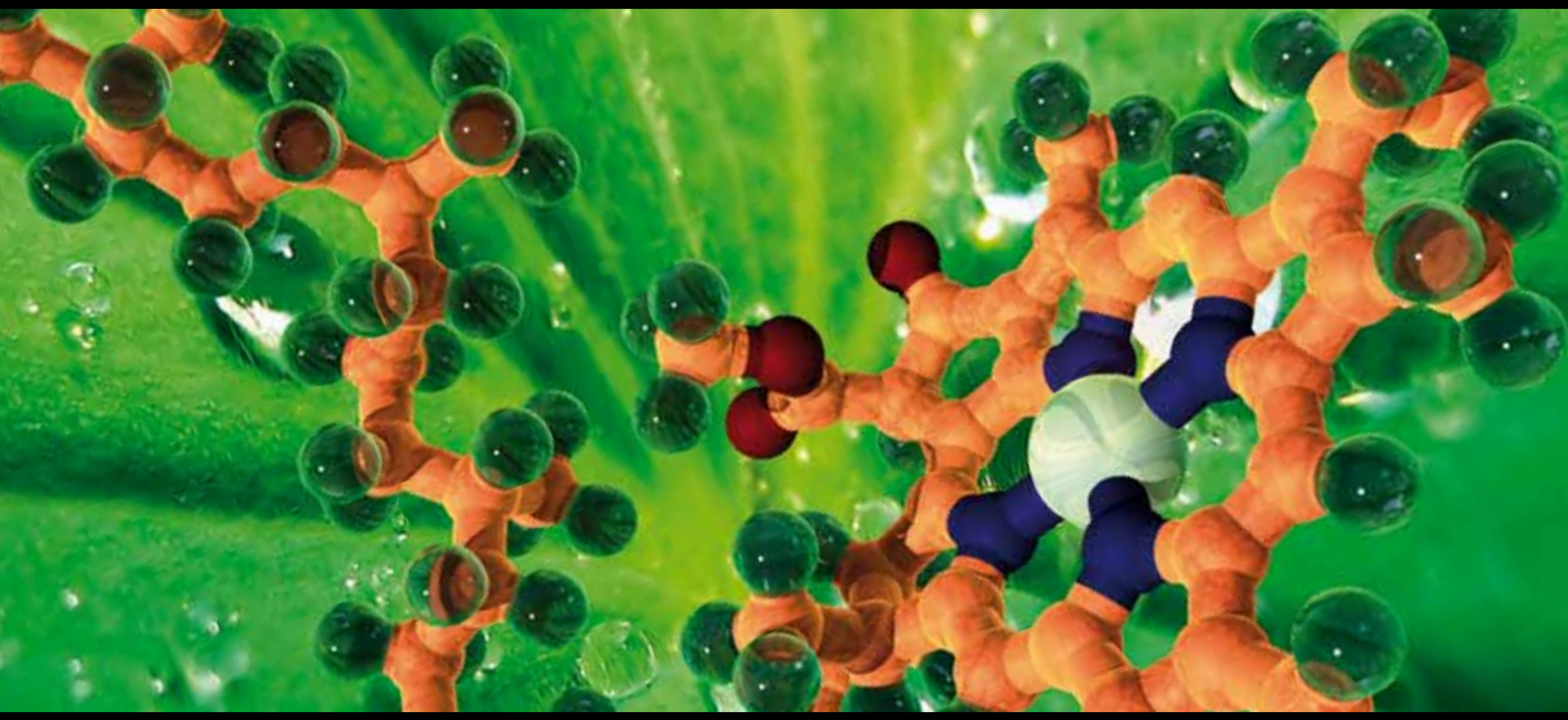
Biochemistry Research International

Glycosaminoglycans Metabolism

Guest Editors: Manuela Viola, Timothy E. L. Douglas, Laura Alaniz, and Barbara Bartolini



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Editorial Board

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Hindawi Publishing Corporation
Biochemistry Research International
Volume 2012, Article ID 245792, 2 pages
doi:10.1155/2012/245792

Editorial
Glycosaminoglycans Metabolism

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Received 2 January 2012; Accepted 2 January 2012

Glycosaminoglycans (GAGs) are unbranched, polysaccharide chains which, with the exception of hyaluronan (HA), are highly sulphated and constitute the glucidic moieties of proteoglycans (PGs) macromolecules. Depending on monosaccharide composition and protein linkage region, as well as sulphation pattern and degree, GAGs can be grouped in four subfamilies named chondroitin/dermatan sulphate (CS/DS), heparan sulphate/heparin (HS/HE), hyaluronan (HA), and keratan sulphate (KS).

GAGs contribute to the PGs native folding and functions, as well as to tissue and organ behavior. In fact, GAGs are involved in stabilization of the fibrillar extracellular matrix (ECM), control of hydration, regulation of tissue, and organism development by controlling cell cycle, cell behavior, and differentiation. HA synthesis is a process carried out on plasma membrane by specific enzymes called Hyaluronan synthases. The other GAGs chains are polymerized in the ER and Golgi compartments by specific enzymes drawn close in a complex named GAGosome. The modification of the synthesis of the GAG portion of PGs or HA can alter significantly the ECM structure and composition, with multiple effects, leading to physiological events such as tissue ageing and pregnancy or pathological events: kidney agenesis, cardiac malformations, abnormal mast cells, somatic overgrowth, lung dysfunction, chondrodysplasia, tumor progression, and fibrosis process.

On the other hand, the same studies about GAGs involvement in cell differentiation have led in the last decade to the use of GAG chains in the preparation of innovative and biocompatible materials. GAGs are incorporated into polymer scaffolds for tissue regeneration in order to improve their physiochemical properties, such as water-binding ability, and to influence cell behavior. Certain biomaterials contain GAGs as their main component. For example, in biomaterials aimed to blood contact, heparin is applied to counteract blood clotting. Furthermore, growth factors are often applied together with scaffolds and GAGs, due to the well known ability of various GAGs to bind specific growth factors and to modulate their activity and bioavailability.

In this special issue on GAGs, we have invited papers that address such issues, both by original work and reviews.

One of the papers of this special issue shows that CS/DS GAGs and HA are differently distributed between pericardium and valves and within heart valves themselves before and after decellularization. Distribution of glycosaminoglycans is also dependent from the vascular district and topographic localization. Data presented suggest that both decellularized porcine heart valves and bovine pericardium represent a promising material for future development of tissue engineered heart valve scaffolds.

The paper by E. Zinellu et al. compares the amount and sulphation characteristic of CS present in plasma of patients with hard and soft carotid arteriosclerotic plaques to the one found in plasma of healthy volunteers. The authors suggest that the differences evidenced in the paper could be used to develop new diagnostic tests for atherosclerosis.

The authors of the paper entitled "*Glycosaminoglycan storage disorders: a review*" reviewed the clinical consequences of alteration in degradation of GAGs. Intralysosomal accumulation of undegraded products causes a group of lysosomal storage disorders known as mucopolysaccharidoses (MPSs). They provide an overview of the molecular

basis, enzymatic defects, clinical manifestations and diagnosis of each MPSs, focusing also on the available animal models and describing potential perspectives of therapy for each one.

Other authors documented the effects on diabetic nephropathy of the treatment with low-molecular-weight heparin (dalteparin, LMW-HE). Patients with type 2 diabetes and with neuro-ischemic foot ulcers were given LMW-HE for a maximum of six months. The authors showed that LMW-HE is beneficial for the outcome of neuro-ischemic foot ulcers and it has no effect on glomerular function despite an increment of excretion of GAGs. Moreover they stated that urinary IgM and IgG (consequence of alterations of the size-selective properties of the glomerular capillary wall) seem to be better markers than albuminuria for detecting and predicting renal injury in the patients. Evidences from this paper contribute to highlight the complex role of the GAG heparin, which can influence directly and indirectly the structure and the function of the glomerular capillary wall and therefore plays a pivotal role in diabetes and kidney diseases.

One of the papers investigates more on the same topic, that is, the importance of GAGs distribution on the endothelial surface, by focusing on a different pathology. Starting from defining the problem of acute coronary syndrome, the authors described the importance of a good reperfusion therapy on the recovery of a proper microcirculation in damaged heart tissue. An adequate capillary circulation is a necessary prerequisite for normal perfusion and organ functioning, and a no-reflow phenomenon is defined as incomplete reperfusion at the microcirculatory level. Under this perspective, the composition of the endothelial glycocalyx in terms of GAGs, PGs, and glycoproteins is significant in defining its role as protective layer of the vascular wall. The no-reflow phenomenon is therefore strictly related to the composition of the glycocalyx, and the authors discuss how the deep knowledge of this layer (drug targeting, ultrastructure, and degradation) could be helpful in the best reperfusion outcome.

Another paper reviews the occurrences, the synthesis, and the catabolism of hyaluronan, followed by a description of HA receptors and the relevance of the chain length in affecting different aspects of cell and tissue behavior. The authors also mention the role of hyaluronan in malignancy and skin, discussing its new recently discovered functions.

In the same field, the paper by M. A. Solis et al. of the issue combines the review of hyaluronan properties and biological activities (strictly dependent on its dimension and receptors) with the research for a perfect microenvironment in which stem cells can be grown and/or differentiated. HA has a pivotal role in development and differentiation of cells, tissue, and organism, and stem cells present most of the HA receptor on their membrane, indicating the perfect match for their appropriate niche biomaterial.

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Hindawi Publishing Corporation
Biochemistry Research International
Volume 2012, Article ID 979351, 10 pages
doi:10.1155/2012/979351

Research Article

Fine Structure of Glycosaminoglycans from Fresh and Decellularized Porcine Cardiac Valves and Pericardium

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Received 30 June 2011; Revised 18 October 2011; Accepted 27 October 2011

Academic Editor: Laura Alaniz

Cardiac valves are dynamic structures, exhibiting a highly specialized architecture consisting of cells and extracellular matrix with a relevant proteoglycan and glycosaminoglycan content, collagen and elastic fibers. Biological valve substitutes are obtained from xenogenic cardiac and pericardial tissues. To overcome the limits of such non viable substitutes, tissue engineering approaches emerged to create cell repopulated decellularized scaffolds. This study was performed to determine the glycosaminoglycans content, distribution, and disaccharides composition in porcine aortic and pulmonary valves and in pericardium before and after a detergent-based decellularization procedure. The fine structural characteristics of galactosaminoglycans chondroitin sulfate and dermatan sulfate were examined by FACE. Furthermore, the mechanical properties of decellularized pericardium and its propensity to be repopulated by in vitro seeded fibroblasts were investigated. Results show that galactosaminoglycans and hyaluronan are differently distributed between pericardium and valves and within heart valves themselves before and after decellularization. The distribution of glycosaminoglycans is also dependent from the vascular district and topographic localization. The decellularization protocol adopted resulted in a relevant but not selective depletion of galactosaminoglycans. As a whole, data suggest that both decellularized porcine heart valves and bovine pericardium represent promising materials bearing the potential for future development of tissue engineered heart valve scaffolds.

1. Introduction

Heart valve disease has a deep impact worldwide related with the large number of valvular replacement operations performed every year. Typical valve substitutes are mechanical prostheses and bioprostheses obtained from cardiac-valvulated conduits (aortic and pulmonary root) or pericardial tissue of porcine and bovine origin. Bioprosthetic valves, although associated with a lower risk of thromboembolism with respect to the mechanical ones, possess limited longevity due to dystrophic calcification consequent to glutaraldehyde (GA) treatment used for preventing rejection [1] and suffer for many of the same degenerative processes that afflict native valves [2]. In the last years, tissue engineering (TE) approaches raised in response to limitations associated with tissue and organ transplantation and with the scarcity of available donors. Three are the components essential for a TE substitute: cells, scaffolds (designed to maintain the cells in a three-dimensional environment), and signals that guide the gene expression and ECM production during tissue development [3]. The typical approach relies on the use of acellular matrix xenografts (mostly porcine and bovine) as scaffold that would ultimately become repopulated with cells from the patient [4]. Repopulation of natural matrix xenografts has been proposed for its greater chance of success with respect to that of biopolymeric scaffolds [5, 6]. The surgical procedures, used to date for the implantation of commercially available devices, are represented by the

transcatheter aortic valve replacement (TAVI) technique and the open chest classical method. The use of TAVI is indeed emerging especially in patients with severe aortic stenosis and multiple comorbidities that might preclude open chest valve replacements [4]. Most of these expandable grafts are built within pericardial tissue.

The relative amounts and distributions of glycosaminoglycans/proteoglycans (GAGs/PGs) have been reported to be different according to the type of mechanical loading [7, 8]. The knowledge of the composition and distribution of the various GAGs and PGs appears to be essential for understanding the relationship between structure and mechanics of heart valve leaflets [9]. The influence of GAGs and PGs on cell migration, proliferation, and differentiation is well known, besides their role in morphogenesis, angiogenesis, wound healing, immune responses, maintenance of tissue viscoelasticity, and resistance to compression and tension [7, 10, 11].

