

Lysosomal Glycosidases in Degradation of Human Articular Cartilage

Janusz Popko¹, Tomasz Guszczyn¹,
Sławomir Olszewski¹, Krzysztof Zwierz²

¹*Medical University, Białystok,*

²*Medical College of the Universal Education Society, Łomża,
Poland*

1. Introduction

Joint diseases cause serious medical problems for several million people world-wide and therefore the World Health Organization has designated years 2000-2010 as the Decade of the Bone and Joint (Popko et al.2011).

Osteoarthritis (OA) is the most common, and increasingly prevalent, human joint disorder (Dieppe, 2000). It has been estimated that in 1990 12 % of Americans, nearly 21 million people had clinical symptoms of osteoarthritis (Lawrence et al., 1998).

Rheumatoid arthritis (RA) affects about 0.3 to 1.5% of the world population(Chikanza et.al., 1998). Juvenile idiopathic arthritis (JIA) is one of the most common rheumatic diseases in children, which causes pain and functional disability. According to a 2008 study performed by the National Arthritis Data Workgroup, there were close to 3000,000 children in the U.S.A. with some form of juvenile arthritis (Giannini et al., 2010).

Lyme arthritis (LA) caused by spirochete *Borrelia burgdorferi*, is increasing in prevalence disease involving the musculoskeletal system, particularly affecting knee joints (Pancewicz et. al.2009).

RA and JIA are chronic autoimmune inflammatory diseases primarily affecting the synovial membrane, leading to joint damage and destruction. OA is the most common joint disorder and a major public health problem in western populations (Lawrence et al. 1998).

Clinical and epidemiological studies on OA have recognized a series of etiologic factors including local factors (such as malformations or joint injuries) and systemic factors (such as overweight, race, gender, or metabolic diseases). OA is associated with a loss of proper balance between synthesis and degradation of the macromolecules that gives articular cartilage its biomechanical and functional properties. Concomitantly in OA, changes occur in the structure and metabolism of the synovium and subchondral bone of the joint.

2. Degradation of human articular cartilage

Progressive destruction of articular cartilage is a common feature of OA, RA, and LA. The articular cartilage from patients with OA and RA has decreased concentrations of proteoglycans and glycosaminoglycans (GAGs), and the size of GAG molecules is also

reduced (Inerot et al.1978). In established joint disease, loss of articular proteoglycans could be more significant than the collagen loss (Mankin & Lippiello, 1970; Popko et al. 1983). Destruction of articular cartilage is a multifactorial process, which is performed extracellularly by concerted action of matrix metalloproteinases (MMPs) and glycosidases (Fig.1).

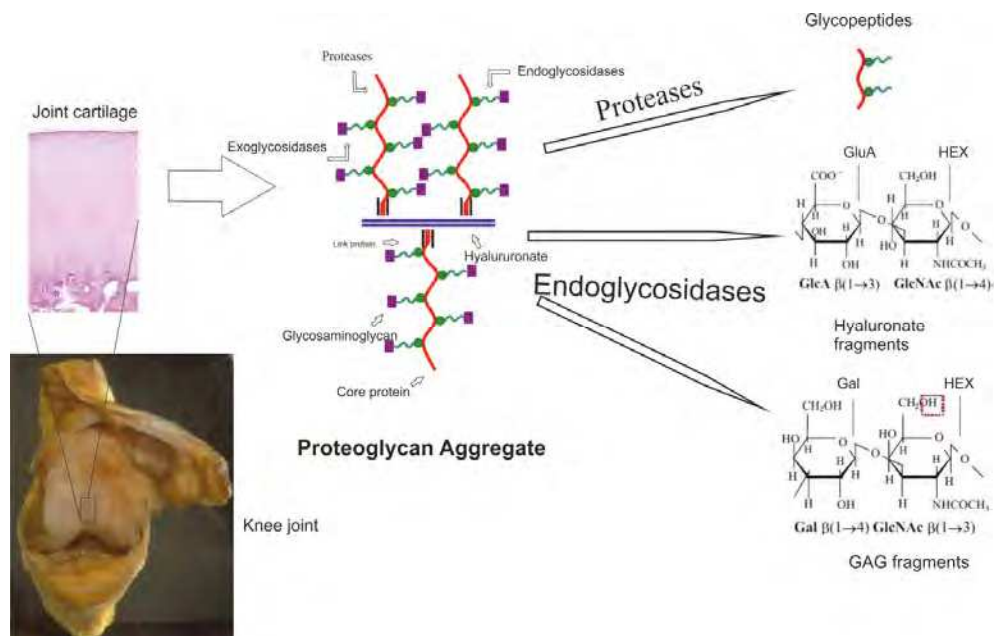


Fig. 1. Cartilage destruction by proteases and glycosidases.

