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Plasma and urinary glycosaminoglycans in the course of juvenile idiopathic arthritis☆

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ABSTRACT

Objectives: The aim of the study was to perform analyses of plasma and urinary glycosaminoglycan isolated from juvenile idiopathic arthritis (JIA).
Methods, results: Chondroitin/dermatan sulfate (CS/DS), heparan sulfate/heparin (HS/H) and hyaluronic acid (HA) were evaluated in samples obtained from JIA patients before and after treatment.
Electrophoretic analysis of GAGs identified the presence of CS, DS and HS/H in plasma of healthy subjects and JIA patients. CS were the predominant plasma GAGs constituent in all investigated subject. The plasma CS level in untreated patients was significantly decreased. Therapy resulted in an increase in this glycan level. However, plasma CS concentration still remained higher than in controls. Increased levels of DS and HA in untreated JIA patients were recorded. Anti-inflammatory treatment led to normalization of these parameters concentrations. Plasma and urinary concentrations of HS/H were similar in all groups of individuals. Urinary CS/DS and HA were decreased only in untreated patients.
Conclusions: The data presented indicate that changes in plasma and urinary glycosaminoglycan occur in the course of JIA. There are probably the expression of both local articular cartilage matrix and systemic changes in connective tissue remodeling.

1. Introduction

The extracellular matrix (ECM) plays a structural role conferring shape and mechanical properties to tissues. Moreover, the matrix, being a highly dynamic network of interacting particles, can regulate cell behavior by modulating adhesion, proliferation and differentiation, as well as signaling dependent on receptors or organization of cell cytoskeleton. These functions are directly related to glycosaminoglycan (GAGs) chains covalently linked to a core protein of proteoglycans (PGs) [1–4]. GAGs are classified into five classes, and they include chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate/heparin (HS/H), keratan sulfate (KS) and nonsulfated hyaluronic acid (HA). They are linear polysaccharide chains that consist of repeated disaccharide units composed of amino sugar and uronic acid or galactose residues [2,3]. It is known that these glycans, due to a high density of negative electric charge, interact with many types of molecules, including enzymes, growth factors, transcription factors or extracellular matrix structural proteins [3]. Although in physiological conditions GAGs are components of a dynamic structure of ECM, which is continually being remodeled, the disturbances of synthesis, modification or degradation of these polysaccharides contribute to development of connective tissue-related disorders, including rheumatoid arthritis [2,5]. Probably, these disorders are also observed in the course of juvenile idiopathic arthritis (JIA). In JIA patients, remodeling of articular cartilage matrix components takes place [6–8]. However, it is not totally obvious whether these changes are only local or whether they include systemic ECM disturbances. It can be assumed that during the development of JIA, the increase of proinflammatory cytokines, including tumor necrosis factor-α

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(TNF-α) or interleukin-1β (IL-1β) as well as growth factors, including transforming growth factor-β (TGF-β) may change the synthesis of GAGs, while the increased activation of proteolytic enzymes, including matrix metalloproteinases (MMP), may intensify the catabolism of tissue PGs [1,6–10]. On the other hand, the inflammatory side effects, including excessive reactive oxygen species (ROS) formation, may contribute to the PGs' core protein oxidation as well as to the partial depolymerization of GAGs chains into fragments that appear in the circulation [6,11].

Due to the fact that tissue alterations of PG/GAGs should be reflected in the GAGs blood profile the aim of the present study was to perform qualitative and quantitative analyses of plasma GAGs isolated from JIA patients. Furthermore, since GAGs blood pattern is related to their glomerular filtration rate, we decided to assess the profile of GAGs excreted in urine of children with JIA.

2. Materials and methods

The study was carried out on the plasma obtained from 30 children of both sexes (19 girls, 11 boys), aged 12–16 years, with newly diagnosed with JIA. All patients were diagnosed and classified according to the International League of Associations for Rheumatology criteria as oligoarthritis or polyarthritis [8]. Moderate disease activity was assessed in all patients, using Juvenile Arthritis Disease Activity Score. The accuracy of diagnosis was confirmed by laboratory analysis (Table 1).