The maintenance of structural and functional ECM integrity is of primary importance for the performance of the valve substitutes; consequently, their depletion or alteration could be responsible for graft deterioration [12].

GAGs are capable of absorbing a large amount of water within the tissue matrix, due to their high concentration of negative charges and hydrophilicity. This reason led to considering them as fundamental components for the mechanical behavior, given the ability to hydrate the spongiosa layer (decreasing the shear stresses associated with cuspal flexure in valve function), and to absorb compressive forces reducing buckling during flexion [13–16]. Furthermore, their high negative charge may reduce the calcification process by chelating calcium ions. These observations suggest that the loss of the GAGs might dramatically compromise the mechanical function, structure, and/or the onset of dystrophic calcification of bioprosthetic heart valves [17, 18].

A loss of GAGs has been described in both fixed tissues, during the preparation of bioprostheses, and in cryopreserved native tissues [19], with possible consequences on the graft performance. Reports characterizing valve GAGs are largely referred to bioprosthetic valves and tissue-engineered heart valves (TEHVs) [14, 16, 19], whereas only few investigations concerned GAG distribution in the native valve [20–22] and their fine structure. The structural properties of GAGs, such as the extent and pattern of sulfation, the charge density, and the epimerization of their uronic acid moiety are thought to be critical for their function and cellular signaling [23–25]. In vivo each of them can be modulated mediating several biological processes that promote the interaction with different ECM molecules and cells. Recently, we reported the impact of detergent based cell removal on structural components distribution and hydration in aortic and pulmonary heart valve conduits highlighting a relevant depletion of GAGs [26]. This study is focused on the fine structural analysis of the most abundant galactosaminoglycans (GalAGs), that is, the chondroitin sulfate (CS) isomers. The aim of the present study was to characterize the distribution as well as the fine structure of GalAGs in native and decellularized porcine cardiac valves and pericardium due to their extensive use as bioprosthetic material in heart valve replacement. Moreover, we tested the mechanical properties of decellularized-treated pericardium and its propension to be repopulated by in vitro seeded fibroblasts.

2. Materials and Methods

2.1. Chemicals. Standard preparations of ΔDi-nonS_{cs}, 2-acetamido-2-deoxy-3-0-(4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid)-4-D-galactose; ΔDi-mono6S, 2-acetamido-2-deoxy-3-0-(4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid)-6-O-sulpho-D-galactose; ΔDi-mono4S, 2-acetamido-2-deoxy-3-0-(4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid)-4-O-sulpho-D-galactose were all purchased from Seikagaku (Tokyo, Japan); ΔDi-nonS_{HA}, 2-acetamido-2-deoxy-3-O-(4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid)-4-D-glucose; ΔDi-mono2S, 2-acetamido-2-deoxy-3-O-(4-deoxy-2-O-sulpho-α-L-threo-hex-4-enopyranosyluronic acid)-D-galactose; ΔDi-di(2,4)S, 2-acetamido-2-deoxy-3-O-(4-deoxy-2-O-sulfo-α-L-threo-hex-4-enopyranosyluronic acid)-4-O-sulfo-D-galactose; ΔDi-di(4,6)S, 2-acetamido-2-deoxy-3-O-(4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid)-4,6-O-sulpho-D-galactose were purchased from Dextra Laboratories. Papain from Papaya latex (EC 3.4.22.2), chondroitin ABC lyase from *Proteus vulgaris* (EC 4.2.2.4), chondroitin AC lyase from *Arthrobacter Aurescens* (4.2.2.5), AMAC (>98%), glacial acetic acid, sodium acetate, chloride acid, DMSO (99.9%), sodium cyanoborohydride, cysteine, sodium chloride, and $EDTANa_2$ were all obtained from Sigma-Aldrich. Acrylamide and N,N′-methylenebisacrylamide were from BioRad, TEMED (99%), and ammonium persulfate (98%) were purchased from Sigma-Aldrich. DEAE Sephacel was from Amersham Biosciences. All other chemicals used were of analytical reagent grade.

2.2. Harvesting and Tissue Analysis. Porcine hearts from 10–12-month-old pigs with weights ranging from 160 to 180 kg were obtained from a local slaughterhouse. Within 2 hours from death, 12 dissected heart valve conduits, aortic and pulmonary roots (AR and PR), as well as a pool of pericardia ($n = 6$), were harvested and decellularized according to a previously described detergent-based procedure [26]. The pool of pericardial tissue was divided into 2 parts. One was considered as a control and the other subjected to decellularization. The valve conduits were divided into 2 groups: 12 ARs and 12 PRs; for each group 6 samples were considered as control and referred to as NT and 6 samples decellularized with Triton X-100 and Sodium Cholate and referred to as TriCol.

NT samples were rinsed in isotonic saline solution and immediately processed. Briefly, TriCol samples were extracted in hypotonic solution using 1% (w/v) Triton X-100 in presence of protease inhibitors (PI) at 4°C. Following treatment in hypertonic conditions, tissues are extracted in 10 mM sodium cholate at room temperature [26, 27]. After extensive washings for detergent removal, each AR and PR sample was cut into three zones corresponding to: aortic and pulmonary wall, sinus area, and leaflet. Fatty adherences

were removed from pericardia by gentle peeling. Wet weight of each valve conduit component and pericardial sample was determined after gently blotting with filter paper (Whatman filter 147 paper number 3). Valve and pericardial minced tissues were dehydrated with 20 volumes of acetone at 4°C for 24 h, defatted with 20 volumes of chloroform: methanol (2 : 1, vol/vol) at 4°C for 24 h, dried for 24 h at 60°C after centrifugation at 3300 ×g for 15 minutes, and finally weighed (dry-defatted tissue (DDT) weight).

2.3. Extraction and Purification of Total GAGs. DDTs (100 mg) were rehydrated for 24 h at 4°C in 0.1 M sodium acetate, pH 6.0, containing 5 mM cysteine, and 5 mM ethylenediaminetetraacetic acid (37 volumes per gram of DDT). Then, papain (0.3 U/mg of DDT) was added to the mixture, which was incubated at 56°C for 48 h under mild agitation. The digest was clarified by centrifugation (9000 ×g for 20 min at +4°C). Digest supernatant was loaded on a (diethylamino)ethyl-cellulose column (0.7 × 6 cm, 2.3 mL), equilibrated with 50 mM sodium acetate, pH 6.0. The column was then washed with 50 mL of the same buffer and eluted with a two-step salt gradient (0.55 and 1.0 M NaCl). Fractions of 1 mL were collected and assayed for hexuronate content by the method of Bitter and Muir, using glucuronolactone as a standard [28]. Total GAG concentrations were estimated by summing the contents of the two elution steps. Fractions containing GAGs from both elution steps were pooled and precipitated using 4 volumes of cold absolute ethanol. The mixture was left overnight at −20°C, and the precipitate was separated by centrifugation, washed twice with ethanol, and then dried.

2.4. Fluorophore-Assisted Carbohydrate Electrophoresis (FACE) Analysis. FACE was used to analyze the quantity and fine structure of the different GAG classes, mainly HA and chondroitin/dermatan sulfates (CS/DS). This technology can quickly provide characteristics about the sulfation and iduronation patterns of GalAGs, providing clues to the identification of particular PGs within valve tissues. The enzymes used for the degradation of the CS isomers belong to the family of chondro/dermato lyases. Chondroitinase ABC cleaves the glycosidic bond between hexosamine and glucuronate (GlcA) so degrading hyaluronan (HA) and all chondroitin sulfate isomers (CS A, CS C, and DS), producing disaccharide units containing α, β-unsaturated hexuronic acids: ΔDi-0S_{HA}, ΔDi-0S_{CS}, ΔDi-mono4S, ΔDi-mono6S. Chondroitinase AC II acts on the same bond, but it is not able to cleave glycosidic bond between hexosamine and iduronate (IdoA), so it completely degrades the CS chains and, to some extent, the DS ones. In the present paper, CS is defined as only containing glucuronate, while DS is defined as containing some quantity of iduronate.

Dried GAGs were dissolved in 100 μL of 100 mM ammonium acetate, pH 8.0. Separate digestions with chondroitin ABC and AC II lyases were performed at 37°C for 24 h, using 0.1 U per 100 μg hexuronic acid. The digestion mixture was boiled for 1 min to inactivate the enzyme, centrifuged at 11000 ×g for 5 min, and vacuum-dried. The free reducing groups exposed by enzyme cleavage can be fluorescently tagged with 2-aminoacridone (AMAC) by reductive amination in presence of sodium cyanoborohydride ($NaBH_3CN$) [29, 30]. This method allows the labeling of the reducing ends of unsaturated disaccharides obtained after enzymatic degradation of GAG chains, improving dramatically the sensitivity of various analytical techniques used for identification and quantitation of GAGs [30, 31].

Briefly, 40 μL of 12.5 mM AMAC solution in glacial acetic acid/DMSO (3 : 17 v/v) was added to the lyophilized sample aliquots, and samples were incubated for 10–15 minutes at room temperature. Then 40 μL of 1.25 M $NaBH_3CN$ in ultrapure water was added to each sample, and the mixtures were incubated at 45°C for 4 hours. After derivatization, 20 μL of glycerol (20% final concentration) was added to each sample prior electrophoresis. PAGE was performed according to Karousou et al. [31], in a Mini-Protean II cell vertical slab gel electrophoresis apparatus (Bio-Rad). Electrophoresis was performed in 0.15 M Tris-borate, pH 8.8, at 400 V and 4°C. Gels were scanned in a UV-light box using a CCD camera (Gel Doc XR System) and analyzed with Quantity One 4.6.3 from Bio-Rad Laboratories. For quantitation of Δ-disaccharides, a CS calibration curve was built using commercial chondroitin sulfate A subjected to chondroitin ABC and AC lyase treatment and derivatization procedure.

The ratios of glucuronate to iduronate containing disaccharides and 4-sulfation to 6-sulfation were calculated.

The GAG class levels from each sample as measured by FACE were normalized to the DDT weight to estimate the tissue concentrations.

2.5. Isolation and Seeding of Bovine Fibroblasts on TriCol-Treated Pericardium. Bovine fibroblasts were isolated from bovine pericardium by 2 mg/mL collagenase II digestion and cultured in DMEM HEPES (Sigma, St.Louis, USA) modification (10% FBS, 1% glutamine, and 1% penicillin/streptomycin) at 37°C and 5% CO_2. Cells were left in culture up to the 3rd passage.

Seeding was performed on 1.77 cm^2 of circle-shaped TriCol-treated pericardium samples in 24-well flat bottom-culture plate (BD, NJ, USA). Suspension of 750,000 cells/cm^2 in 1 mL DMEM HEPES (Sigma, St.Louis, USA) modification (10% FBS, 1% glutamine, and 1% penicillin/streptomycin) was distributed onto each pericardial sample. After 7 days of culture in incubator, the specimens were collected and processed for histological evaluation.

2.6. Histological Staining. To assess the presence, surface spreading and penetration of fibroblasts after seven days in vitro culture on TriCol-treated pericardium, specimens were embedded in Optimal Cutting Temperature Medium (OCT Bioptica, Milano, Italy), sectioned at 8 μm and stained for hematoxylin and eosin (H and E) (Rapid Frozen Section Kit, Bioptica, Milano, Italy).

2.7. Mechanical Testing. Stress-strain tests of native and decellularized bovine pericardium were performed in a tensiometer Zwich Z0.5 (Brugger HSG/ETK). For both native and TriCol-treated pericardium, bone-shaped samples

Table 1: Total GAG content (μg hexuronate/mg DDT) in the selected areas of cardiac valves and in pericardial tissue (before and after TriCol treatment).

	Aortic valve			Pulmonary valve			Pericardium
	Leaflet	Sinus	Wall	Leaflet	Sinus	Wall	
NT	17.50 ± 5.55	18.21 ± 11.57	5.74 ± 3.72	9.11 ± 5.02	5.66 ± 0.94	4.18 ± 1.09	2.34*
TriCol	4.88 ± 3.43	5.61 ± 2.16	2.74 ± 0.60	4.61 ± 1.23	2.62 ± 0.95	2.82 ± 0.87	1.44*

* pooled samples.

were excised from the pericardial area facing the left ventricle anterior wall and with the main axis orthogonal to the interventricular septum. The area of the specimens free from the grips was a rectangle with a length of 34 mm and a width of 6 mm. The thickness of the specimen was measured with a digital thickness gauge and used to calculate the cross-sectional area. The mechanical tests were performed in physiological solution at RT, and for every sample an elongation of 30 mm/m was applied bringing the tissue to rupture. Data of elongation and force applied were collected every second.