Of the metalloproteinases, collagenase (MMP-1) in particular, appears to be responsible for the degradation of interstitial collagens. The gelatinases (MMP-2 and MMP-9) degrade the denatured forms of collagens, acting in synergy with MMP-1. The stromelysins (MMP-3) have broader substrate specificity for non-connective tissue proteins. Membrane-type MMPs (MT-MMP-1 and MT-MMP-3) have been detected at sites of destruction in rheumatoid arthritis (Pap et al., 2000).

Protease action increases the accessibility of cleavage sites for endo- and exoglycosidases (Ortutay et al. 2003) by production glycopeptides (Fig.1).

Endoglycosidases (hyaluronidases, chondroitinases, keratanases, etc.) cleave glycosidic linkages inside glycosaminoglycan or oligosaccharide chains of the proteoglycans or release oligosaccharide chains from protein cores (Styplukowska et al. 2004).

In contrast to endoglycosidases, lysosomal exoglycosidases: N-acetyl- β -hexosaminidase (HEX), β -galactosidase (GAL), β -glucuronidase (GluA), α -mannosidase (MAN) and α -fucosidase (FUC), release monosaccharides from the non-reducing terminals of oligosaccharide chains of glycoproteins, glycolipids and proteoglycan glycosaminoglycans of synovial tissue, articular cartilage and synovial fluid. N-acetyl- β -hexosaminidase (HEX) is present as two isoenzymes HEX A and HEX B which both release terminal N-acetylhexosamines, whereas HEX A also hydrolyzes hexosamines in acidic oligosaccharides

as in G_{M2} gangliosidases (Zwierz et al. 1999; Pennybacker et al. 1996; Sharma et al. 2003; Itakura et al. 2006). β -galactosidase (GAL) releases terminal galactose (Czartoryska 1977), from the non reducing terminal of oligosaccharide chains of glycoproteins, glycolipids and keratan sulfate. Mannose is liberated from N-linked sugar chains of glycoproteins, as well as a variety of synthetic and natural β -mannosides, by α -mannosidase (MAN) (Czartoryska 1977). Lysosomal α -fucosidase (FUC) (Li, C., Qian et al. 2006) is involved in the degradation a variety of fucose-containing oligosaccharide chains of glycoproteins and glycolipids and β -glucuronidase (GluA) cleaves glucuronic acid residues from the non-reducing terminal of glycosaminoglycans (GAGs) (Marciniak et al. 2006).

3. The characterization and function of lysosomal glycosidases

The main exoglycosidases in tissues, serum and synovial fluid of humans are:

N-acetyl- β -hexosaminidase (HEX), β -glucuronidase (GluA), β -galactosidase (GAL), α -mannosidase (α -MAN), and α -fucosidase (FUC). N-acetyl- β -hexosaminidase (EC 3.2.1.52, HEX, NAG) is the most active enzyme of the lysosomal exoglycosidases (Popko et al. 2006). HEX has several isoenzymes: A, B, S, C, I₁, I₂. HEX A and S are thermostable and B, P, I₁, I₂ thermostable. In humans there are two major isoenzymes of hexosaminidase: HEX A ($\alpha\beta$), and HEX B ($\beta\beta$). Both isoenzymes recognize terminal N-acetylglucosamine and N-acetylgalactosamine, but only HEX A recognizes 6-sulfated residues of these sugars. HEX A represents (an average) 48% of total HEX activity in serum, and 52% of total HEX activity in synovial fluid (Popko et al. 2006).

The hexosaminidase S (HEX S) is of minor importance, as it constitutes less than 0.02% of HEX activity (Ikonne et al. 1975), and can be detected in patients with Sandhoff disease (Yamanak et al. 2001). The function of the HEX S is not well understood, but it is probably involved in the degradation of GAGs.

The protein moiety of lysosomal exoglycosidases is synthesized in the rough endoplasmic reticulum, and transported to lysosomes through the endoplasmic reticulum and Golgi apparatus (Zwierz et al. 1999). Some of the lysosomal enzymes are secreted from the cell into the extracellular fluid. Another route for the secretion of lysosomal enzymes is from the lysosomes via the endosomes and Golgi compartment to the cell surface and extracellular fluid. The release of exoglycosidases is regulated by a small Ras-related GTP-binding protein Rho p21 (Rho proteins control the polymerization of actin into filaments and govern the organization of body filaments into specific types of structures). The release of exoglycosidases from mast cells has shown to be induced by an IgE mediated increase in intracellular Ca^{2+} (Zwierz et al. 1999).

Exoglycosidases activity of knee synovial fluid and serum of healthy humans is presented in Fig. 2.