The tests were repeated in the same patients after therapy, when clinical improvement was observed (11.60 ± 0.21 months after therapy beginning). Clinical improvement was determined by the ACR Pediatric 30 criteria. Treatment with stable doses of non-steroidal anti-inflammatory drugs, oral glucocorticoids (at maximum dose of 1 mg of prednisone equivalent per kilogram per day, with gradual dose reduction) and methotrexate (≤15 mg per square meter of body-surface-area once a week) was used.

Blood samples obtained from 30 age and body mass index-matched healthy individuals were used as controls. The clinical data of healthy individuals are shown in Table 1.

The plasma obtained both from healthy individuals and JIA patients were divided into portions and stored at −80 °C till the initiation of the study. The determination of one parameter was completed within one day, consequently the inter-assay variation was insignificant.

Informed consent was obtained from teenage participants under age 16 according to the ethical guidelines of the Helsinki Declaration. In cases of all under-aged patients, parents or legal guardians signed the informed consent. The Local Ethics Committee of Medical University of Silesia approved the research protocol used in this study.

2.1. Extraction and determination of plasma glycosaminoglycans

The determinations of plasma GAGs were performed by the multistage extraction and purification using papaine hydrolysis and alkali elimination, according to the method previously described [12]. The total amount of GAGs was determined by the hexuronic acid assay, with analytical sensitivity of 0.5 mg/L and calibration range from 0.5 to 50 mg/L. The coefficient of an intra-assay variation was less than 6%. This method does not measure the concentration of KS, which do not contain hexuronic acids in their structure [13]. Hence, quantitative evaluation of KS was not carried out in our study.

2.2. The assay of plasma sulfated glycosaminoglycans

Samples of isolated plasma sulfated GAGs, i.e. CS, DS and HS/H (10 μg of hexuronic acid) were submitted to electrophoresis on cellulose acetate, before and after the use of agents eliminating particular GAG types. The following GAGs digestion agents were used: chondroitinase ABC, chondroitinase B and combination of heparinase I with heparinase III. Digestion was carried out according to the manufacturer's instructions. All enzymes were obtained from Sigma Aldrich (USA). Chondroitinase ABC was used to remove CS and DS and retain HS/H. Chondroitinase B was used to remove DS and retain CS and HS/H. In turn heparinase I and heparinase III were used to remove HS/H and retain CS and DS.

2.3. Electrophoresis of glycosaminoglycans

Total plasma GAGs, and particular types of GAG were subjected to cellulose acetate electrophoresis employing the method elaborated in our laboratory, previously described [12]. The obtained electrophoregrams were subjected to a densitometric analysis, by using a computerized gel documentation system G:BOX (Syngene, USA). On basis of the observed results, proportional relationships between total GAGs and particular types of sulfated GAG were calculated.

The identity of electophoretic bands was confirmed by comparison of electrophoretic patterns of plasma GAG samples submitted to electrophoresis without any previous treatment and depolymerized with agents specifically eliminating particular GAG types. Treatment of isolated plasma GAGs with chondroitinase ABC made possible to destroy CS, DS and retain HS/H. Comparison of

Table 1
Clinical characteristics of control subjects and JIA' disease patients.

Parameter	Control subjects (n = 30)	Untreated JIA (n = 30)	JIA′ disease after attainment of clinical improvement (n = 30)
Age (years)	8.67 ± 4.12	7.27 ± 4.49	8.21 ± 4.01
Sex, female/male	17/13	19/11	19/11
WBC (10^3/μl)	7.32 ± 2.19	10.04 ± 4.03	7.14 ± 2.31
RBC (10^6/μl)	4.95 ± 0.35	4.48 ± 0.41	4.62 ± 0.36
Hb (g/dl)	13.85 ± 0.94	11.58 ± 1.38	12.97 ± 1.16
Ht (%)	40.96 ± 3.18	35.35 ± 3.61	37.17 ± 7.39
PLT (10^3/μl)	283.47 ± 73.83	405.47 ± 129.10	351.20 ± 93.78
Total cholesterol (mM)	4.12 ± 0.87	4.61 ± 1.42	4.25 ± 1.65
Glucose (mM)	4.56 ± 0.38	4.11 ± 1.16	4.40 ± 0,98
Creatinine (μM)	80.01 ± 12.66	52.97 ± 10.23	64.54 ± 15.47
CRP (mg/l)	1.24 ± 1.59	20.25 ± 24.00	2.76 ± 0.57
ESR (mm/h)	10.50 ± 7.03	42.00 ± 27.021	13.11 ± 7.21
ANA	–	56% (positive)	56% (positive)
RF	–	100% (negative)	100% (negative)