2.8. Statistical Analysis. Data are reported as mean ± standard deviation or total numbers and relative frequencies. For comparison between two groups, Student's t-test was performed. A value of $P \leq 0.05$ was considered statistically significant. The analyses were performed with Microsoft SPSS 11.0 and SigmaStat 3.11.0 software.

3. Results

In the three conduit tissues (leaflet, sinus, and wall) of both NT AR and PR, the differential concentration of total GAGs (normalized to DDT) was in turn deeply different from that in pericardium (Table 1). Particularly, in AR, the GAG content of leaflet and sinus was similar and three times higher than that of arterial wall. However, in PR GAG content of leaflet was twice that found in both sinus and pulmonary arterial wall. Otherwise, by comparing the two valvulated conduits, GAG concentration in AR leaflet resulted twice that in PR leaflet while that in aortic wall was similar to that in pulmonary artery wall. In turn the GAG content of pericardium accounted to about half that in the arterial wall of both valvulated conduits.

TriCol-based decellularization procedure produced a relevant loss of GAGs. Relative to native aortic valve, the total GAG content in TriCol-decellularized specimens was reduced by 72% in leaflet, 69% in sinus, and 52% in arterial wall. For pulmonary valve, total GAG reduction was by 49% in leaflet, 53% in sinus, and 33% in wall. In pericardial samples, decellularization reduced total GAG concentration by 39%.

As already reported in a previous paper [26], GAG distribution was partly different in each different portion (leaflet, sinus, and wall) of NT AR and PR. In both conduits chondroitin sulfate (CS) isomers and hyaluronan were present in the greatest proportion, the hyaluronan exhibiting a decreasing gradient from leaflet to arterial wall. Moreover, in leaflets and sinuses of both valves CS isomers

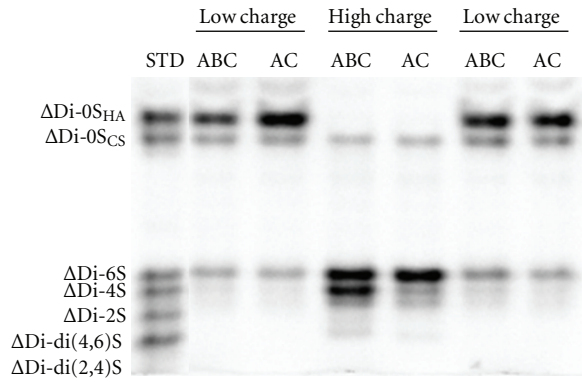


Figure 1: Representative FACE separation of unsaturated AMAC-labeled disaccharides obtained from low- (0.55 M NaCl) and high-charged (1 M NaCl) GAGs after depolymerization with Chase ABC and AC.

comprised a slow migrating CS, as assessed by acetate cellulose electrophoresis of free intact polysaccharides.

In pericardium, the GAG class present in the greatest proportion was dermatan sulfate (DS), with very low amounts of hyaluronan, as assessed by discontinuous electrophoresis of intact chains (data not shown).

3.1. GAG Structural Analysis. Since the relative proportion of HS in sinuses and leaflets of native cardiac valves and in pericardium was very low, we focused on the structural characterization of CS isomers purified from NT and TriCol samples. FACE analysis allows us to detect both mono- and di-sulfated CS-derived disaccharides, also discriminating between the nonsulfated forms released from CS isomers and those from HA (Figure 1). The distributions of Δ-disaccharides in porcine cardiac valves were similar in each selected portion of aortic and pulmonary conduits, while pericardial tissue contained an abundance of ΔDi-mono4S and ΔDi-diS with very low proportions of nonsulfated and 6 sulfated Δ-disaccharides (Figure 2).

There were no significant changes in Δ-disaccharides distributions in each sample following decellularization, even if in aortic leaflet and pericardium the loss seems to affect mainly HA.

The data analyses on ABC/ACII depolymerization revealed that in valve leaflets and sinuses CS was the galactosaminoglycan present in the greatest proportions, while pericardial tissue had almost exclusively DS (Figure 3).

Statistical analysis of the FACE results relative to GalAG structural characterization in native and decellularized valve

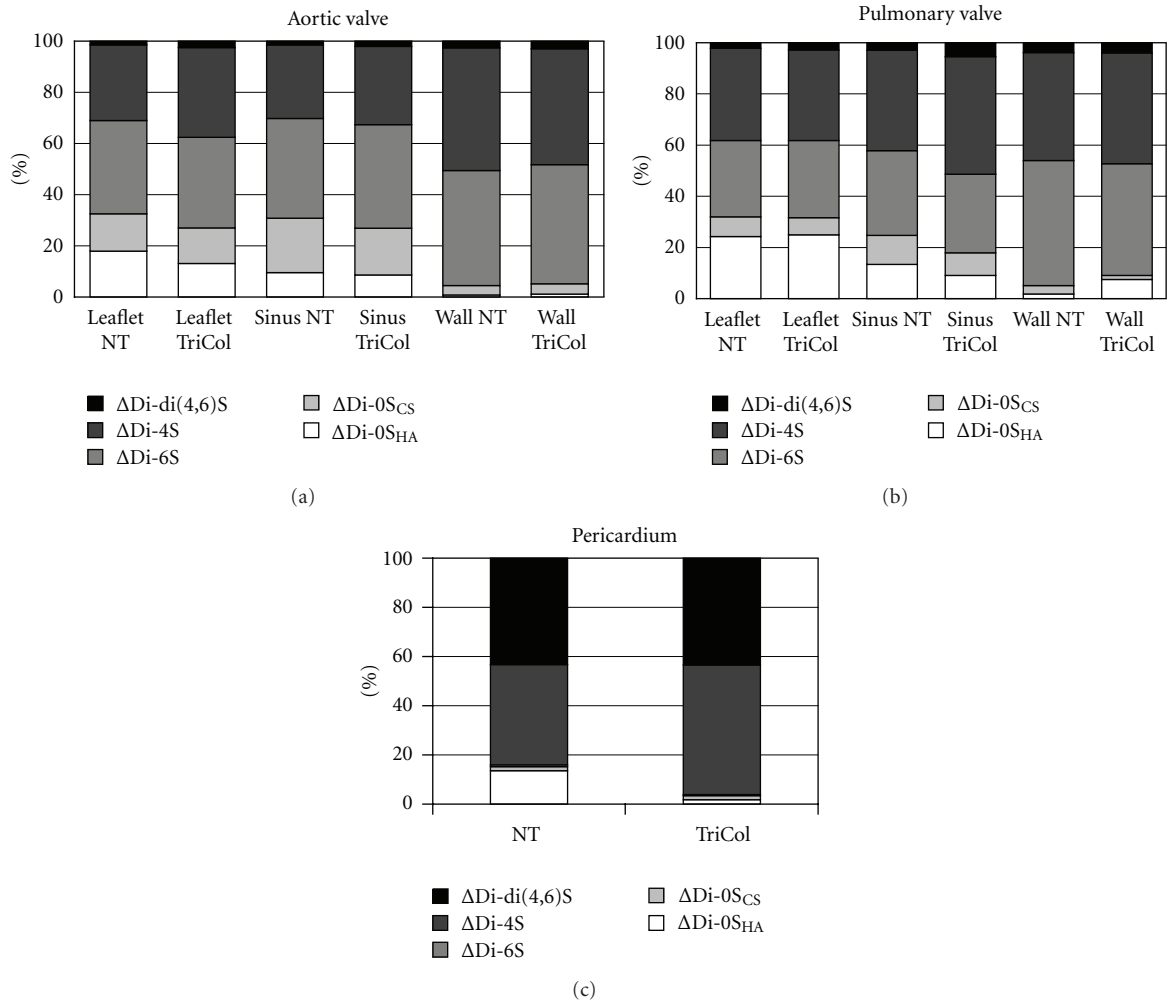


Figure 2: Relative Δ-disaccharides content in fresh (NT) and decellularized (TriCol) porcine heart valves and pericardium, as calculated from FACE data.

and pericardium indicated that the depletion in total content does not produce significant changes in the distribution of CS isomers. The percentages of nonsulfated disaccharides from CS isomers are reported in Table 2. Interestingly, significant differences in the under-sulfation degree were detected comparing native aortic and pulmonary valve conduits, referred to leaflet and sinus.

Additional data like epimerization and sulfation patterns of CS isomers were calculated comprehensively on native and treated samples (Table 3). Aortic leaflet and sinus showed a higher proportion of CS respect to the pulmonary ones, a value that was the opposite to that in the artery wall, while in the pericardial tissue, as already stated, the major CS isomer was DS.

3.2. Histology. H and E staining highlighted the removal of resident cells from bovine pericardium after TriCol treatment (Figure 4(b)) compared to native tissue (Figure 4(a)). The collagen fibers of the resulting matrix exhibited many void areas surrounding the bundles exhibiting a more defined wavy pattern (even if partially shrinked) with respect to the native sample. Seven days after seeding the fibroblasts did adhere to, crowded the pericardial surface and started to colonize the underlying detergent-treated matrix (Figure 4(c)). Moreover, the collagen fiber bundles apparently resumed morphological features approaching those of the native sample.

The propensity of TriCol-treated porcine heart valve leaflet to be repopulated by in vitro seeded cells has been previously reported [27, 32].

4. Mechanical Testing

A typical stress-strain diagram of native and decellularized pericardial samples is reported in Figure 5. Load-bearing fibrous component of treated pericardium appeared to be tensioned at lower strain with respect to those of native sample as having been partly reoriented in the direction of the stress. Nevertheless in its linear part the slope of the diagram was similar in both preparations even if the load at rupture of TriCol samples accounted to about half that of native ones.

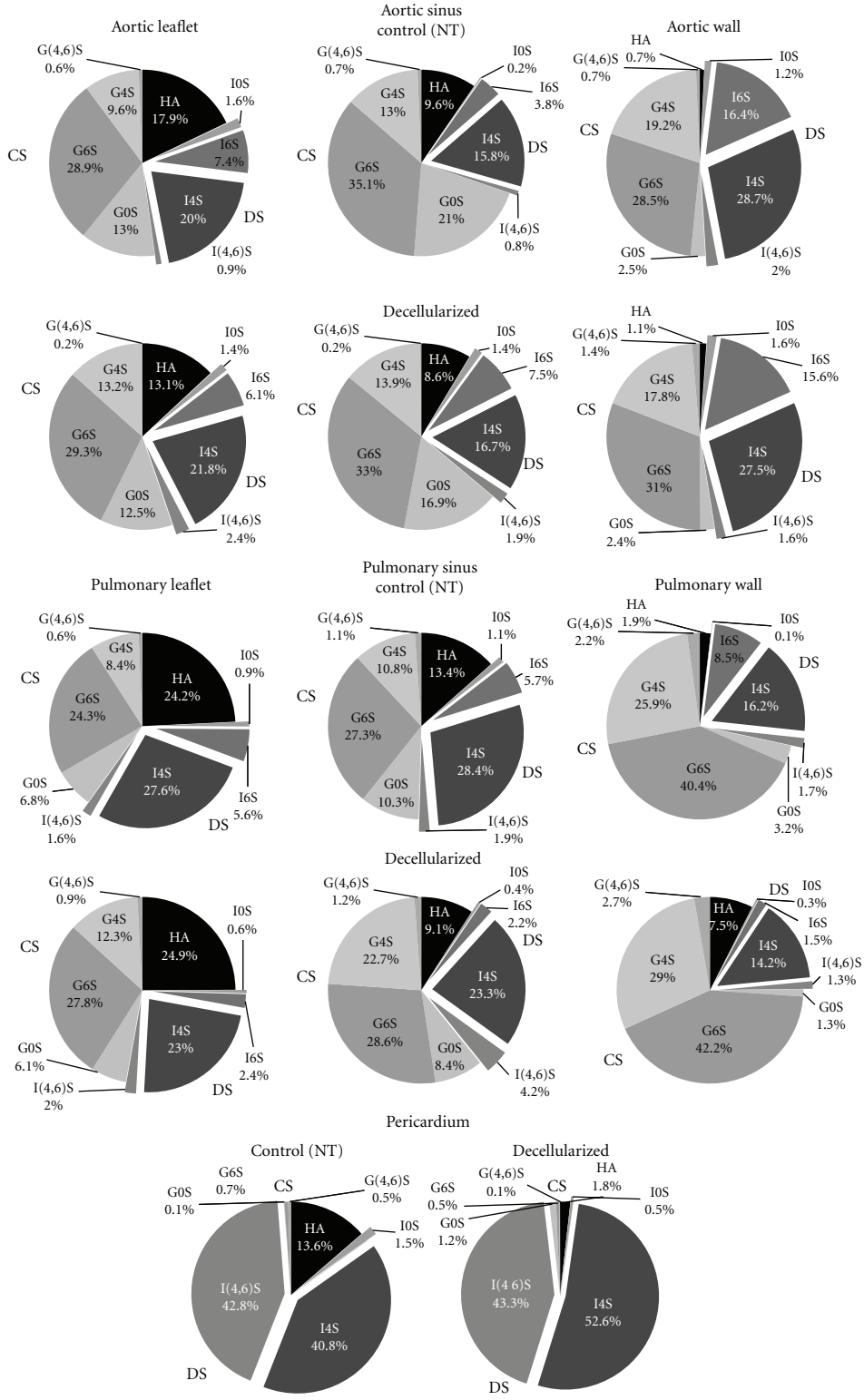


Figure 3: Distribution of glycosaminoglycans (GAGs) in NT and TriCol-treated porcine valves and pericardium, as calculated from FACE data. G0S: nonsulfated GlcA-containing disaccharides, G6S: 6-sulfated GlcA-containing disaccharide, G4S: 4-sulfated GlcA-containing disaccharide, G(4,6)S: disulfated GlcA-containing disaccharide, I0S: nonsulfated IdoA-containing disaccharide, I6S: 6-sulfated IdoA-containing disaccharide, I4S: 4-sulfated IdoA-containing disaccharide, I(4,6)S: disulfated IdoA-containing disaccharide.

