Expression of Matrix Metalloproteinase-2 and 9 in the Medial Collateral Ligament Epiligament in Rat Knee

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ABSTRACT

Background: In this study, we describe and compare the different expression of matrix metalloproteinase-2 and 9 (MMP-2 and 9) in the epiligament (EL) of the medial collateral ligament (MCL) of the rat knee. **Methods:** Twelve 8-month old male Wistar rats were used in this study. The MCL and the external surface of the surrounded EL were precisely dissected. The description of the structure of EL and the expression of MMP-2 and 9 were implemented by light microscopic and imunohistochemical analysis. **Results:** In the ligament, a classic morphological structure was found. The expression of the MMP-2 and MMP-9 was fSound in the adventitia of the blood vessels and in the fibroblast in the EL. The reaction of MMP-2 was more intensive in comparison to MMP-9. **Conclusion:** We present one of the first study of immunohistochemical localization and distribution of the enzyme MMP-2 and 9 in EL tissue and furthermore we describe and compare the enzyme activity of MMP-2 and 9 in the EL.

Keywords: *Epiligament, Immunohistochemistry, Matrix metalloproteinase-2 and 9, Medial collateral ligament, rat.*

INTRODUCTION

In 1990 Bray et al.[1] in his work "*Fine vascular anatomy of adult rabbit knee ligaments*" defined for the first time the term "epiligament" (EL). This structure has been described as a "surrounding adherent connective tissue removed simultaneously with the ligament but which was grossly distinguishable from ligament tissue proper".

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After this first description, however, only few studies have investigated the EL. [2-4] The medial collateral ligament (MCL) is the most commonly injured ligament structure of the knee joint used to compare normal healing and non-healing in ligaments. The incidence of this injury has increased in recent years and represents a commonly encountered problem in modern sports medicine. [4-6] Our previous data concerning the injury of MCL in the rat knee model, presented that the understanding of the healing process in the EL tissue is essential in understanding the normal recovery in ligament tissue.[4]

Matrix metalloproteinases (MMPs), also known as matrixins, are a large group of zinc-dependent proteases responsible for cleaving and rebuilding connective tissue components such as collagen, elastin, gelatin and casein. MMPs play an important role in the degradation of extracellular matrix (ECM), a process that takes place during developmental stages

such as growth and morphogenesis. Due to their physiological functions, high levels of MMPs activity are observed in diseases and pathological processes involved in connective tissue degradation such as inflammation and cancer.[7] In recent years, MMPs have gained considerable attention in many studies on normal tissue events, inflammation, and disease processes, and this enhanced interest is probably due to several factors. For the most part, MMPs are produced or activated when needed, and expression of these enzymes provides a reliable indicator of ongoing tissue remodeling. Thus, for investigators, these enzymes are models of gene products that are accurately regulated and precisely targeted to specific extracellular substrates by a wide variety of cells during numerous normal tissue processes, such as wound healing, bone resorption, and morphogenesis. In contrast, exuberant production of MMPs is a hallmark of many destructive diseases and of many disease-related processes, such as inflammation, metastasis, and angiogenesis, and aberrant regulation of MMPs production is thought to be a primary mechanism contributing to disease progression and injury. [7] MMPs family members have been classified into different but closely related subgroups with fairly close characteristic features. 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.[8] MMP-2 and 9 has been found to be involved in many cellular processes such as tissue remodelling, repair and basement membrane degradation and it has been proved that is involved in the healing of acute tears, tumour invasion, neovascularization and metastases.[9]

There is no literature data concerning the localization of MMP-2 and 9 in the MCL EL in the rat knee. Therefore, the aim of this study was to investigate for the first time in literature the presence, expression and localization of MMP-2 and 9 in MCL EL.

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 animals were sacrificed after overdose of ether. After skin incision, the overlying connective tissue was dissected to expose the knee's MCL. The MCL and the external surface of the surrounded EL were precisely dissected, and then the pieces were immediately fixed. The light microscopy of the EL tissue was presented on semi-thin sections stained with 1% methylene blue, azure II and basic fuchsin, after previous fixation with 3% glutaraldehyde and 1% osmium tetroxide.