Results are expressed as mean ± SD; WBC, white blood cell; RBC, red blood cell; Hb, hemoglobin; Ht, hematocrit; PLT, platelet; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; ANA, antinuclear antibodies; RF, rheumatoid factor.

Table 2
The distribution pattern of plasma and urinary glycosaminoglycans in the healthy controls and juvenile idiopathic arthritis' patients.

Parameter	Control group (n = 30)	Non-treated JIA patients (n = 30)	JIA patients after treatment and attainment of clinical improvement (n = 30)
Plasma CS *(hexuronic acids, mg/L)*	95.88 ± 35.14	40.11 ± 15.07[b]	76.91 ± 10.26[a,d]
Plasma DS *(hexuronic acids, mg/L)*	25.15 ± 6.99	36.58 ± 12.71[a]	29.30 ± 8.79[c]
Plasma HS/H *(hexuronic acids, mg/L)*	20.06 ± 13.17	18.87 ± 11.15	19.21 ± 11.30
Plasma HA *(ng/mL)*	35.59 ± 9.10	54.50 ± 25.23[b]	32.42 ± 10.26[d]
Urinary CS/DS *(mg/g of creatinine)*	3.47 ± 0.92	2.82 ± 1.04[a]	3.20 ± 1.12
Urinary HS *(mg/g of creatinine)*	0.86 ± 0.37	1.12 ± 058	0.78 ± 0.35
Urinary HA *(mg/g of creatinine)*	0.018 ± 0.007	0.013 ± 0.004[a]	0.014 ± 0.006

Results are expressed as mean ± SD; [a]$p<0.05$, [b]$p < 0.001$ compared to control group; [c]$p < 0.05$, [d]$p < 0.001$ compared to untreated JIA patients; JIA, juvenile idiopathic arthritis; CS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate; H, heparin; HA, hyaluronic acid.

electrophoretic patterns of intact material containing CS, DS and HS/H with those obtained after chondroitinase ABC digestion allowed localizing CS and DS in electrophoregrams. Digestion of isolated GAG samples with chondroitinase B was performed in order to remove DS. Material resistant to chondroitinase B but susceptible to chondroitinase ABC was identified as CS. HS/H were identified as material susceptible to heparinase I and heparinase III digestion.

2.4. Measurement of plasma and urine hyaluronic acid level

HA levels were measured in duplicate using a TECO®Hyaluronic acid test kit provided by TECOmedical AG (Sissach, Switzerland) according to the manufacturer's instructions. The analytical sensitivity was at 13.3 ng/mL. The appropriate low and high control samples were used for quality control procedure. The intra-assay variation of the HA levels was less than 6%.

2.5. The assay of urinary CS/DS and HS concentration

The concentration of sulfated GAGs i.e. CS/DS and HS in urine samples was determined with the use of a microplate method and the Blyscan-Sulfated-Glycosaminoglycan-Assay from Biocolor Ltd. (Northern Ireland, UK). The reaction between GAGs present in the samples and a stain reagent, i.e. 1,9-dimethylmethylene blue (DMB), was used in the assay. This reaction results in a colored complex (GAGs-DMB) from which GAGs are released after adding a dissociation reagent. Then, GAGs were quantified spectrophotometrically and total concentration of glycans in urine was determined with analytical sensitivity of 0.5 μg/100 μL and calibration range from 0.5 to 5 μg/100 μL. The coefficient of intra-assay variation was less than 5.6%.

