatured collagen as well as intact collagen type IV found in the structure of basal membranes.[11] Other substrates of MMP-9 include collagen V, VII, X and XIV, gelatin, entactin, aggrecan, elastin, fibronectin, osteonectin, and plasminogen.[11]

Currently, there is no literature data concerning the localization of MMP-9 in the EL during the period of healing after acute ligament injury. Accordingly, the aim of this study was to conduct an immunohistochemical study of the expression and localization of MMP-9 in the EL of the MCL on the 8th, 16th and 30th day after grade III injury in an experimental rat knee model.

MATERIALS AND METHODS

Twelve 8-month-old male Wistar rats, ranging in weight from 350 g to 400 g, were used for this study with the approval of the University Committee on Animal Resources. All animals received humane care in compliance with the "Principles of laboratory animal care" formulated by the National Society for Medical Research and the "Guide for the care and use of laboratory animals" prepared by the National Institute of Health (NIH publication No. 86–23, revised 1996). The experimental animals were divided in four groups, each group containing three subjects. The last group of animals underwent no injury and served as intact controls.

Nine rats were anesthetized by intraperitoneal injection using a mixture of 5mg/kg b.w. Xylasine and 45mg/kg b.w. Calypsol. Their hind limbs were then shaven and washed with betadine solution. Under sterile conditions a small incision (10 mm) was made in their skin on the knee joint of the left hind limb over the site of MCL. After skin incision, the overlying connective tissue was dissected to expose the MCL. Then, a 1-mm gap in the mid-substance was surgically created and the gap was left without suturing. The skin incision was closed using 5-0 Ethibond suture. The right knees of the animals were preserved intact. After surgery, the rats were allowed free cage activities. No infections or complications were observed. The animals were sacrificed as follows: three on the eight, three on the sixteenth, and three on the thirtieth day after surgery, with intracardiac injection of Thiopental. The injured ligaments were carefully removed without disturbing the scar region.

Immunohistochemistry for MMP-9

Tissue samples were fixed in 10% buffered formalin for 24 h and after that were dehydrated in increasing concentrations of ethanol. Alcohol was removed using cedar oil until samples became translucent. Samples were rinsed in xylene and embedded in paraffin. Paraffin sections, 5 µm in thickness, were mounted on adhesive slides. Sections were deparaffinized, dehydrated and washed in Tris-buffered saline (pH 7.4) with 0.05% Tween-20 (TTBS). Endogenous peroxidase activity was blocked with 3% H2O2 for 10 min at room temperature. The sections were rinsed in

TTBS and nonspecific binding sites were blocked with Super Block for 5 min. Primary polyclonal antibody for MMP-9 at a dilution 1:1000 were added and sections were incubated overnight at 4ºC, rinsed in TTBS and incubated with biotinylated goat anti-rabbit IgG for 10 min at room temperature. Sections were rinsed as before and incubated with streptavidin-HRP for 10 min at room temperature. Antibody binding was visualized using 3,3´-diaminobenzidine tetrahydrochloride as chromogen for 10 min. Sections were counterstained with Mayer's haematoxylin, dehydrated in graded series of ethanol, cleared in xylene and cover-slipped with Canada balsam. As a negative control, the primary antibody was replaced with isotype-matched control antibody. All sections were stained immunohistochemically under the same conditions.

Photomicrographs of representative fields of the immunohistochemical staining were obtained using Olympus CX 21 microscope fitted with an Olympus C5050Z digital camera (Olympus Optical Co, Ltd).

RESULTS

Our observations in control animals revealed an interesting contrast between the distribution of MMP-9 in the EL and the ligament substance. A positive immunohistochemical reaction for the enzyme was noted predominantly in the EL as opposed to the ligament substance. The adventitia of the blood vessels in the EL also exhibited a distinct positive staining for MMP-9. In the ligament substance, intense staining for MMP-9 was noted in the fibroblasts [Figure 1a]. On the eight day after injury, the immunohistochemical study demonstrated an intensive reaction for MMP-9 across the entire EL scar tissue, characterised by a mostly homogenous pattern of distribution, which was significantly higher that the one observed in the group of control animals [Figure 1b]. Thus, it became apparent that the EL is the main donor of MMP-9 in the EL-ligament complex during this early period of healing and tissue recovery.

The highly positive immunohistochemical expression of MMP-9 on the sixteenth day was again localised predominantly in the EL granulation tissue. However, staining was less intense and the homogenous pattern of distribution described on the eight day after injury was slightly altered, with higher expression being noted in the perivascular zones in the EL and lower expression in the surrounding EL tissue. The positive reaction for the enzyme in the ligament substance was very low [Figure 1c].

On the thirtieth day after MCL injury an intensive reaction for MMP-9 was detected on the border between the EL and the ligament, again with predominant expression of the enzyme in the EL, particularly in the perivascular zones [Figure 1d]. These data revealed the key role of MMP-9 during ligament healing after acute injury.