The exoglycosidases activity, is higher in synovial fluid than in serum. Levels of the HEX activity are constant in serum of healthy humans up to 40 years of age, whereas in older people (more than 40 years of age) the level of HEX activity significantly increases.

The substrates for exoglycosidases in articular cartilage include cell surface and extracellular matrix glycoproteins as well as glycosaminoglycans: chondroitin 4-sulfate, chondroitin 6-sulfate, hyaluronic acid, keratan sulfate, and dermatan sulfate (Winchester 1996; Stypułkowska et al. 2004).

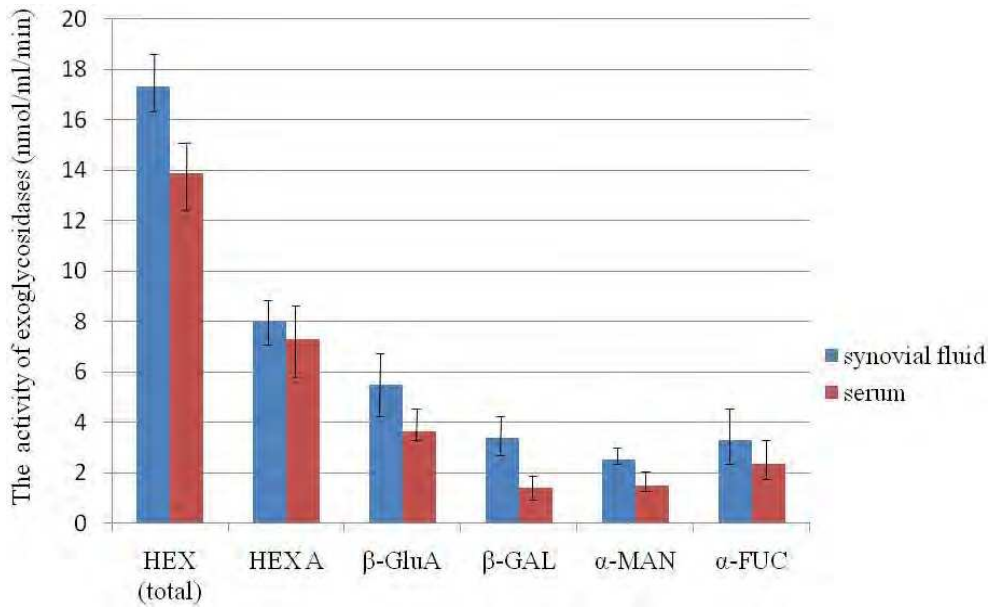


Fig. 2. Activity of exoglycosidases (nmol/ml/min) in synovial fluid and serum of healthy humans.

Exoglycosidases degrade glucoconjugates within the lysosome at an optimum pH ranging from 4.3 to 5.5 (Zwierz et. al. 1989; Marciniak et. al. 2006). Investigation of the pH dependence showed that HEX is active at pH 4.2 to 5.6 with optimum at pH 4.7, and β -glucuronidase is active between pH 3.4 and 5.6, with optimum activity at pH 4.5 (Marciniak et al. 2006).

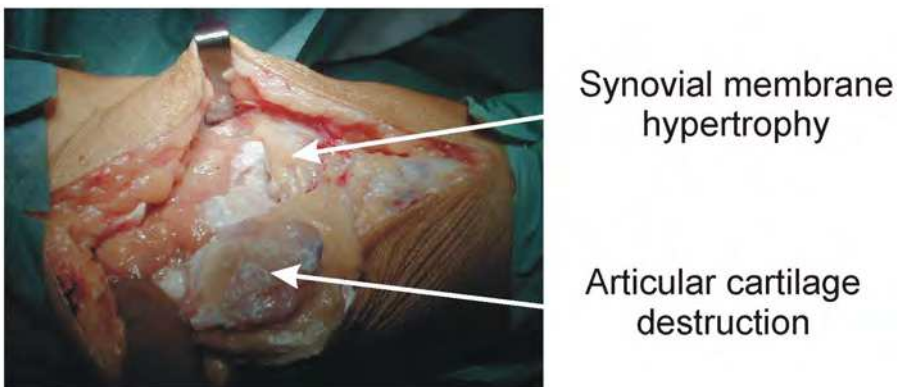


Fig. 3. Typical knee of a patient with RA. Showing destruction areas of articular cartilage and hypertrophy of synovial membrane.

4. The localization of exoglycosidases in joint tissues

Pugh and Walker (Pugh 1961), using histochemical techniques, reported that the source of HEX activity in synovial fluid is from cells of the synovial membrane. Others (Shikhman et al. 2000; Ortutay et al. 2003) have suggested that chondrocytes of RA patients activated by IL 1 β (interleukin-1 β) may be a source of HEX activity in synovial fluid. Relating to this, it has been observed that damage to the joint reduces the volume of cartilage and increases a proliferation of synovial membrane (Fig. 3).