For the quantitative measurement of CS/DS, isolation procedure of these glycans was performed. It consisted of removing the HS fraction from the samples received at an earlier stage, containing a total pool of urinary GAGs. Degradation of HS was performed with nitrous acid treatment. Use of isolation procedure allowed also quantitate HS as a percentage difference between sulfated GAGs concentration in urine sample with and without nitrous acid treatment. Urine GAG concentrations were expressed in terms of g of creatinine. Creatinine concentration was determined with the use of kit from BioSystems S.A. (Barcelona, Spain). The intra-assay CV was lower than 3%.

2.6. Statistical analysis

A statistical analysis was carried out using Statistica 10.0 package (StatSoft, Cracow, Poland). The normality of distribution was verified with the Shapiro–Wilk test. The data obtained were expressed as mean values and standard deviation. Since the variables were normally distributed, the parametric Student's t-test was used to evaluate the differences between untied variables. To compare the same parameters in each patient, before the treatment and after the restoration of clinical improvement, the paired Student's t-test was used. The Pearson's correlation coefficient was employed for the statistical analysis of correlations between two variables. P-values of less than 0.05 were considered significant.

3. Results

We found that JIA results in qualitative and quantitative changes in plasma GAGs. The results are presented in Table 2. CS were predominant GAGs constituents in plasma of all investigated subjects. It was observed that the CS concentration decreased in plasma of untreated JIA patients. The untreated patients had a more than 2-fold lower ($p < 0.001$) mean concentration of CS than the controls. It was also proven that the used therapy brought about a significant increase ($p < 0.001$) in CS concentration compared with the pre-treatment concentration. However, JIA patients after clinical improvement had plasma CS content still markedly ($p < 0.05$) decreased as compared to the values of the healthy subjects. Plasma concentration of DS was significantly increased in patients with untreated JIA. Compared to the control values, the mean increase in DS levels was 45% ($p < 0.05$), and 25% ($p < 0.05$) compared to treated JIA patients. The blood concentration of DS in treated children with JIA corresponded to its concentration in the control group. In the case of HS/H, the concentrations of these glycans were similar in all three groups of studied individuals. A quantitative assessment of HA revealed significantly higher ($p < 0.001$) levels of the parameter in the plasma of untreated JIA patients than in the control group. Compared to the control values, the mean increase in plasma HA was 53%. Antiinflammatory treatment led to a significant decrease ($p < 0.001$) in HA concentration vs. pre-treatment situation. The pharmacological therapy normalized the concentrations of mentioned GAGs.

As shown in Table 2, in JIA patients the concentrations of particular urinary GAGs were characterized by different trends of alterations. Thus, when CS/DS and HA levels decreased significantly in urine of untreated children with JIA, the concentration of HS was found to be unchanged in JIA patients when compared to the controls. The patients had a 19% lower ($p < 0.05$) level of CS/DS and a 28% lower ($p < 0.05$) level of HA than the controls. Antiinflammatory treatment led to a normalization of the concentrations of urinary CS/DS and HA.

The analysis of the relations between an inflammatory indicator which is routinely assayed, i.e. CRP, and particular plasma and urinary GAGs concentration, revealed the relationship between these parameters only in children with newly diagnosed and untreated JIA. The obtained values were as follows: CRP and plasma CS ($r = -0.63, p = 0.036$), plasma DS ($r = 0.59, p = 0.031$), plasma HA

($r = 0.68, p = 0.01$), urinary HA ($r = -0.56, p < 0.001$), respectively. We recorded insignificant relationships between CRP and plasma HS/H ($r = 0.05, p = 0.861$), urinary CS/DS ($r = -0.29, p = 0.421$) as well as urinary HS/H ($r = 0.37, p = 0.42$) in untreated patients with JIA. Furthermore, a correlation analysis, revealed that in the untreated JIA patients there was a significant correlation between plasma HA level and urinary HA ($r = 0.47, p = 0.023$). No correlation was recorded between plasma CS/DS level and urinary CS/DS ($r = 0.01, p = 0.901$) as well as between plasma HS/H and urinary HS ($r = 0.04, p = 0.91$). Similarly, in the treated JIA patients there was a significant correlation only between plasma HA level and urinary HA ($r = 0.42, p = 0.043$).