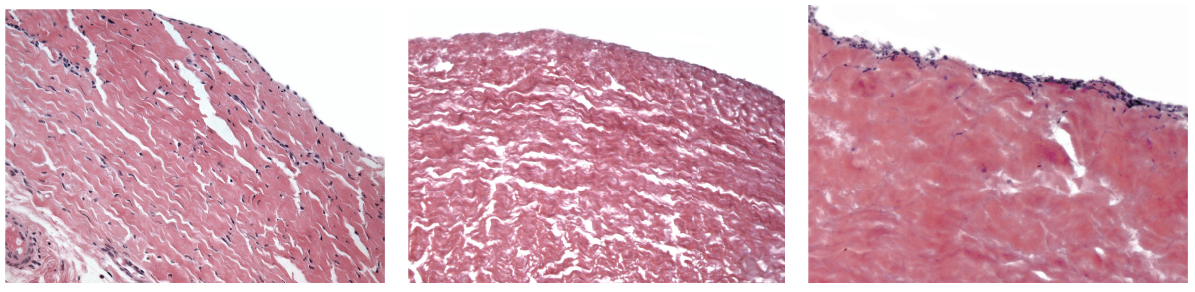
Table 2: Percentages of nonsulfated disaccharides from CS isomers in the examined tissues (means ± SD). Significant differences ($P < 0.05$) are reported in bold.

	ΔDi-nonsulfated/total Δ-disaccharides		
	NT	TriCol	
Pericardium	0.019	0.017	
Aortic valve			
	NT	TriCol	*P*
Leaflet	0.177 ± 0.031	0.150 ± 0.024	0.228
Sinus	0.237 ± 0.031	0.158 ± 0.056	0.061
Artery wall	0.028 ± 0.016	0.048 ± 0.040	0.404
Pulmonary valve			
	NT	TriCol	*P*
Leaflet	0.100 ± 0.017	0.090 ± 0.020	0.463
Sinus	0.129 ± 0.021	0.086 ± 0.029	0.070
Artery wall	0.029 ± 0.019	0.018 ± 0.004	0.380
	Aortic versus pulmonary		
	NT	TriCol	
Leaflet	**0.005**	**0.008**	
Sinus	**0.001**	0.117	
Artery wall	0.938	0.274	

Table 3: Levels of epimerization and sulfation of CS isomers (means ± SD).

	CS/DS	CS	DS	Total GalAGs (CS + DS)
Aortic valve	GlcA/IdoA	4S/6S	4S/6S	4S/6S
Leaflet	1.666 ± 0.645	0.375 ± 0.072	2.340 ± 0.699	0.823 ± 0.121
Sinus	2.611 ± 0.908	0.397 ± 0.072	2.359 ± 1.175	0.724 ± 0.077
Artery wall	1.446 ± 0.843	0.644 ± 0.111	2.221 ± 0.910	0.971 ± 0.074
Pulmonary valve				
Leaflet	1.180 ± 0.404	0.412 ± 0.087	2.605 ± 0.759	1.304 ± 0.525
Sinus	1.476 ± 0.673	0.589 ± 0.429	3.995 ± 0.665	1.273 ± 0.190
Artery wall	3.133 ± 0.777	0.681 ± 0.103	1.982 ± 0.760	0.905 ± 0.074
Pericardium*	0.017	*	*	80.289

* Single data regarding C4S/C6S and D4S/D6S for pericardium were very high, as 6-sulfation was near to 0.



(a) (b) (c)

Figure 4: H and E staining of native bovine pericardium (a); TriCol-decellularized bovine pericardium before (b) and after 7 days of bovine fibroblasts seeding (c). Magnification 20x.

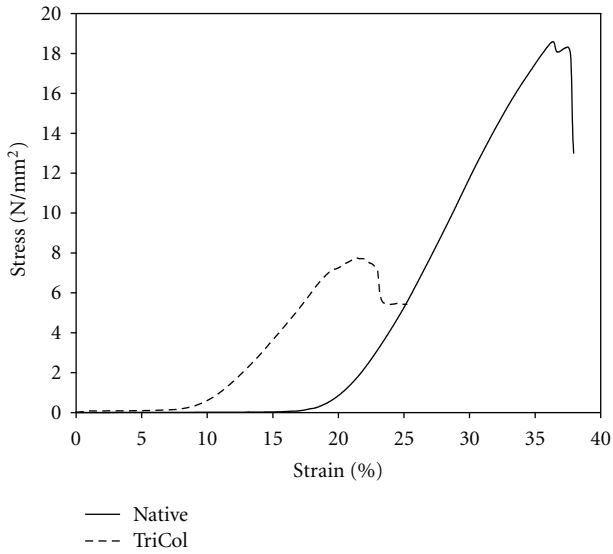


Figure 5: Stress-strain diagram of native and TriCol-decellularized bovine pericardium strips. See Section 2.

The mechanical properties of TriCol-treated valvular leaflets from pigs have been previously reported [27] to be not significantly different from those of untreated samples although circumferential samples exhibited 20% higher extensibility and trended to lower (about 10%) stiffness.

5. Discussion

Recent decellularization studies comprising our combination of ionic and nonionic detergents showed excellent cell removal capacity with preservation of the major structural ECM molecules although ionic detergents like sodium-dodecyl-sulfate (SDS) and sodium deoxycholate (DOC) might somehow modify the resulting scaffold matrix [33, 34]. Particularly, the adopted TriCol procedure is reported to thoroughly remove the α-gal antigen responsible for the iperacute rejection of xenogenic grafts and possibly for the chronic inflammation elicited by bioprosthetic devices treated with glutaraldehyde [35].

However, the loss of GAGs following decellularization could have deep effects on the structure of the ECM. In fact, the extraction of the GAGs could affect the mechanical behavior of the valves by introducing flexural rigidity, thickness decrease and favoring an increase in tissue Ca^{2+} content. All of these changes in the ECM composition might lead to problems in valve functionality or directly in valve failure. In this paper, fluorophore-assisted carbohydrate electrophoresis (FACE) was used to provide detailed information regarding GAG distribution as well as quantity and fine structure of various GAGs in porcine vascular tissues (aortic valve, pulmonary valve, and pericardium) used as xenografts for heart valve implants.

This study showed that the content and distribution of GAGs within native aortic valve, pulmonary valve, and pericardium are deeply different. In valve conduits both levels and composition of GAGs were region-specific. The leaflets, which experience compression, contain the highest concentration of GAGs, with an abundance of HA and under-sulfated CS. The quantification and characterization of GAGs in porcine valve conduits by FACE largely agree with our previous results obtained by analyzing intact polysaccharides [26]. The pericardial tissue contains relatively fewer GAGs but higher proportions of dermatan-4-sulfated and dermatan oversulfated. Particularly the level of oversulfation is strikingly higher than that already reported for oversulfated DS from other porcine tissues (e.g., up to 10–20% in skin) which in turn exhibit significant HC-II-mediated inhibition of thrombin activity [36]. Noteworthy, the oversulfation level found here is comparable to that of DS from marine ascidians (50–70%) also studied for its thrombin inhibitory activity [37]. All that could be the rationale behind the still unexplained low thrombogenicity potential of pericardial valve xenografts making the chronic anticoagulation treatment unnecessary in such patients [38]. Moreover, this finding is in good agreement with published data which described the presence of a low-molecular-weight dermatan sulfate proteoglycan in bovine pericardial tissue [39]. The abundance of particular GAGs and PGs can vary according to different biological needs of the tissues. In example, the dermatan sulfate (mostly 4-sulfated) PGs decorin and biglycan regulate the formation and orientation of collagen fibrils and hence tissue tensile strength, whereas the hyaluronan (HA), which is not covalently bound to a core protein, entraps large amounts of water to create a swelling force [40, 41]. It has been speculated that their ability to hydrate the spongiosa layer serves to decrease the shear stresses during valve function, and the presence of negatively charged GAG molecules may reduce calcification by chelating calcium ions, thereby preventing hydroxyapatite nucleation [13, 14, 42]. Moreover, it is thought that decellularization treatment extracts more easily hyaluronan and chondroitin/dermatan-6-sulfate, which exist in tissues as part of the aggregate of hyaluronan and versican PG [43]. These observations suggest that the loss of GAGs may be crucial for the development of new bioprostheses.

Our results showed that there was a relevant extraction of GAGs following TriCol decellularization procedure, but it was not selective, with the only exception of HA from aortic leaflet and pericardium. Distribution of PGs and GAGs in vascular tissue has been reported to be complex, district- and layer-specific, associated with different mechanical environments [11], and could have important implications for heart valve tissue engineering and bioprosthesis development. Although some of these compositional differences may appear quite subtle, such as the degree and position of sulfate and the degree and position of 5′epimerization on sulfated GAG chains, these fine structural distinctions may have important biological roles such as binding sites for other matrix components or for signaling cell differentiation in addition to their possible influence on the scaffold thrombogenicity. The possibility to assess carefully the ΔDi-mono4S and ΔDi-mono6S content in tissues is important considering that modifications of the ΔDi-mono4S/Δdi-mono6S ratio and the ratio of nonsulfated to sulfated GAGs

have been described in aging and disease. The C4S/C6S ratio has important biological implication; in humans, it decreases progressively in pulmonary artery, iliac artery, and aorta [44]. Moreover, the relative content of C4S decreases about fivefold in human aorta [45] and cerebral arteries [46] with aging and C4S/C6S ratio decreases particularly with atherosclerosis development [47].

Furthermore, this ratio might gather more information about the mechanical behavior and hence, be used to hypothesize the type of PGs found in the valves associated to GAG class concentrations and fine structure characteristics [10, 11].

H and E staining showed that TriCol procedure did in fact remove the cells from the native pericardium while the resulting matrix revealed to be suitable for cell repopulation. The fibroblasts adhered to the pericardial surface and started, after 7-day culture, to spread into the underlying extracellular matrix. Removal of cells did modify partly the mechanics of the native pericardium while being otherwise compatible with the features expected for the making of valve substitutes although the ultimate mechanical behaviour of repopulated scaffolds has still to be explored. As a whole both TriCol-decellularized porcine heart valves and bovine pericardium appeared a promising material bearing the potential for future development of tissue-engineered heart valve scaffolds able to be recellularized by the patient own cells.

Future studies are expected to examine the inherent complexity within valve tissues, due to the histological layers, mechanical forces, matrix composition and effects of aging, and also the functional characteristic of different GAGs that could have impact on the function of normal valves and on the choice of the candidate cardiovascular tissue to produce the best scaffold for the development of a tissue-engineered heart valve.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

The authors wish to thanks Mr. Edward James Buratto from the University of Melbourne for the scientific review of this paper and Nvt Jotec (Hechingen, Germany) for the kind disposal of instrumental facilities for mechanical testing. This research was supported by Fondazione Banco di Sardegna, Sassari, Italy, and from Fondazione Cassa di Risparmio di Padova e Rovigo (Italian Heart Project).