Immunohistochemistry for MMP-2 and MMP-9

Tissue samples were fixed in 10% buffered formalin for 24 h, 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. Two to three serial paraffin sections, 5 µm in thickness, were mounted on slides previously coated with chrome-gelatine. 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% H_2O_2 for 10 min at room temperature. The sections were rinsed in TTBS and nonspecific binding sites were blocked with Super Block (ScyTek Inc., USA) for 5 min. Primary polyclonal antibody for MMP-2 and MMP-9 (Sigma Co., St. Louis, MO) 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 (UltraTek Rabbit, ScyTek Inc., USA) for 10 min at room temperature. Sections were rinsed as before and incubated with streptavidin-HRP (UltraTek HRP, Scy Tek Inc., USA) for 10 min at room temperature. Antibody binding was visualized using 3,3´-diaminobenzidine tetrahydrochloride (ScyTek Inc., USA) 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

Normal morphology of the EL of the MCL in rat knee

The EL of MCL is quite different from the ligament substance. The external surface of the EL of the MCL was comprised of fibroblasts, fibrocytes, adipocytes, and neuro-vascular bundles as well as numerous multidirectional collagen fibers [Figure 1a, b]. The EL is relatively abundant of blood vessels. In contrast, to the EL, the ligament tissue was poorly vascularized, composed of fascicles. In the ligament, a classic morphological structure was found. These fascicles were formed by longitudinal groups of collagen fibers. Each fascicle appeared hypocellular and the cells were aligned interspersed between bundles of collagenous fibers. In contrast to a ligament, the collagen fibers in the midsubstance of the external surface of the MCL EL had uniformly small diameters and were organized in bundles with different orientations. There were also chaotically orientated small groups of collagen fibers.

Expression of MMP-2 and MMP-9 in the MCL EL in rat knee

The most interesting finding from our study was the distribution of MMP-2 and MMP-9 in the EL and the ligament substance. It should be pointed out that the positive reaction of this enzyme was predominantly localized in the EL compared to the ligament substance. The adventitia of the blood vessels in the EL also showed a distinct positive staining for MMP-2 and MMP-9. The fibroblasts in the ligament also stained positive for MMP-2 and MMP-9 [Figure 2a, b]. It should be also pointed out the more intensive reaction of MMP-2 in comparison to MMP-9.
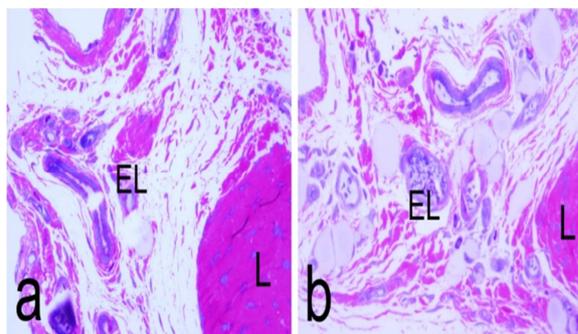
Figure 1a, b: Normal morphology of the external surface of the MCL EL tissue, presented by fibroblasts, fibrocytes, adipocytes, surrounded by collagen fibers, and scarce blood vessels. Magnification x 400.
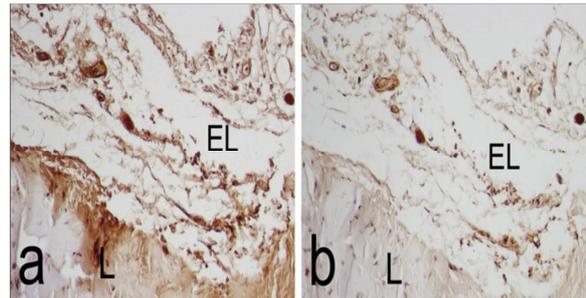
Figure 2a, b: (a) Immunohistochemical localization of MMP-2 of the MCL EL tissue; (b) Immunohistochemical localization of MMP-9 of the MCL EL tissue Magnification x 200.