Profiles of the exoglycosidases in the synovial membrane of the knee joint of patients with RA, JIA and a control group are presented in Fig. 4.

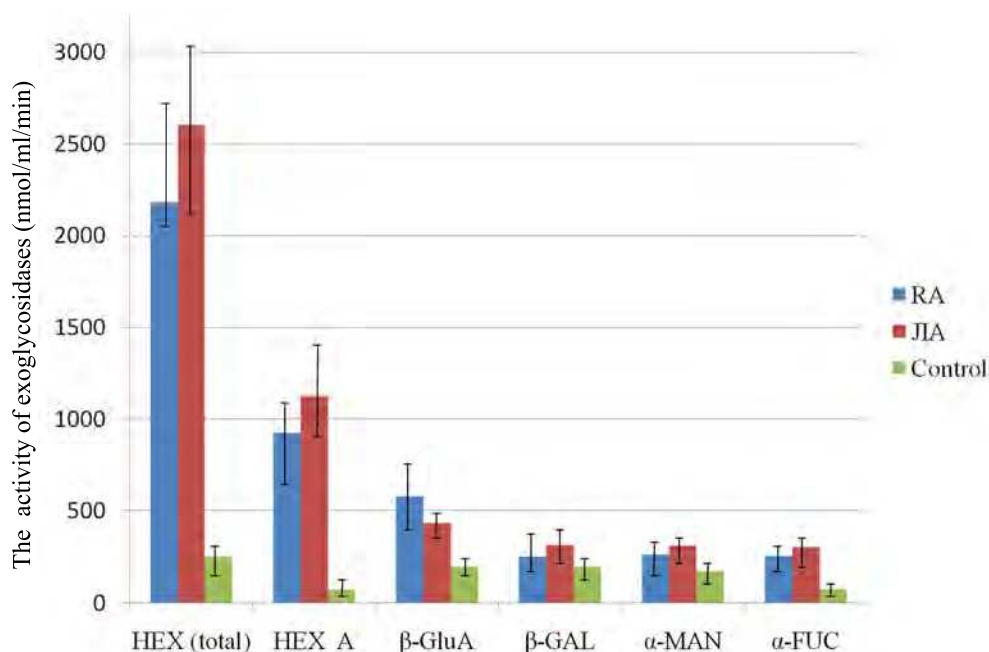


Fig. 4. The activity of exoglycosidases in the synovial tissue of the knee joint of patients with RA and JIA.

Normal and inflamed synovial tissues have similar patterns of exoglycosidases activity with a significant predominance of HEX activity (Popko et.al.2006). HEX activity in the synovial tissue of RA and JIA patients was approximately 10-fold higher than in the synovial tissue of the reference group. The increase in activity of GluA, GAL, MAN and FUC in synovial tissue of RA and JIA patients (in comparison with reference groups) was moderate, i.e. no more than doubled.

Synovial fibroblast-like cells and chondrocytes may be regarded as a source of mediators of joint destruction in RA and JIA. Synoviocytes and chondrocytes secrete proteolytic enzymes and exoglycosidases, especially HEX, that are crucial for the degradation of cartilage. The destructive phenotypes of the synovial fibroblasts-like cell and chondrocytes in RA are probably regulated by inflammatory cytokines released by the pannus connected to the cartilage.

The high activity of exoglycosidases in synovial tissue of RA and JIA patients (Popko et al.2006) suggest the value of synovectomy in treatment of rheumatoid diseases (Fig. 5).



Fig. 5. Arthroscopic synovectomy a knee of patient with JIA.

We recommend the usefulness of synovectomy in patients with RA and JIA, as the removal of diseased synovial tissue (Fig. 5) could slow down the destructive process of the joint cartilage, and allow regeneration of a normal synovial membrane.

5. Exoglycosidases activity in cell cultured synoviocytes

The patterns of compartmental distribution of exoglycosidases activity in cultured synovial cells of RA and JIA patients are presented in Fig. 6.

The activity of HEX (total), HEX A, and Glu A in the intracellular compartment in cultured synoviocytes of RA and JIA patients is over 3-fold higher than in the extracellular compartment. In contrast, no activity of GAL, MAN and FUC in the extracellular compartments of these cultured synoviocytes was detectable. It is most likely that these exoglycosidases were released extracellularly in small quantities, i.e. below the limits of detection of our colorimetric procedure. Shikhman et al. (2000) have demonstrated similar results in cultured human articular chondrocytes. The high level of activity of exoglycosidases in the intracellular compartment of cultured synoviocytes indirectly confirms the suggestion that the degradation of glycosaminoglycans predominantly takes place in the intracellular compartment. However, Woynarowska et al. (1992) reported data indicating the contribution of extracellular HEX activity in glycosaminoglycan degradation.