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Hindawi Publishing Corporation
Biochemistry Research International
Volume 2012, Article ID 480529, 7 pages
doi:10.1155/2012/480529

Research Article

Increased Urine IgM and IgG_2 Levels, Indicating Decreased Glomerular Size Selectivity, Are Not Affected by Dalteparin Therapy in Patients with Type 2 Diabetes

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Received 29 August 2011; Revised 8 November 2011; Accepted 22 November 2011

Academic Editor: Barbara Bartolini

Fifty-four type 2 diabetic patients with neuroischemic foot ulcers were randomised to treatment with 5000 IU of dalteparin, ($n = 28$), or physiological saline, ($n = 26$), once daily until ulcer healing or for a maximum of 6 months. Thirty-three patients had normo-, 15 micro-, and 6 macroalbuminuria. The urinary levels of IgM and IgG_2 were elevated in 47 and 50 patients, respectively. Elevated urinary levels of IgM and IgG_2 indicate decreased glomerular size selectivity. Urine IgM levels were associated with IGF-1/IGFBP-1 and IGFBP-1 levels. Dalteparin treatment increased urinary levels of glycosaminoglycans ($P < 0.001$) and serum IGFBP-1 ($P < 0.05$) while no significant effects were seen in any of the other studied parameters. In conclusion, dalteparin therapy in patients with type 2 diabetes had no effects on urinary levels of albumin, IgM, or IgG_2 despite significantly increased glycosaminoglycans in urine. Elevated urinary levels of IgM and IgG_2 might be more sensitive markers of renal disease than albuminuria in patients with type 2 diabetes and antihypertensive therapy.

1. Introduction

Albuminuria is a marker of diabetic nephropathy and a strong predictor of widespread vascular damage [1]. The Steno hypothesis held that genetically based disturbances in the production or sulphation of heparan sulphate (HS) lead to a reduction of sulphated and negatively charged HS glycosaminoglycan (GAG) side chains. Negatively charged HS GAG side chains are normally found in the extracellular matrix and vascular basement membranes. High blood glucose levels lead to lower activity of the enzymes involved in GAG metabolism and sulphation of HS [2]. A reduction of negatively charged HS GAG may induce an increased transvascular permeability of negatively charged plasma proteins, which promotes vascular and glomerular changes [1, 3–5]. Positive effects of heparin on diabetic nephropathy have been shown in experimental studies [6–8]. In humans with diabetes, several studies have shown a reduction of urinary albumin excretion during treatment with unfractioned heparin, low-molecular-weight heparins (LMWH), or oral treatment with sulodexide, suggesting that these compounds can improve GAG metabolism and sulphation of HS [3]. Thus, in patients with type 1 diabetes, treatment with unfractionated heparin, sulodexide or LMWH decreased the albumin excretion rate [9, 10], whereas in type 2 diabetes, the effect on albuminuria seems less consistent [3]. In a study by

Nielsen et al., three weeks of daily injections of the LMWH tinzaparin had no effect on albuminuria in patients with type 2 diabetes [11]. We have earlier reported an improved outcome of chronic neuroischemic foot ulcers in patients with diabetes during long-term treatment with dalteparin [12]. The beneficial effects of dalteparin on ulcer outcome involved an inhibitory effect on thrombin generation and improved haemostatic and microvascular functions [13]. The described effects of dalteparin may be beneficial not only for outcome of neuroischemic diabetic foot ulcers but also for other complications, such as diabetic nephropathy. Thus, the aim of this ancillary study was to investigate the effect of treatment with the LMWH dalteparin on proteinuria in patients with diabetes and severe vascular complications. The selectivity of the glomerular filter was studied by analyzing the urinary excretion of molecules of different size and charges [14–17], that is, IgM was analysed for determination of the size, and IgG_2 and IgG_4 for determination of the neutral and negative charges of the glomerular filter, respectively. The glomerular mesangial matrix turnover was assessed by measuring the urinary excretion of cytokine transforming growth factor beta 1 (TGFβ1) [18]. Furthermore, we analyzed insulin-like growth factor 1 (IGF-1) and IGF-binding protein 1 (IGFBP-1) since the IGFBP-1 [19] and IGF1 have been shown to be associated with diabetes nephropathy independent of the degree of albumin [20]. It has been speculated that low IGF-1 activity may induce apoptosis or loss of podocytes and thus lead to glomerulosclerosis [21].

2. Subjects and Methods

2.1. Subjects. Of the previously described 87 diabetic patients [12] with peripheral arterial occlusive disease (PAOD) and chronic foot ulcer, 54 type 2 diabetic patients who completed the urine collections were included in the present study. All patients were treated with 75 mg aspirin once daily since at least four weeks before randomization and throughout the study period.

2.2. Methods. Prospective, double-blind, and placebo-controlled multicenter study to evaluate the effects of dalteparin (Fragmin, Pfizer) primarily on healing of neuroischemic foot ulcers [12] and secondarily on haemostatic and microvascular functions [13], and renal excretion of proteins. The patients were randomized to treatment with 0.2 mL daily subcutaneous injections of dalteparin (25000 U/mL) or physiological saline until ulcer healing or for a maximum of six months.

Timed urine collections from three consecutive nights before and at the end of treatment were stored at -20°C and analyzed at the Renal Laboratory, Lund. Microalbuminuria was defined as a mean value of the urine collections of 20 to 200 μg/min or u-albumin/creatinine ratio of 3–30 mg/mmol. An excretion below these levels was defined as normo- and an excretion above as macroalbuminuria.

Urine albumin [22], total GAG [23], IgM [24], IgG_2, and IgG_4 [25] were analyzed as previously described. Biologically active TGFβ1 was analyzed with a commercially available assay (Emax Immunoassay System, Promega Corp., Madison, WI, USA). U-creatinine was analyzed with an enzymatic method (EKTACHEM, Clinical Chemistry Slide, Johnson & Johnson Clinical Diagnostics, Rochester, NY, USA). HbA_{1c} was analyzed by an immunoturbidimetric method (UNIMATE 3 HbA_{1c}, Roche Diagnostics). HsCRP and S-AA were measured using particle-enhanced immunonephelometric methods (BN, Dade Behring). IGF-I [26] and IGFBP-1 [27] were determined in serum by radioimmunoassays (RIAs).

2.3. Statistical Methods. Data are shown as mean and SD and skewed variables as median (minimum and maximum values). For differences within subjects we used Friedman's test, with Wilcoxon signed-rank test as post hoc test. The chi-square test was used to compare differences in the distribution of categorical variables. For testing of differences between subject groups, the Mann-Whitney U test was used. P values below 0.05 were considered significant (2-tailed). The statistical program SPSS was used.

2.4. Ethical Considerations. The study protocol was approved by the local ethics committee of each centre and the Swedish Medical Products Agency. Written informed consent was obtained from all patients.

3. Results

3.1. Patient Characteristics. Fifty-four patients with type 2 diabetes were able to leave timed urine collections from three consecutive nights before and at the end of treatment period. All patients had PAOD, peripheral neuropathy, and chronic foot ulcers. Seven patients in the dalteparin and 10 in the placebo group had suffered from myocardial infarction, and two patients in the placebo group had undergone leg amputation. Except for more ex-smokers in the placebo group, the baseline patient characteristics were not different between the two groups (Table 1). Levels of HbA_{1c} at baseline (Table 1) and at the end of treatment period (dalteparin: 7.0 (4.9–10.8)%; placebo: 6.3 (4.6–8.7)%) were not significantly different between the groups. Ten patients in the dalteparin group and 11 in the placebo group had micro- or macroalbuminuria (Table 1). Thirty-six patients, including 23 patients with normoalbuminuria, were on antihypertensive treatment (Table 1).

3.2. Treatment Period. The treatment period with dalteparin was not significantly different from the treatment period in the placebo group. It lasted for median 26 and range 8 to 26 weeks.

3.3. Renal Parameters. At baseline, 33 patients had normo-, 15 micro-, and 6 macroalbuminuria. Thirty-six patients, including 23 patients with normoalbuminuria, were on antihypertensive treatment (Table 1). Ten patients in the dalteparin group and 11 in the placebo group had micro- or macroalbuminuria (Table 1). Forty-seven patients showed elevated urinary levels of IgM (Figure 1), while 50 patients had elevated urinary levels of IgG_2, both indicating decreased

Table 1: Baseline characteristics of 54 patients randomized to dalteparin or placebo.

	All	Dalteparin	Placebo
	$N = 54$	$N = 28$	$N = 26$
Age (years)	75 (54–90)	73 (57–86)	75 (54–90)
Gender (male/female)	37/17	17/11	20/6
Smoker/ex-smoker/nonsmoker (n)	9/14/31	4/3/21*	5/11/10
HbA_{1c} (%)	6.7 (5.0–11.0)	6.9 (5.1–11)	6.9 (5.0–9.6)
Diabetes duration (years)	17 ± 9	17 ± 10	16 ± 8
Tablets/insulin/tablets + insulin/diet (n)	10/31/8/5	4/18/3/3	6/13/5/2
Antihypertensive treatment (n) (ACE/β/Ca/diuretic/other)	10/13/5/22/4	5/6/3/14/0	5/7/2/8/4
Systolic blood pressure (mmHg)	158 ± 22	160 ± 22	155 ± 22
Diastolic blood pressure (mmHg)	80 ± 11	78 ± 9	82 ± 12
P-Creatinine (μmol/L)	83 (53–160)	83 (57–130)	84 (53–160)
GFR (mL/min)	74 (17–218)	74 (34–190) ($n = 22$)	80 (17–218) ($n = 22$)
Albuminuria (normo/micro/macro):			
Baseline (n)	33/15/6	18/8/2	15/7/4
At endpoint (n)	35/12/7	18/7/3	17/5/4

* $P < 0.05$ versus placebo. Data are given as mean ± SD, or as median and minimum-maximum values. GFR: glomerular filtration rate: creatinine clearance.

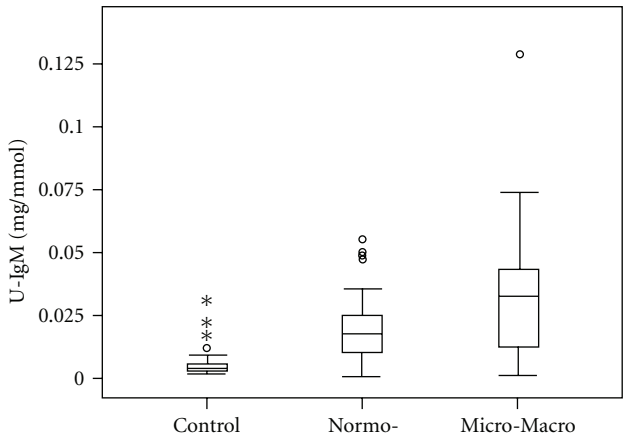

Figure 1: Box-plot of levels of IgM in control subjects compared with patients with normo- and micro- or macroalbuminuria at baseline ($P < 0.001$). Normo- versus micro- or macroalbuminuria ($P = 0.05$).

glomerular size selectivity. Twelve patients had a ratio of IgG_2/IgG_4 less than 1 indicating decreased charge selectivity, while 8 patients had urine levels of GAG less than or equal to 2 mg/mmol.

Urinary GAG increased from 2.43 (0–8.65) mg/mmol at baseline to 3.40 (1.25–8.0) mg/mmol during dalteparin therapy ($P < 0.001$), while GAG levels were not significantly changed in the placebo group (baseline: 2.53 (0–8.99) mg/mmol). All other urinary parameters, including glomerular filtration rate (GFR), were not significantly different between dalteparin- and placebo-treated patients at baseline or at the end of treatment (Tables 1 and 2; data at end of treatment not shown). Baseline levels of systolic blood pressure, HbA_{1c}, S-creatinine, S-HsCRP, S-AA, S-IGF, S-IGFBP-1, U-GAG, and U-IgG_2/IgG_4 were not significantly different between patients with normo- and micro- or macroalbuminuria (Table 2), and no associations were found between urinary GAG, HbA_{1c}, and blood pressure levels and the urinary parameters. Urine levels of IgG_2 and IgG_4 were higher in patients with micro- or macroalbuminuria than in those with normoalbuminuria ($P < 0.05$) (Table 2). The dalteparin-induced increase in urinary GAG was independent of the degree of albuminuria, and no gender differences were found (data not shown). No significant effects of dalteparin treatment were seen on the urinary excretion of proteins in either patients with normoalbuminuria, or in patients with micro- or macroalbuminuria (Tables 3 and 4).

3.4. Comparisons with Data from Control Subjects. In comparison with control subjects [28], the urinary levels of IgG_2 were higher in the patients with micro- or macroalbuminuria while normal in those with normoalbuminuria. Levels of IgG_4 were normal, while IgG_2/IgG_4 ratios, and IgM and TGFβ1-values [18] were increased irrespective of the level of albuminuria (for reference values, see Table 2).