4. Discussion

The GAGs present in the blood are derived from many different cells and tissues including the platelets, liver or cartilage. CS represent the major GAGs in circulation of adults, while DS, HS/H and HA occur in smaller amounts [3,12]. KS are present in blood only in trace amounts, which results from their specific localization in tissue [13]. Similar proportions of plasma GAGs were observed by us in blood of healthy children. Whereas in JIA children we recorded differences represented by a decrease in CS and an increase of DS and HA levels. The level of HS/H remained constant during the course of JIA. However, we cannot compare our results with those obtained by other researchers since the profile of plasma GAGs has not been estimated in children with JIA so far. An exception an increase of HA level in JIA children was found [14,15]. The authors suggest that serum HA measurement is useful for diagnosing systemic and polyarticular JIA [14,15]. We are of similar opinion since we proved that treatment leading to the achievement of clinical improvement in children simultaneously contributes to normalization of this GAG concentration. It is also difficult to compare our results with those obtained in adults with rheumatoid arthritis [5]. Despite the clinical similarities between both diseases, the young age of patients with JIA could be the main factor responsible for observed changes of different GAG types in plasma. Our previous studies demonstrated that the plasma concentration of the various GAG types is dependent on the age of the subject [16].

The profile of plasma GAGs which is observed in young patients seems to reflect different sensitivity of particular glycan fractions to the effects of factors leading to JIA. Besides genetic predispositions, a significant role in JIA development is played by autoimmune disorders enhanced by proinflammatory cytokines (IL-1, IL-6, TNF-α) and growth factors (TGF-β), infectious agents (*streptococcus, mycoplasma pneumonia, parvovirus B19)* or hyperactivity of agents degrading articular structures (MMP, ROS) [6,9,10,17–19]. The listed cytokines and growth factors, which are agents stimulating biosynthesis of GAGs, seem to have a special influence on high plasma levels of HA and DS in untreated JIA children [4,7,8]. The IL-1β, TNF-α and -β are involved especially in the up-regulation of the expression of HA by nuclear factor-κB pathway, while IL-6 and TGF-β is more affiliated with the synthesis of DS [4,9,20,21]. At the same time, these factors were shown to significantly increase the expression of ECM components' degrading proteases [1,22,23]. Exactly the changes in CS appear to be more related to tissue degradation of their proteoglycan cores, which occurs due to the effect of MMPs, especially MMP-3, aggrecanases, especially aggrecanase-1, as well as ROS [6,11,23,24]. In the course of JIA, the activity of the proteolytic enzymes is increased, and oxidative stress is also observed [6,19].

Clinical consequences of changes in plasma GAG concentrations in JIA patients result not only from alterations in the ECM structure and function but, due to multifunctionality of glycans, these consequences can be diversified. For instance, due to ability to modify resistance to infectious diseases by dermatan sulfate, this glycan might be a favorable factor for JIA development. On the one hand, this hypothesis is based on the fact that the JIA development is linked to infections and a post-infectious period [8,19]. On the other hand, DS appears to be involved in infection through mechanisms dependent on microbial adherence. Furthermore, free DS binds to and inactivates the neutrophil-derived cationic antimicrobial peptide α-defensin and cathelicidins, i.e. small cationic peptides essential for resistance to infection [25,26]. Increased susceptibility to infections in children with immunological predisposition may lead to manifestation of JIA. Here demonstrated high blood concentration of DS in untreated JIA patients, which correlates positively with CRP, could favour, according to the above mentioned mechanism, manifestation of the disease. What is more, high concentrations of plasma HA detected in patients also seem to facilitate the JIA development. It is known that tissue HA undergoes rapid degradation at inflammation sites resulting in the accumulation of lower molecular weight HA fragments, which then enter the blood [27]. It has been reported that low molecular weight degradation products of HA are potent inflammatory agents [4,27–29]. Low molecular weight but not native HA has been shown to stimulate the expression of IL-1β, TNF-α, and insulin-like growth factor in murine bone marrow-derived macrophages [29]. HA fragments have also been shown to elicit the upregulation of inducible nitric oxide synthase in macrophages and the stimulation of cyclooxygenase-2 (COX-2) and prostaglandins in epithelial cells [28].