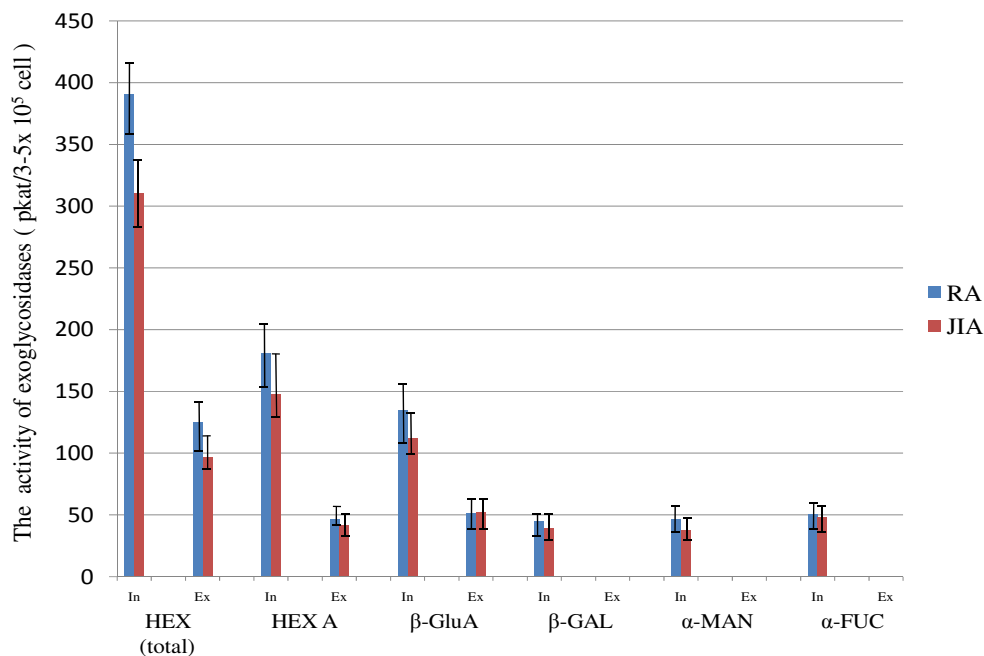


Fig. 6. Exoglycosidases activity of RA/JIA synovial fibroblast in the intra (In) / extracellular (Ex) compartment, expressed as pKat/3-5x10⁵ cells.

IL-1 and TNF- α (tumor necrosis factor- α) are key proinflammatory cytokines whose concentration significantly increases in rheumatoid synovial fluid and joint tissues.

We have established a profile of exoglycosidases in cell cultures stimulated by IL-1 β of inflamed (RA, JIA), and post-injury human synoviocytes (Popko et al. 2008) (Fig. 7).

Stimulation by IL-1 β cultured synoviocytes taken from patients with ACL, JIA and RA causes much higher increases in activity of HEX and HEX A than remaining exoglycosidases (Fig. 7). On Fig. 7 one can see that the increase in HEX activity after IL-1 β stimulation is more pronounced in the intracellular compartment of synoviocytes derived from rheumatoid patients than in ACL-injury, amounting 189.44 % increase (in comparison to untreated cultures) in synoviocytes from JIA, and 127.97 % in synoviocytes of RA patients. We noted a 201.18 % increase in HEX-A activity (in comparison to untreated cultures) in stimulated synoviocytes from JIA, and a 128.03 % increase in synoviocytes of patients with RA. In extracellular compartment of cultured rheumatoid synoviocytes, stimulation by IL-1 β cause only 33.4-72.44 % increases in HEX and HEX A activities. In extracellular compartment of cultured synoviocytes derived from injured knees of ACL patients, after stimulation by IL-1 β the highest increase (121.80 %) was observed in HEX A activity. The mechanism of selective stimulation of HEX by IL-1 β is not known. Shikhman et al. (2000) suggested that cytokines are involved in secretion of HEX from chondrocytes and stated that IL-1 β could selectively up-regulate HEX synthesis and facilitate intra-compartmental transport of HEX from lysosomes/endosomes into the extracellular space, by modifying the mannose-6-phosphate receptor system.