3.5. Inflammatory Parameters, IGF-1 and IGFBP-1. The levels of hsCRP, SAA, S-IGF-1, and S-IGFBP-1 were similar in the dalteparin and placebo groups at baseline and during the treatment period (data not shown), except for S-IGFBP-1 which increased in patients with micro-macroalbuminuria in comparison with placebo-treated patients (Tables 3 and 4). No associations were found with any of the urinary parameters or HbA1c levels. S-IGF-1 was negatively associated with systolic BP at entry ($r = -0.304$, $P = 0.048$, $n = 43$). SAA and hsCRP were negatively associated with systolic BP at endpoint ($r = -0.294$, $P = 0.038$, $n = 50$ and $r = -0.292$,

Table 2: Baseline values in patients grouped with normo- or micro- and macroalbuminuria.

	Normoalbuminuria $N = 33$	Micro- and macroalbuminuria $N = 21$
Age (years)	74 (54–90)	75 (61–86)
Diabetes duration (years)	15 ± 9	19 ± 8
Gender (male/female)	21/12	16/5
Systolic blood pressure (mmHg)	150 (115–210)	160 (135–215)
Diastolic blood pressure (mmHg)	80 (60–100)	85 (60–105)
S-HbA_{1c} (%)	6.5 (5.0–9.9)	6.9 (5.1–11.0)
S-Creatinine (μmol/L)	81 (53–160)	85 (65–128)
S-Hs CRP (mg/L)	9.4 (0.9–118)	2.7 (0.3–78.2)
S-AA (mg/L)	5.5 (1.2–415)	5.1 (1.7–127)
S-IGF-1 (μg/L)	134 (47–384)	115 (49–269)
S-IGFBP-1 (μg/L)	41 (15–310)	60 (8–313)
U-Glycosaminoglycan (mg/mmol)[a]	2.7 (0–8.7)	2.6 (0–11.1)
U-IgG_2 (mg/mmol)[b]	0.18 (0–8.1)	0.85 (0–99)*
U-IgG_4 (mg/mmol)[c]	0.06 (0–7.7)	0.27 (0–28.7)*
U-IgG_2/IgG_4[d]	3.1 (0.04–31.0)	3.3 (0.76–10.5)
U-IgM (mg/mmol)[e]	0.02 (0–0.06)	0.03 (0–0.13)
U-TGFβ1 (ng/mmol)	3.2 (1.1–379)	4.5 (1.4–16.5)

* $P < 0.05$ versus normoalbuminuria. Data are given as median and range (min-max). Urine data are the ratio between urine protein and urine creatinine. [a]Reference values for U-GAG: 2.9 (2.0–4.4) mg/mmol [25]; [b]U-IgG_2: 0.19 ± 0.14 mg/mmol; [c]U-IgG_4: 0.35 ± 0.25 mg/mmol; [d]U-IgG_2/IgG_4: 2.3 ± 0.7; [e]U-IgM: 0.002 ± 0.001 mg/mmol [28]. IGFBP-1 (15–45) [29, 30].

Table 3: Diabetic patients with normoalbuminuria: effects of treatment on urinary indices.

	Dalteparin		Placebo	
	Baseline $n = 18$	At endpoint $n = 18$	Baseline $n = 15$	At endpoint $n = 15$
U-Albumin (mg/mmol)	0.81 (0.07–2.39)	0.77 (0.06–4.97)	0.81 (0.13–4.09)	0.80 (0.19–6.73)
U-IgG_2 (mg/mmol)	0.19 (0–8.14)	0.14 (0–7.79)	0.18 (0.06–2.64)	0.16 (0.01–3.79)
U-IgG_4 (mg/mmol)	0.05 (0–7.68)	0.04 (0–1.08)	0.07 (0.02–2.51)	0.06 (0.02–4.63)
U-IgG_2/u-IgG_4	3.49 (0.04–31)	2.20 (0.37–44.59)	2.38 (0.41–8.58)	2.63 (0.54–18.7)
U-GAG (mg/mmol)	2.43 (0.86–8.65)	2.85 (1.32–8)*#	3.32 (0–6.45)	2.53 (0.93–8.99)
U-IgM (mg/mmol)	0.02 (0–0.06)	0.02 (0–0.05)	0.02 (0–0.05)	0.02 (0–0.05)
TGF-β1 (mg/mmol)	3.2 (1.1–379)	5.17 (1.47–21.3)	3.4 (1.4–24.6)	3.32 (1.7–28.9)
GFR (mL/min)	70 (34–190) ($n = 14$)	65 (33–163) ($n = 13$)	99 (17–218) ($n = 12$)	86 (20–334) ($n = 12$)
IGFBP-1 (μg/L)	42 (21–310)	49 (27–315)	38 (15–98)	53 (15–97)

Data are given as the median (with minimum and maximum values in parentheses) of the ratio between urinary concentrations of substance and u-creatinine. * $P < 0.05$ versus placebo; # $P < 0.05$ versus baseline. GFR: glomerular filtration rate: creatinine clearance.

$P = 0.036$, $n = 52$; resp.). No differences were found between normo- and micro- or macroalbuminuric patients (Table 2). However, urine IgM/creatinine ratio was correlated to IGF1/IGFBP1 ($r = -0.36$, $P = 0.008$, $n = 54$) and IGFBP1 ($r = 0.34$, $P = 0.013$, $n = 54$).

4. Discussion

The results of the present study show that six months of treatment with the LMWH dalteparin had no effect on glomerular function, inflammatory parameters, or urinary levels of proteins despite an increased urinary excretion of GAG. Our results extend the findings of an earlier study showing that three weeks of LMWH treatment had no effect on albuminuria in patients with type 2 diabetes [11]. These findings are in contrast to the effect seen in type 1 diabetic patients showing a reduced albuminuria during one-to-three month treatment with either unfractionated heparin or LMWH [9, 10]. The reason for this discrepancy in effects of heparins on urinary excretion of proteins between patients with type 1 and type 2 diabetes is unclear and cannot be explained by the present study. However, the structure of the heparin

Table 4: Diabetic patients with micro- or macroalbuminuria: effects of treatment on urinary indices.

	Dalteparin		Placebo	
	Baseline	At end point	Baseline	At end point
	$n = 10$	$n = 10$	$n = 11$	$n = 11$
U-Albumin (mg/mmol)	8.5 (0.9–435)	11.3 (1.5–311)	23.2 (2.1–187)	7.9 (0.9–273) ($n = 10$)
U-IgG_2 (mg/mmol)	0.46 (0–20.7)	0.83 (0.13–35.1)	2.99 (0.02–99.4)	2.60 (0–70.0)
U-IgG_4 (mg/mmol)	0.21 (0–3.94)	1.02 (0.04–7.16)	0.50 (0.03–28.7)	0.15 (0–53.7) ($n = 10$)
U-IgG_2/u-IgG_4	1.88 (1–10.5)	3.05 (0.33–9.16)	5.57 (0.8–9.7)	4.47 (0.35–24.1)
U-GAG (mg/mmol)	2.31 (0–4.52)	3.97 (1.25–6.1)$^{*\#\#}$	2.70 (0–11.1)	2.49 (0–5.19) ($n = 10$)
U-IgM (mg/mmol)	0.03 (0–0.05)	0.03 (0.01–0.12)	0.03 (0–0.13)	0.02 (0–0.14)
TGF-β1 (mg/mmol)	4.44 (1.4–15.9)	4.09 (2.14–14.12)	4.5 (1.8–16.5)	3.5 (1.19–22.44) ($n = 10$)
GFR (mL/min)	74 (36–140) ($n = 8$)	76 (18–208) ($n = 9$)	71 (38–107) ($n = 10$)	67 (34–113) ($n = 10$)
IGFBP-1 (μg/L)	66 (8–313)	105 (23–219)* ($n = 9$)	60 (20–130)	46 (10–161)

Data are given as the median (with minimum and maximum values in parentheses) of the ratio between urinary concentrations of substance and u-creatinine. $^{*}P < 0.05$ versus placebo; $^{\#\#}P < 0.01$ versus baseline. GFR: glomerular filtration rate: creatinine clearance.

molecule might be of importance since mixed compositions of sulphated GAG and heparan sulphate, for example, danaparoid [31], seemed to be more effective in type 2 diabetic patients. Another compound sulodexide which is a mixture of glucuronyl glycos aminoglycan and dermatan sulphate in an early study seemed to be effective [32], while a later double-blind randomized study showed that the drug was unable to decrease urine albumin excretion in patients with type 2 diabetic nephropathy and microalbuminuria [33].

The levels of total urinary GAG increased during treatment with dalteparin, which may be due to restitution of glomerular GAG or simply by an increased urinary excretion of dalteparin [34]. The low-molecular-weight heparin dalteparin is composed of strongly acidic sulphated polysaccharide chains with an average molecular weight of 5000 and about 90% of the material within the range 2000–9000. An earlier study by our group showed normal excretion of GAG in normoalbuminuric type 1 diabetic patients, while the levels were decreased in micro- and macroalbuminuric patients [25]. In contrast, the present study showed no significant differences in the levels of GAG in patients with normal or increased urinary excretion of albumin.

Normally, the urinary levels of IgG_2, IgG_4, and IgM are undetectable. In the present study, more patients had increased levels of urinary IgG_2, IgG_2/IgG_4 ratio, or IgM than patients who had micro- or macroalbuminuria. The loss of negative charges of the glomerular capillary wall causes the "effective" small pore radius vis-à-vis negatively charged macromolecules to increase to ~4.5 nm, which allows the passage of albumin. Larger proteins, such as IgG (mol radius 5.5 nm) or IgM (mol radius 12 nm), are still unable to pass across this pathway. IgG passes the glomerular capillary walls through the large pores, while IgM can permeate the glomerular capillary wall solely through the shunts [28]. Thus, increased transport of IgG indicates increased density of large pores, while increased concentration of urine IgM indicates increased density of shunts in the glomerular capillary wall [28]. In the present study, 47 patients had detectable levels of IgM, while only 21 patients had albuminuria; thus, renal disease was found in patients not detected by analysis for urine albumin. Since peripheral arterial occlusive disease is a marker of widespread vascular disease, one could expect that more patients in the present study would have had albuminuria. One reason for the low number may be antihypertensive medication, which was common in the present study. Thus, albuminuria may be a less sensitive parameter for evaluating nephropathy in patients on antihypertensive treatment. LMWH had no effect on the other urinary parameters studied, that is, IgG_2/IgG_4, IgG_2 or IgM. In recent years new technologies of genomic analysis and proteomic approaches have detected several new markers for renal disease like neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1), and podocin [35–37]. However, the substances have not been proved to be of significant prognostic value and thus the findings have not resulted in improvement of the management of diabetic nephropathy [38, 39].

We have previously found higher renal excretion of IgM, IgG_2, and IgG_2/IgG_4 in type 2 than in type 1 diabetic patients with overt nephropathy despite similar degree of albuminuria [28]. Thus, proteinuria in type 2 diabetic patients may be caused by an alteration of the size selective properties of the glomerular capillary wall, including the occurrence of nondiscriminatory "shunt pathways," rather than by charge selectivity [15]. We have previously found increased excretion of IgM to be a poor prognostic factor [40]. The IGFBP-1 gene has been suspected to be protective for nephropathy [19], possibly through altered IGFBP-1 binding to IGF-1

with local effect in the kidney. In the present study in patients with vascular disease we found increased excretion of IgM, and thus these patients may be at increased risk. We furthermore found a positive association between IGFBP-1 and excretion of IgM indicating that high IGFBP-1 may be associated with glomerular damage. Thus, we were able to confirm decreased levels of IGF-1 and increased levels of IGFBP-1 in type 2 diabetes patients with nephropathy [20]. Furthermore, IGFBP-1 increased to significantly higher levels in patients treated with dalteparin than in placebo-treated ones. The reason for these increased levels is not known but may be due to reduced proteolysis of IGFBP-1. In line with a study by Sharma et al. [18], the present study showed increased urinary levels of TGFβ1 in patients with type 2 diabetes. However, the levels of TGFβ1 were also unaffected by dalteparin treatment.

In conclusion, the present study showed no effects of dalteparin on the glomerular filter despite increased S-IGFBP-1 levels and urinary levels of GAG. Thus, the study indicates that proteinuria in type 2 diabetic patients may be caused by an alteration of the size-selective properties of the glomerular capillary wall. IgM and IgG_2 seem to be better markers than albuminuria for severe vascular disease.

Abbreviations

IgG: Immunoglobulin G
TGFβ1: Transforming growth factor β1
IGF-1: Insulin-like growth factor 1
IGFBP-1: Insulin-like growth factor binding protein 1.

Acknowledgments

The authors would like to thank Ann-Christin Salomonsson, Microcirculation Laboratory, Karolinska Institute, Department of Clinical Sciences, Danderyd Hospital, Stockholm, and Åsa Pettersson, Renal Laboratory, Lund University Hospital, for skilful assistance and Inga-Lena Wivall (Department of Molecular Medicine and Surgery, Karolinska Institute) for help with the analyses of IGF-1 and IGFBP-1. Grants were received from the Pharmacia Corporation, the Swedish Heart-Lung Foundation, the Stockholm County Council, and the R&D Department of the Public Health and Medical Services Committee and the Swedish Research Council (VR 04224). This study was supported by an unrestricted grant from Pharmacia Corporation, who also supplied the dalteparin and placebo injections. Pharmacia Corporation had no input into the study design, its execution, or interpretation of the findings.