DISCUSSION

Numerous studies have investigated the structure and healing process of the collateral ligaments of the knee in animal models. The EL consists of fibroblasts, fibrocytes, adipocytes, neurovascular bundles, and a multitude of collagen fibres, disposed in different directions. The EL of MCL ligament tissue is more vascularized and composed of ligament hypocellular collagen fascicles. The cells are interspersed between bundles of collagenous fibres. In the transition area the cells have a rounded shape. There are many single cells and groups of cells on the lateral side of the EL.[10-18] Herein, we present expression and localization of MMP-2 and 9 in EL and described changes between them. The primary roles of MMPs are to breakdown and remove ECM molecules from the tissue. However, it has been increasingly evident that the breakdown of ECM or cell surface molecules alters cell–matrix and cell–cell interactions and the release of growth factors bound to the ECM. A number of non-ECM molecules also become potential substrates of MMPs.[19] Gelatinases include MMP-2 and MMP-9 proteins. MMP-2 (Gelatinase A) and MMP-9 (Gelatinase B) are known to cleave native collagen type I, IV, V, VII, X, XI and XIV, elastin, fibronectin, osteonectin.[20] Modulation of cell–matrix interactions occurs through the action of unique proteolytic systems responsible for hydrolysis of a variety of ECM components. MMPs are also involved in wound healing, a tissue-remodelling process which involves the migration and differentiation of connective tissue cells. The role of MMPs in angiogenesis is also wide and complex. Many MMPs are produced by endothelial cells and have been described as important for the formation of new blood vessels in both physiological and pathological conditions.[7]

The ligaments are described as poorly vascularized connective tissue, composed of fascicles.[21] In contrast, the EL is more cellular than the ligament and composed of different types of cells, and contains abundant blood vessels and nerves, as revealed by our study.[21, 22] The fat cells in the EL appear to be typical adipocytes that make up white adipose tissue.[22] According to Chowdhury et al.[23] these cells metabolize and store lipids and may function as a packing material that could confer distinct material properties to the EL. As with the epitenon, the EL cells may be involved in differentiation, phagocytosis and collagen synthesis, and thus take part in ligament healing.[21] Vessels in the EL were randomly dispersed in a loose connective tissue matrix and nerve bundles often accompanied the blood vessels in the EL, but apparently not all blood vessels formed part of a neuro-vascular bundle.[23, 24] Light microscopic investigations revealed that the general cellular morphology of EL was similar to that seen in synovium. This is consistent with the hypothesis that the EL is a specialized form of synovium.[21]

MMPs play an important role in the degradation of ECM, a process that takes place during developmental stages such as growth and morphogenesis. Due to their physiological functions, high levels of MMPs activity are observed in diseases and pathological processes involved in connective tissue degradation such as inflammation and cancer. MMP-2 is known to cleave native collagen type I. Other studies have shown that MMP-2 binds to intact collagen to prevent autolytic inactivation. In addition to gelatine and other forms of denatured collagen, MMP-9 cleaves a number of other physiological substrates. Many MMPs are produced by endothelial cells and have been described to be important for the formation of new blood vessels in both physiological and pathological conditions.[25] In a study of Zhou et al.[26] the expression and localization of different types of MMPs, including MMP-2 and 9 in MCL were described. They concluded that numerous MMP family members that are expressed in various quantities in the MCL might be involved in the different matrix remodelling process as well as the different healing ability of MCL.[26] Furthermore Ishiguro et al. [27] was observed MMP-9 positive cells in the perivascular area, while MMP-2 positive cells frequently was seen between irregular collagen bundles. Similar results we describe in our study, in addition, we have identified and a more intense expression of MMP-2. MMPs primary role is to breakdown and remove ECM molecules from the tissue. However, it has been increasingly evident that the breakdown of ECM or cell surface molecules alters cell–matrix and cell–cell interactions and the release of growth factors bound to the ECM. The ability of MMPs to degrade structural components of ECM and basement membranes has supported their direct implication in these processes.[25] The role of MMPs in tissue remodelling has also been studied by others.[7, 19, 20, 26, 27] The role of MMPs in angiogenesis is also broad in scope and complex. Many MMPs are produced by endothelial cells and is important in the formation of new blood vessels in physiological conditions. In addition to physiological processes, MMPs effects are also observed in a number of pathological processes such as arthritis, Alzheimer's disease, atherosclerosis, vascular disease, gastritis ulcer disease, central nervous system disease, liver cirrhosis, tumours and their metastasis. MMPs can be studied from different perspectives as markers of some cancer, neurodegenerative, immune and cardiovascular diseases.[25]

CONCLUSION

In conclusion, herein we present the first study of immunohistochemical localization and distribution of the enzyme MMP-2 and 9 in EL tissue. In addition, we describe and compare the enzyme activity of MMP-2 and 9 in the MCL. Changes in the expression of the enzyme MMP-2 and 9 in different components of the EL plays a major role in the physiological and pathophysiological process.

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