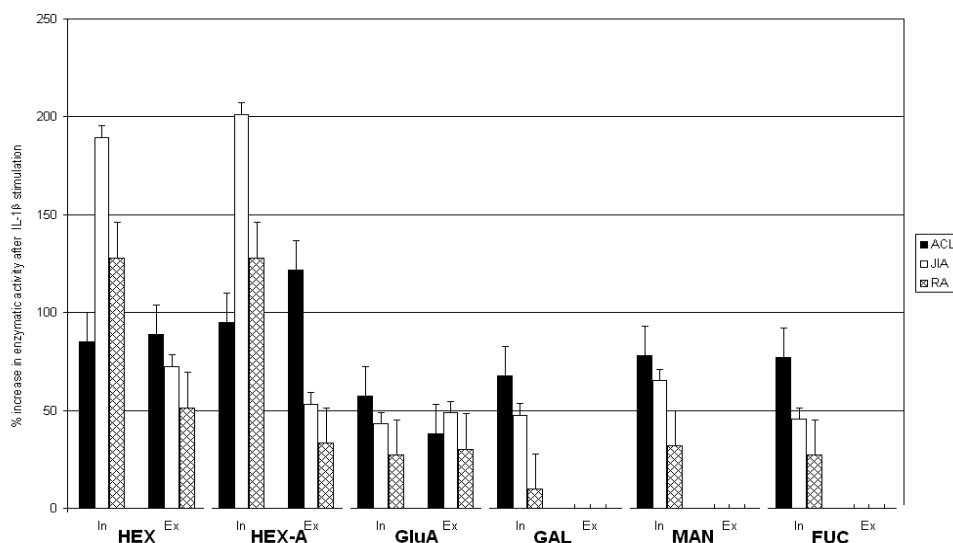


Fig. 7. Effect of interleukin 1 β (IL-1 β) on intra-(In) and extracellular (Ex) activity of lysosomal exoglycosidases in cultured human synovial cells from patients with anterior cruciate ligament (ACL) injuries, juvenile idiopathic arthritis (JIA), and rheumatoid arthritis (RA). Synoviocytes were stimulated with IL-1 β for 24h at 37°C. After IL-1 β stimulation, cultured cells and extracellular fluid were analyzed for exoglycosidase activities. The effect of IL-1 β was expressed as the enzymatic activity in stimulated versus unstimulated cultures.

Rheumatoid synoviocytes exhibit altered morphology and show certain similarities to tumor cells (Mor et al. 2005). Our data confirmed the observation that synoviocytes obtained from patients with JIA and RA are more active in the synthesis and secretion of exoglycosidases than those obtained from patients with injured knees or healthy joints (Popko et al.2008). (Popko et al.2008).

6. Activity of lysosomal exoglycosidases in serum and synovial fluid of patients with RA and JIA

Information concerning the activity of exoglycosidases in patients with joint diseases is ambiguous and limited to a few, mostly old, publications (Bartholomew 1972; Stephens et al.1975; Ganguly et al. 1978; Berenbaum et al. 2000; Sohar et al. 2002). Serum HEX activity was higher in 35 % of the RA patients than in healthy controls (Berenbaum et al. 2000). Berenbaum et.al. (2000) found that the serum HEX concentration was significantly higher in destructive RA than in inflammatory RA. Since RA patients with high serum HEX activity have more erosions, than those with inflammatory RA, but have no differences in CRP levels, it is possible that HEX is a marker of erosions.

Lysosomal enzymes in human polymorphonuclear leukocytes are ubiquitous, biologically active molecules, that can degrade macromolecules such as proteins, glycosaminoglycans, nucleic acids, and lipids (Sohar et al.2002). Sohar et.al. (2002) have suggested that the increase of HEX activity in the serum of RA patients may be caused by an increase in HEX

activity of RA leukocytes. Patients with long-standing RA had higher activity of lysosomal glycosidases in their leukocytes than those with disease of shorter duration.

We suggest that of the exoglycosidases, only HEX activity measurement in synovial fluid and serum has practical value (Popko et. al. 2006). The specific activity of HEX and its isoenzyme A in serum and synovial fluid from patients with different arthropathies is presented in Fig. 8.