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Hindawi Publishing Corporation
Biochemistry Research International
Volume 2012, Article ID 346972, 11 pages
doi:10.1155/2012/346972

Review Article

Hyaluronan Regulates Cell Behavior: A Potential Niche Matrix for Stem Cells

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Received 15 July 2011; Revised 24 September 2011; Accepted 18 November 2011

Academic Editor: Manuela Viola

Hyaluronan is a linear glycosaminoglycan that has received special attention in the last few decades due to its extraordinary physiological functions. This highly viscous polysaccharide is not only a lubricator, but also a significant regulator of cellular behaviors during embryogenesis, morphogenesis, migration, proliferation, and drug resistance in many cell types, including stem cells. Most hyaluronan functions require binding to its cellular receptors CD44, LYVE-1, HARE, layilin, and RHAMM. After binding, proteins are recruited and messages are sent to alter cellular activities. When low concentrations of hyaluronan are applied to stem cells, the proliferative activity is enhanced. However, at high concentrations, stem cells acquire a dormant state and induce a multidrug resistance phenotype. Due to the influence of hyaluronan on cells and tissue morphogenesis, with regards to cardiogenesis, chondrogenesis, osteogenesis, and neurogenesis, it is now been utilized as a biomaterial for tissue regeneration. This paper summarizes the most important and recent findings regarding the regulation of hyaluronan in cells.

1. Hyaluronan Properties

Hyaluronan (also known as hyaluronate or hyaluronic acid, HA) is a nonsulfated linear polysaccharide present in the extracellular matrix of every vertebrate's tissue. It is a member of the glycosaminoglycan (GAG) family and is synthesized at the inner leaflet of the plasma membrane [1] as a large, unbranched polymer of repeating disaccharides of glucuronic acid and *N*-acetylglucosamine. It has a simple chemical structure but differs from other glycosaminoglycans in its high molecular weight, lack of sulfate groups, and absence of covalent attachment to core proteins [1]. It was assumed that its major functions were in joint lubrication, tissue homeostasis, and holding gel-like tissues together. However, the high molecular weight of hyaluronan enables it to acquire a viscous characteristic that goes beyond these functions. Indeed, hyaluronan coregulates numerous physiological processes including embryonic development, inflammation, tissue regeneration, cell migration, and proliferation. Some reports [2, 3] have also suggested that hyaluronan plays some role in cancer invasion and in the promotion of angiogenesis.

Many functions of hyaluronan depend upon its interaction with cell surface receptors, including cluster determinant 44 (CD44), receptor for hyaluronan-mediated motility (RHAMM), lymphatic vessel endothelial hyaluronan receptor (LYVE-1), hyaluronan receptor for endocytosis (HARE), liver endothelial cell clearance receptor (LEC receptor), and toll-like receptor 4 (TLR4) [1]. Although it is well established that hyaluronan-induced signaling occurs through receptor interactions, its signal transduction mechanism in cells has not been fully characterized.

Stem cells have the unique abilities of self-renewal and pluripotency. These characteristics therefore play essential roles in organogenesis during embryonic development and tissue regeneration. The potential utility of stem cells for tissue regeneration and treatment of many formerly incurable diseases has motivated scientists to discover the key influences of stem cell behavior. The identification of hyaluronan

in many locations where stem cells are present raised the possibility that hyaluronan influences these potent cells. Indeed, emerging studies [4–6] have proven the veracity of this hypothesis, and hyaluronan is now being studied as a key component that may be manipulated to induce desired stem cell behaviors.

2. Hyaluronan Receptors and Signaling

Hyaluronan interacts with cell surfaces in at least two ways. It can bind cell surface receptors, hyaladherins, such as CD44, LYVE-1, HARE, layilin, TLR4, and RHAMM, to induce the transduction of a range of intracellular signals, either directly or by activating other proteins. Through signal-transducing receptors, hyaluronan influences cell proliferation, survival, motility, and differentiation and might have roles in cancer pathogenesis. The detail signaling mechanisms for each receptor is reviewed as follows.

2.1. HARE. HARE, also known as stabilin-2, is a transmembrane receptor protein that contains a C-type lectin-like HA-binding module, which enables binding and endocytosis of hyaluronan ligand. The function of this receptor has been regarded to be involved in the mediation of normal turnover process of hyaluronan and other GAGs, such as chondroitin sulfate, from the circulatory system. Hyaluronan is internalized via a clathrin-coated pit pathway, leading to hyaluronan degradation in the lysosome [7].

2.2. Layilin. Similar to HARE, layilin is a transmembrane protein homologous to C-type lectins, which is located at the membrane ruffle. It acts as a membrane docking site for talin and can also specifically bind hyaluronan [8, 9] through its lectin-like domain. Similar to CD44, layilin extracellularly binds HA and induces binding of cytoskeletal proteins such as talin and ERM complex through its cytoplasmic domain. After receiving the HA signal, layilin interacts with merlin, a protein of the ERM superfamily, at the amino terminus and modulates cell cytoskeletal structure [10].

2.3. RHAMM. RHAMM is a ubiquitous protein which is present in the nucleus, cytoplasm, and cell plasma membrane [11–13]. RHAMM can be alternatively spliced to produce molecules of different sizes. When the membrane-bound RHAMM interacts with hyaluronan, kinase activity of c-Src, FAK, or PKC is activated [13, 14]; the intracellular RHAMM domain is capable of binding kinase or cytoskeletal components [11], forming a cytoskeleton regulating complex and triggering cytoskeletal rearrangement. Disruption of hyaluronan-RHAMM interaction using either protein mutation or antisense mRNA treatment significantly reduces cell motility rate and formation of stable focal adhesions [14], supporting the model of RHAMM-mediated regulation of FAK activity. It has been reported that RHAMM binding of epiregulin, a novel ligand of EGFR, activates downstream tyrosine kinase-mediated autophosphorylation and regulates cell survival and proliferation of human cementifying fibroma [15] cells (HCF).

2.4. LYVE-1. LYVE-1 is a type I integral membrane protein containing a link domain, similar to the proteolytic hyaluronan binding domain of the Link protein superfamily [16]. LYVE-1 is identified as an endocytic receptor of hyaluronan, specifically expressed in the lymphatic endothelium. LYVE-1 has been widely used as a marker for investigation of lymph vessel structure in both benign and malignant prostate tissues and hence applicable in cancer-related studies. In HS-578T human breast cancer cells, adhesion to COS-7 cells was enhanced by overexpression of LYVE-1. This phenomenon was reduced by enzyme digestion treatment of HS-578T cell surface with bacterial *Streptomyces hyaluronidase* prior to adhesion experiment, suggesting that LYVE-1 enhances tumor cell adhesion through interaction with hyaluronan. A recent study pointed out that in human primary effusion lymphoma (PEL) tumor cells, when LYVE-1 and EMMPRIN/CD147 coexpressed with drug transporter protein, breast cancer resistance protein/ABCG2 (BCRP), EMMPRIN induced upregulation of BCRP, and LYVE-1 colocalized with BCRP on the cell surface, suggesting that LYVE-1 takes part in facilitating hyaluronan and EMMPRIN-mediated chemoresistance.

2.5. CD44. CD44-mediated cell interaction with hyaluronan has been implicated in a variety of physiological events including cell-cell and cell-substrate adhesion, cell migration, cell proliferation, and hyaluronan uptake and degradation [17]. A proposed signaling pathway activated by the CD44 receptor is depicted in Figure 1. Hyaluronan is often bound to CD44 isoforms, which are ubiquitous, abundant, and functionally important cell surface receptors [18] in coordinating intracellular signaling pathways (e.g., Ca^{2+} mobilization [19], Rho signaling [20], PI_3-kinase/AKT activation [21], NHE1-mediated cellular acidification [22], transcriptional upregulation, and cytoskeletal function [23]) and generating the concomitant onset of tumor cell activities (e.g., tumor cell adhesion, growth, survival, migration, and invasion) and tumor progression. CD44-hyaluronan interaction in MSC migration can be found in the rat MSC line Ap8c3 and mouse $CD44^{-/-}$ or $CD44^{+/+}$ bone marrow stromal cells. Adhesion and migration of MSC Ap8c3 cells [24] to hyaluronan was suppressed by anti-CD44 antibody and by CD44 small interfering RNA (siRNA). In some tumor studies, hyaluronan binding to tumor cells promoted Nanog protein in association with CD44 followed by Nanog activation and the expression of pluripotent stem cell regulators such as Rex1 and Sox2. Nanog also formed a complex with the signal transducer and activator of transcription protein 3 (Stat-3) in the nucleus leading to Stat-3-specific transcriptional activation and MDR1 (P-glycoprotein) multidrug transporter gene expression [25].

Scientists have long noticed superficial similarities between stem cells and cancer cells. We believe that CD-44 mediated cell interaction with hyaluronan will provide valuable new insights into previously understood aspects of solid tumor malignancy. In fact, human breast cancer cells strongly expressing CD44 along with low or no expression of CD24 effectively formed tumors. These cells possess the

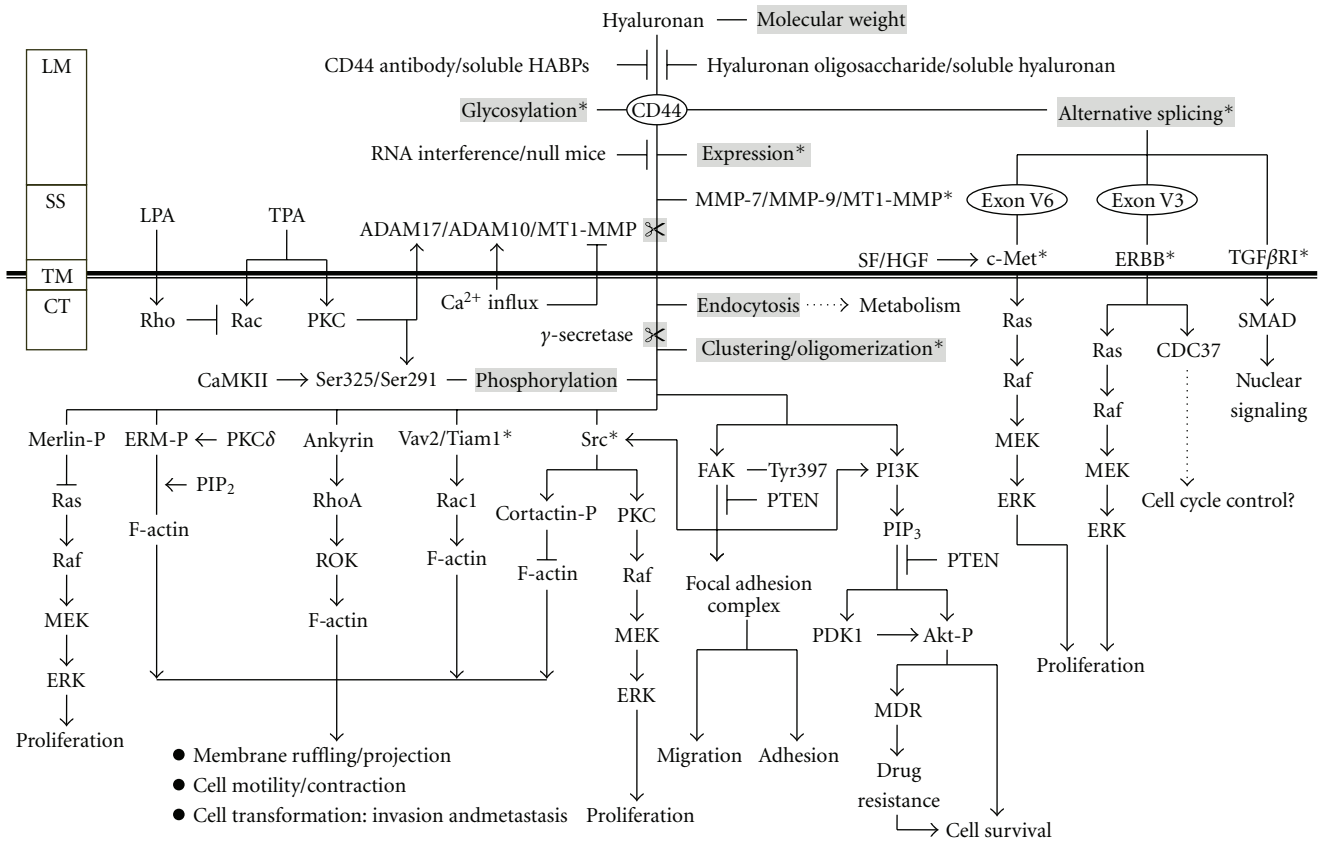

Figure 1: Signaling through CD44: hyaluronan binding to a receptor such as CD44 or RHAMM will cause a conformational change in the receptor. In the case of CD44, γ-secretase cleavage of the intracellular fragment can lead to phosphorylation of cellular components such as CaMK (calmodulin kinase). Clustering of the intracellular portion of CD44 with cellular proteins such as merlin, Src, and PKC leads to downstream activation of the Raf/MEK/ERK pathway, enhancing cell proliferation. Alternatively, activation with ankyrin, ERM, or Vav2/Tiam1 leads to F-actin activation and cytoskeletal rearrangement, membrane ruffling, and cell motility. Hyaluronan signaling also regulates cell migration and adhesion through interaction of PI3K and activation of the focal adhesion complex. PI3K activation by hyaluronan signaling, when incorporated with the Akt pathway, may also lead to MDR and cell survival.