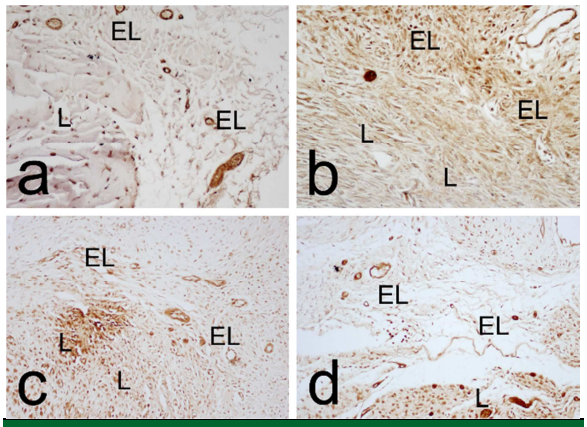

Figure 1: (a) MMP-9 expression in the epiligament (EL) and ligament (L) controls; (b) MMP-9 expression on the 8th day after injury; (c) MMP-9 expression on the 16th day after injury; (d) MMP-9 expression on the 30th day after injury. Magnification x 200.

DISCUSSION

Although the evolution of changes in the ligament substance after acute injury has been well studied, the changes undergone by the EL and its role in ligament repair are yet to be fully elucidated.[14-16] Therefore, in the present study, we describe the changes in the immunohistochemical expression of the enzyme MMP-9 in the EL-ligament complex throughout the initial 30 days after grade III injury of the MCL.

MMPs play a key role in the breakdown and removal of ECM molecules from the tissue. However, it has been increasingly evident that the breakdown of ECM or cell surface molecules can influence cell–matrix and cell–cell interactions and lead to the release of growth factors bound to the ECM.[3,11] More specifically, processes such as morphogenesis and tissue regeneration require ECM and basal membranes degradation to allow for the migration of different cells participating in tissue remodeling.[11,17,18] MMP-activity is also implicated in the process of angiogenesis. Endothelial cells synthesise different MMPs which apparently are important for the

formation of new blood vessels in both physiological and pathological conditions.[19] MMPs function as an irreplaceable factor in wound healing, a tissue-remodelling process which requires both the migration of various connective tissue cells and the formation of new blood vessels.[20] The role of MMPs has also been studied in other abnormal processes and diseases such as Alzheimer's disease, atherosclerosis, diseases of the central nervous system (CNS), liver cirrhosis, arthritis, vascular pathology, tumours and their metastases.[11] MMP-9 in particular can aid cancer cells in escaping the host immune response against them by suppressing the proliferation of T-lymphocytes through inhibition of the signal pathway mediated by IL-2.[21]

The unique structure and properties of the EL have been studied extensively over the years.[22-30] Unlike the ligament, the EL is built of various types of cells and displays an abundance of blood vessels and nerve fibres.[9,31] Earlier studies described the presence of only two types of cells in EL - spinous-shaped fibroblasts and fat cells (adipocytes).[32] These spinous-shaped cells were believed to play an important role in the synthesis and maintenance of the ECM, particularly the synthesis of collagen fibres.[32] Adipocytes were described as typical white adipose tissue cells which metabolise and store lipids and serve as packing material.[32] More recent data point out that the fully-developed EL is made up of two distinctive layers - one containing packed fat cells, fibroblasts, blood vessels and nerve fibres, and a second one, built of elongated and spindle-shaped fibroblasts and single blood vessels.[33] These newly-described types of fibroblasts were also reported to have the characteristics of collagen-synthesising cells.[33] EL cells are apparently involved in differentiation, phagocytosis and collagen synthesis, and thus take part in ligament healing.[9] Collagen fibres in the EL have a uniform small diameter and form bundles with alternating orientations.[9,32,34] Blood vessels were randomly distributed and often formed bundles with nerve fibres, though not all vessels were accompanied by nerve fibres.[32,35] Overall, light microscopic studies of the EL deduced that its cellular morphology resembles that of the synovium,[33] which supports the idea of the EL functioning as a specialized form of synovium.[9]

It is a well-known fact that the MCL heals faster than the anterior cruciate ligament (ACL) of the knee, and typically, injuries of the MCL can be treated conservatively.[3,36,37] One hypothesis states that this is most likely due to the specific characteristics of the EL of the MCL but the role of the stem cells of the MCL which are intrinsically different from those of the ACL has also been proposed.[3,36] A study of Zhou et al. compared the expression and localization of several types of MMPs in the MCL and ACL and concluded that numerous MMP family members that are found in various quantities in the EL of the MCL might be associated with the different healing potential of the MCL.[38] In the present study, we observed that fibroblasts in the EL of the MCL normally generated low levels of MMP-9. On the 8th and 16th day after grade III injury, which represents the early periods of healing, however, high levels of MMP-9 were expressed across the entire EL. As healing progressed, MMP-9 expression diminished and on the 30th day after injury was observed predominantly on the border between the EL and the MCL and in the perivascular zones in the EL.

CONCLUSION

In conclusion, herein we present the first immunohistochemical study of the expression and distribution of the enzyme MMP-9 in the EL of the MCL. In addition we describe the enzyme activity of MMP-9 in the early recovery period after grade III ligament injury to the MCL and track the changes in enzyme activity on the 8th, 16th and 30th day after damage. This study reveals that the changes in MMP-9 activity in the period after injury are associated with the process of ligament healing, in which the EL plays a key role.

Acknowledgements

This research was supported by Grant No. 19/2016 of the Medical University of Sofia, Bulgaria.

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How to cite this article: Iliev A, Georgiev GP, Kotov G, Dimitrova LN, Malinova L, Rashev P, et al. Immunohistochemical Study of Matrix Metalloproteinase-9 in Medial Collateral Ligament Epiligament in Rat Knee after Grade III Injury. Acad. Anat. Int. 2017;3(1):20-25.

Source of Support: Nil, **Conflict of Interest:** None declared.