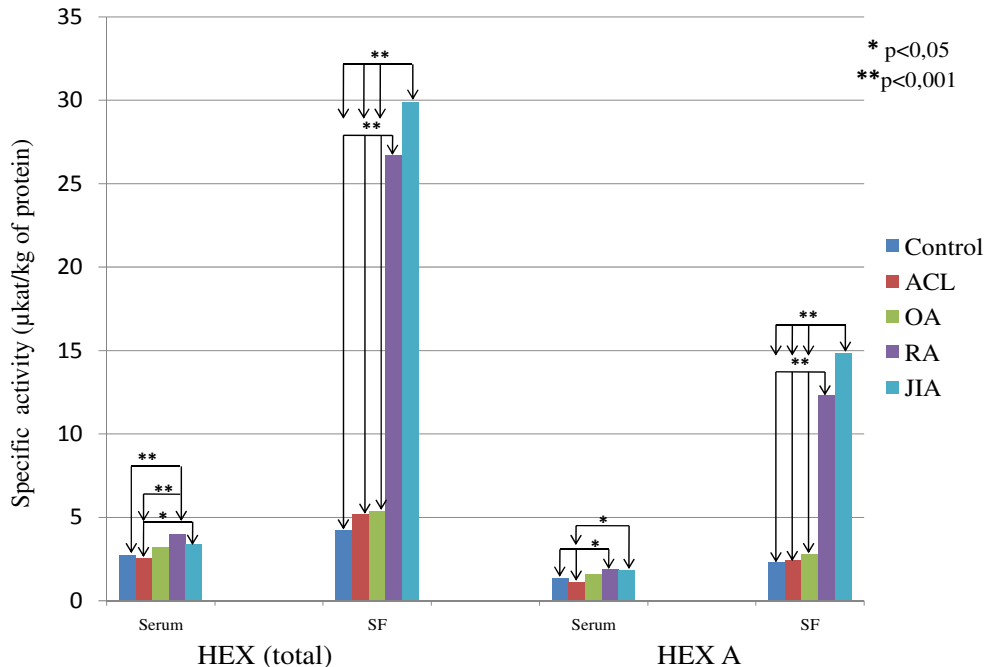


Fig. 8. Specific activity of N-acetyl- β -hexosaminidase and its isoenzyme A in serum and synovial fluid ($\mu\text{kat}/\text{kg}$ of protein).

In the serum of RA patients, the specific activity of HEX was significantly increased in comparison to control (Fig. 8). This increase of HEX activity in the serum of RA patients may depend on the increase in HEX activity of RA leukocytes (Sohar et al. 2002). In the serum of patients with JIA and OA, we observed a moderate increase in HEX activity, i.e. 25.46% and 17.3 % respectively. In the case of patients with ACL injury, the specific activity of HEX and its isoenzymes in serum behaved similarly as in the control.

In the synovial fluid of JIA and RA patients, we found a significant increase in the specific activity of HEX and its isoenzymes, in comparison to HEX activity in synovial fluid from OA and ACL injuries. This suggests that release of HEX to synovial fluid is greatly enhanced by the autoimmune-inflammatory process in knee joint cavity.

The activity of HEX in synovial fluid of patients with JIA and RA was significantly higher than the activity in OA and ACL patients. Additionally, we have found that in JIA and RA patients, the specific activity of HEX in synovial fluid is 6-8 times higher than in serum

from the same patients. This results suggest that HEX in synovial fluid derives mainly from articular tissues or articular leukocytes and not from serum. Therefore, activity of HEX in synovial fluid better reflects the situation in the joint cavity than activity of HEX in serum.

The differences between specific activities of HEX (activities calculated per 1 kg of protein) in synovial fluid of RA and JIA patients in comparison to OA and ACL patients (Fig. 9) are even more evident than differences in concentration of HEX (activities calculated per volume of synovial fluid) in the same situations.

HEX-JIA, RA & OA - Marker

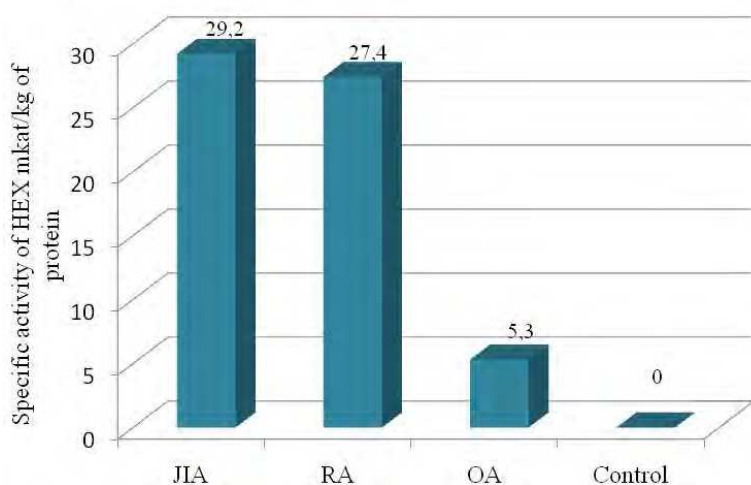


Fig. 9. The specific activity of HEX in synovial fluid of patients with rheumatoid arthritis.

Significant increase of specific HEX activity in synovial fluid of JIA patients (in comparison to OA and control) may be of diagnostic value in children with prolonged exudates in the knee joint, who are resistant to pharmacological and physiotherapeutical treatment. In these cases we advise determining specific activity of HEX in the synovial fluid, where values above 10-13 $\mu\text{kat}/\text{kg}$ of protein suggest rheumatoid disease. It is worthy of note that specific activity of HEX in synovial fluid of patients with RA demonstrates a broad standard deviation which probably depends on destructive or inflammatory processes in the joint. However, significantly elevated HEX activity indicated an inflammatory or autoimmunological process within the joint.