In contrast, the alteration of CS during JIA appear to be more complex than the alterations of the remaining glycan fractions, and more strongly related to degradation processes than to an increased biosynthesis. The changes in concentration of the CS may reflect the defense potential against inflammatory factors and oxidative stress leading to JIA. It is known that the CS significantly reduce inflammatory symptoms and delay the progression of cartilage destruction by reduction of the activity of MMP-1, -3, -13 and aggrecanases-4 and -5 [30–32]. The anti-inflammatory effects of CS depend on inhibiting the expression and activity of pro-inflammatory enzymes – COX-2, phospholipase A2, nitric oxide synthase-2, and reducing the concentrations of cytokines – TNF-α, IL-1β as well as on inhibiting ROS [12,30–32]. Hence, we can conclude that a decreased CS concentration, negatively correlating with CRP, may reflect both, depletion of these GAGs due to their anti-inflammatory and antioxidant action, and their increased degradation. The latter process occur probably much earlier in children, even before manifestation of clinical symptoms of JIA. Increased degradation of tissue CS, which is not compensated by the rate of synthesis process, results in disturbances of the cell signaling process which involves interactions with cytokines, growth factors or ROS [2,3,30]. As a result, the JIA can manifest itself. The multiplicity effects of CS support the hypothesis that these GAGs could be a beneficial agent slowing down development of diseases with an inflammatory component, such as JIA.

Transformation pathways of ECM components in the course of JIA appear to be complex. Treatment of inflammation, which simultaneously alleviate symptoms by reducing pain and inflammation, did not contribute to the normalization of CS concentrations in patients' blood. These results indicate that the metabolism of the GAGs seems to be also associated with autoimmune processes. In the systemic circulation and in the synovial fluid of rheumatoid arthritis patients, the presence of GAG-specific antibodies was revealed [33,34]. Their synthesis is up-regulated possibly because of the extensive release of cartilage molecules. It is concluded that during the active phase of the disease, the levels of anti-GAG antibodies are reduced because of binding of these

antibodies to GAGs released from the degrading cartilage [33]. As a result, the pool of plasma GAGs may also diminish.

Another mechanism of alterations of particular types of GAGs concentration in the blood could be related to impaired excretion of the molecules in urine [6,26]. However, disturbed excretion of GAGs in urine was not observed in JIA patients. What is more, we did not prove any relations between the concentration of sulfated plasma GAGs and their renal excretion rate. The exception is a lower rate of HA excretion in untreated children, correlating positively with plasma HA concentration. Differently from us, in adult patients with rheumatoid diseases was demonstrated increased urinary HA excretion [35,36]. Considering the strong effect of age on GAGs excretion in the urine [37], it is difficult to compare our findings with the results of the cited above work. The observed urinary HA levels in JIA patients, which might result from enhanced degradation of this type of GAG, taking place with hyaluronidases action in the liver or kidneys and the spleen. Although several of these enzymes are expressed in the mentioned organs [27,28], their enhanced expression in children with JIA have not been proven.

Our results indicate that in the course of JIA qualitative and quantitative changes in plasma and urinary glycosaminoglycans occur. There are probably the expression of both local articular cartilage matrix and systemic changes in connective tissue remodeling and may play a complex role in the pathogenesis of the disease.

Financial disclosure

No financial interests to disclose.

Conflict of interests

The authors declare that they have no competing interests.

The Authors: Katarzyna Winsz-Szczotka, Kornelia Kuźnik-Trocha, Katarzyna Komosińska-Vassev, Grzegorz Wisowski, Anna Gruenpeter, Iwona Lachór-Motyka, Bogusław Żegleń, Wojciech Lemski, Krystyna Olczyk of the manuscript, entitled: “Plasma and urinary glycosaminoglycans in the course of juvenile idiopathic arthritis” submitted to *Biochemical and Biophysical Research Communications* declare any conflict of interest.

Authors' contributions

All authors participated and were involved in the conception and design drafting the manuscript or revising it critically for intellectual content, and approved the final version of the paper before submission.

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