7. Assay of exoglycosidase activity

Samples of the synovial fluid are easily taken from the knee joints during diagnostic or therapeutic arthrocentesis or at arthroscopy. Synovial fluid is aspirated from a lateral infrapatellar approach with a 21G needle (Fig. 10).



Fig. 10. Synovial fluid aspiration from the knee.

To obtain reliable results of exoglycosidase determinations, patients with inflammatory arthritis should avoid steroid drug treatment at the time of arthrocentesis, and for two days before arthrocentesis. About 1 ml of synovial fluid is sufficient for assays of exoglycosidase activity and protein concentration. Samples of synovial fluid are collected in plastic tubes and centrifuged at $10,000\times g$ for 30 min, separated from the cell pellet, and stored at $-70^{\circ} C$ before use. As substrates for exoglycosidase activity we use p-nitrophenyl derivatives of appropriate sugars purchased from Sigma, St.Louis, Mo, USA (HEX, GAL, MAN, FUC), and Fluka Chemie GmbH (GluA).

The activities of HEX (E.C. 3.2.1.52), β -glucuronidase (E.C. 3.2.1.31), β -galactosidase (E.C. 3.2.1.23), α -mannosidase (E.C. 3.2.1.24), and α -fucosidase (E.C. 3.2.1.51) are determined by simple and inexpensive methods (Marciniak et. al. 2006). Before exoglycosidases determinations, the samples of synovial fluid are diluted with 0.1 M of the appropriate buffer and incubated with excess of substrate for 60 min at $37^{\circ} C$. The reaction is stopped by adding 0.2 M borate buffer, pH 9.8. HEX A activity is calculated as the difference between the total HEX activity and HEX B activity.

Spectrophotometric measurements of released 4-nitrophenol were carried out at 405 nm using a microplate reader Elx800™. The concentration of enzymatic activity of the appropriate exoglycosidase was expressed as nanomoles of p-nitrophenol released per minute per ml in synovial fluid and specific activity was expressed in $\mu\text{kat}/\text{kg}$ of proteins of synovial fluid. The concentration of exoglycosidase activity indicates the ability of the specified volume of synovial fluid to release the quantity of monosaccharide indicated. The specific activity relates a particular exoglycosidase activity to total protein concentration, and shows the proportion of exoglycosidase protein to the total protein content of an articular sample of synovial fluid.

8. Conclusions

Although many publications (Shinmei et al. 1992; Lohmander et al. 1995; Saxne & Heinegard 1995; Myers 1999; Ortutay et al. 2003) have described increased release of markers of cartilage, bone, and synovial metabolism into joint fluid, serum, and urine in rheumatoid arthritis, the significance of several of these markers remains elusive. Up to now, no markers have yet been formally validated to monitor rheumatoid diseases of the joints. We hypothesize that determining HEX activity in the synovial fluid of patients with suspected idiopathic juvenile arthritis has diagnostic value. Elevation of HEX activity in synovial fluid to greater than 10 μ kat/kg of protein, suggests rheumatoid disease.

Despite its huge public health impact, the conservative treatment of joint diseases, particularly of OA, is limited to a few types of medication which provide primarily symptomatic relief. Inhibition of hexosaminidase activity may represent a potentially novel strategy for treating RA and OA. Liu et al. (2001) have synthesized and investigated a series of iminocyclitols designed as transition-state analogue inhibitors of extracellular human hexosaminidase. Our team (Olszewski et al. 2010) is focusing on pyrimethamine which contributes to the regulation of HEX gene expression in synovial cells.

9. Acknowledgment

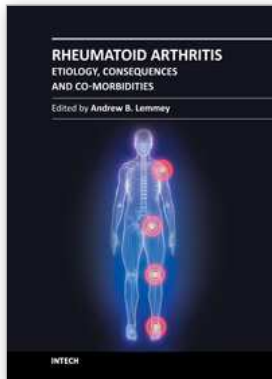
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The purpose of this book is to provide up-to-date, interesting, and thought-provoking perspectives on various aspects of research into current and potential treatments for rheumatoid arthritis (RA). This book features 16 chapters, with contributions from numerous countries (e.g. UK, USA, Japan, Sweden, Spain, Ireland, Poland, Norway), including chapters from internationally recognized leaders in rheumatology research. It is anticipated that Rheumatoid Arthritis - Etiology, Consequences and Co-Morbidities will provide both a useful reference and source of potential areas of investigation for research scientists working in the field of RA and other inflammatory arthropathies.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
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Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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