ability to differentiate, proliferate, and self-renew, comparable to normal stem/progenitor cells; hence, $CD44^+/CD24^{low/-}$ cells were considered to possess the stem/progenitor cell phenotype [26]. Later, this idea was confirmed when epithelial mesenchymal transition (EMT) traits were observed to correlate with the $CD44^+/CD24^{low/-}$ stem cell phenotype in human breast cancer [27]. Another marker, ALDH1, further divides the $CD44^+CD24^{low/-}$ cell population into 2 fractions, in which the $ALDH1^+CD44^+CD24^{low/-}$ cells are highly tumorigenic [28]. Forced expression of CD24 in $CD44^+CD24^{low/-}$ cells resulted in decreased MEK/MAPK signaling, reduced cell proliferation, and enhanced DNA damage-induced apoptosis through attenuated NF$\kappa\beta$ signaling [29]. In contrast, siRNA silencing of CD44-encoding genes in $CD44^+CD24^{low/-}$ cells enhanced sensitivity to doxorubicin, suggesting CD44 as a suitable target for treating cancer via gene therapy [30].

This new understanding of hyaluronan/CD44-mediated oncogenic signaling events may have important clinical utility and could establish CD44 and its associated signaling components (hyaluronan/CD44-mediated Nanog-Stat-3) as important tumor markers for early detection and evaluation of oncogenic potentials. This could also serve as groundwork for the future development of new drug targets that may inhibit hyaluronan/CD44-mediated tumor metastasis and cancer progression.

3. Hyaluronan Constitution in the Stem Cell Niche

After the term "niche" was first proposed in 1978 [31], important related studies in cell biology emerged, including a focus on the mysterious microenvironment that supports stem cells. The stem cell niche is not solely the location where these cells are present but involves the surrounding cellular components of the microenvironment and the signals emanating from the support cells [32]. This niche requires an environment that fosters a delicate balance between self-renewal and differentiation. Absence of this balance immedi ately triggers inappropriate differentiation. Indeed, a decrease in proliferation potential of bone-marrow-derived hematopoietic stem cells (HSC) has been observed in

the absence of a "niche" environment [33]. Hematopoiesis, the process by which HSC differentiate into hematopoietic cells in order to generate different blood cell types, is greatly influenced by the surrounding microenvironment. The actual mechanism by which HSCs interact with the niche environment remains largely unknown; however, many studies have shown that the HSC niche is important for attracting and anchoring HSCs. Hyaluronan, being a critical component of the HSC microenvironment and widely distributed in mesenchymal tissue, is thought to take part in the postnatal hematopoietic niche since it is required for in vitro hematopoiesis [34]. Hyaluronan degradation leads to an arrest in HSC proliferation that is necessary for commitment to the maturation of hematopoietic cells [34].

In vertebrates, hyaluronan appears to regulate cell transformation and migration at several embryonic stages, as early as gastrulation. During embryogenesis, the accumulation and organization of hyaluronan plays a role in the epithelial-mesenchymal transition, a critical step in early embryogenesis for differentiation of pluripotent embryonic stem cells (ESC) to mesenchymal stem cells (MSC) for further formation of different tissues [35]. In general, endogenously produced hyaluronan contributes to differentiation of hESC to mesodermal lineage, but more specifically to hematopoietic cells [36]. The likely source of hyaluronan during early embryonic developmental stages is hyaluronan synthase (Has) 2 [37]. Many cardiac or skeletal development anomalies are due to inactivation or upregulation of hyaluronan synthetases. It seems that interaction of hyaluronan with ESC is fundamental to early embryonic development. Failure of this interaction results in abnormal tissue formation. Hence, hyaluronan has a central regulatory role during embryogenesis and morphogenesis [38, 39]. At later stages, Has1, Has2, and Has3 are expressed in both undifferentiated and differentiated hESC [36]. During embryonic development, hyaluronan mostly interacts with cells via the RHAMM and CD44 receptors [15, 34]. High-molecular-weight hyaluronan induces CD44 association with MEKK1 in epicardial cells to promote epithelial mesenchymal transition and differentiation [40]. However, more recent studies have observed that the HA-CD44 signaling pathway is largely associated with activation of ERK, by which the EGF receptor and downstream molecules such as Raf, MEK, and ERK1/2 are activated to promote cell proliferation [15]. ERK and its downstream molecules cyclin D1 and E2F also affect cell cycle progression [41].

In addition to collagen, alginate, and fibrin, hyaluronan is one of the extracellular matrix molecules being used to develop scaffolds for in vitro studies of stem cells, mainly due to its great abundance in the stem cell niche environment. Human ESCs may be maintained in an undifferentiated state by utilizing hyaluronan hydrogels [42], once more indicating the importance of the interactions provided by hyaluronan.

4. Influence of Hyaluronan on Stem Cell Behavior

MSCs are naturally sensitive to their environment, responding to chemical, physical, and mechanical features of their matrices or substrates, as well as the spatial/temporal presentation of biochemical cues [43]. Hyaluronan influence in behaviors such as adhesion, proliferation, differentiation, and migration occurs through several newly discovered genetic signaling mechanisms that involve binding to specific cellular receptors.

4.1. Effect of Hyaluronan on Mitochondrial Function. The effect of hyaluronan in cell behavior involves a direct influence in cell metabolic activity including bioenergetics. Few studies have underscored this phenomenon in stem cells; however, the first report suggesting an influence of hyaluronan on mitochondrial properties was obtained through studies of chondrocytes from osteoarthritis (OA) patients, who exhibit disrupted cellular behavior [44]. The fact that abundant levels of hyaluronan are present in normal and healthy joint areas and that these levels are dramatically diminished in OA patients [44] suggests that hyaluronan abundance may be a somewhat chondroprotective. Because oxidative stress, disrupted mitochondrial respiration, and mitochondrial damage promote aging, cell death, functional failure, and degeneration in a variety of tissues [45], including joint regions, it is possible that the putative chondroprotective role of hyaluronan may occur through the preservation of mitochondrial function. This was proven true when incubation of primary human chondrocytes from OA patients with hyaluronan significantly increased mtDNA integrity, improved ATP levels, and increased cell viability under normal conditions [44]. Moreover, hyaluronan ameliorated the negative effects of reactive oxygen and nitrogen species on mtDNA integrity, mtDNA repair, ATP production, and cell viability, all of which chondrocytes are exposed to during OA. Anti-CD44 antibody at saturating concentrations abolished the protective effects of hyaluronan, which suggests the mechanism is mediated by this receptor. CD44 promotes apoptotic resistance in colonic epithelium via a mitochondria-controlled pathway [46]. In addition, expression of CD44 in some cell types, such as stem cells, may provide the means to internalize hyaluronan by endocytosis, and one of the functions of internalized hyaluronan is to protect DNA from oxidative metabolism [47]. In this study, high-molecular-weight hyaluronan in culture medium prevented H_2O_2-induced H2AX phosphorylation in 2 cell types. In contrast, the effect of low-molecular-weight hyaluronan was somewhat less pronounced. Indeed, there is evidence that some glycosaminoglycans such as chondroitin-4-sulfate and hyaluronan inhibit lipid peroxidation caused by oxidative stress and thereby decrease inflammatory reactions mediated by oxidants [48]. These conditions mimic the situation in vivo when cells are growing within an intercellular matrix containing hyaluronan and are exposed to an exogenous oxidizing agent. This supports the proposition that one of the biological functions of hyaluronan is to provide protection against cellular damage caused by radicals produced by oxidation or ionizing radiation.

Stem cells thus may have several diverse mechanisms to protect the integrity of their DNA, such as by maintaining low metabolic activity to ensure minimal oxidative damage, having a highly effective efflux pump that rapidly removes

genotoxic agents from the cell [49], and possibly allowing internalization of hyaluronan, which protects DNA from oxidants. These mechanisms may be coordinated with each other and with cell cycle status. However, information is sparse concerning how exactly CD44 may affect mitochondrial function. Stem cell mitochondria have recently come under increased scrutiny because new information has revealed their role in numerous cellular processes, beyond ATP production and apoptosis regulation, suggesting mitochondria to serve as a cell fate or lineage determinant [50–52]. Thus, hyaluronan influence on mitochondrial properties may at least to some extent affect stem cell self-renewal and differentiation. Unfortunately, no ongoing studies have been published thus far.

4.2. Cell Migration. Observations made by a very successful research group in Boston in the beginning of the 1970s revealed that hyaluronan accumulation coincides with periods of cellular migration [53]. This event may occur through the physiochemical properties of hyaluronan or via direct interaction with cells. When hyaluronan is synthesized and released to the extracellular environment, its physiochemical characteristics of viscosity and elasticity contribute to local tissue hydration. This results in weakening of cell anchorage to the extracellular matrix, allowing temporal detachment to facilitate cell migration and division [54]. Chemically, this can be explained by the hyaluronan structure. In dilute solutions, where the domain that is occupied by each hyaluronan molecule expands because of mutual repulsion between the carboxyl groups inside its structure, a large volume will eventually be occupied with water trapped inside it. This property provides resilience and malleability to many tissues so that in hyaluronan-rich areas internal pressure may cause the separation of physical structures and create "highways" for cell migration; during fetal development, the migration path through which neural crest cells migrate is rich in hyaluronan [55]. Another example is the migration of mesenchymal cells into the cornea following increased hyaluronan deposition, hydration, and concomitant swelling of the migratory pathway [56]. Beyond the physiochemical interactions with hyaluronan, cells may be able to mediate, direct, and control their migration and locomotor mechanism through specific interactions via cell surface hyaluronan receptors. The principal cell surface receptors include CD44, RHAMM, and ICAM-1 [17]. RHAMM in particular forms links with several protein kinases associated with cell locomotion, for example, extracellular signal-regulated protein kinase (ERK), $p125^{fak}$, and $pp60^{c-src}$ [17]. Increased cell movement in response to hyaluronan can also be demonstrated experimentally in other cell types, and cell movement can be inhibited, at least partially, by degradation and/or blocking of hyaluronan receptor occupancy.

4.3. Cell Cycle and Proliferation. The formation and repair of mature hard tissue require cell proliferation. Hyaluronan levels have been shown to have a direct influence on this event. Cell proliferation is activated by hyaluronan, which increases volume and surface area for cell migration and cellular activities, and stimulates receptor-mediated events. Hyaluronan can form a pericellular coat, settle into a cell-poor space, and facilitate cell detachment from the matrix and mitosis in response to mitogenic stimulators such as preinflammatory mediators and growth factors [57]. Human fibroblasts synchronized with colchicine or cytochalasin showed increased hyaluronate synthesis at the time of cell rounding during mitosis but declined sharply as cells entered G_1-phase and resumed the spread morphology [57]. It is reasonable to suggest that basal levels of hyaluronan synthesis during the G_1, S, and G_2 phases could be used for cell migration; during mitosis, synthase activity could be activated at all cellular contact areas to cause detachment and rounding. The high local concentration of hyaluronan causes release of endogenous growth factors and stimulates cell-cell interaction, resulting in faster cell proliferation during early stages of in vitro culture [58]. Although hyaluronan facilitates cell detachment, it has not been shown to possess direct mitogenic activity. However, by facilitating cell mitosis in response to mitogenic factors that are abundant during the early phases of tissue repair, hyaluronan may also have an important, although indirect role in cell proliferation [17].

Many reports have experimentally confirmed this idea, demonstrating the acceleration of stem cell proliferation by hyaluronan. In previous studies using mouse adipose-derived stem cells (mADSCs), we showed that supplementation of the culture medium with minute amounts of high-molecular-weight hyaluronan increases the growth rate of mADSCs at early passages, contributes to the extension of their lifespan with a marked reduction of cellular senescence during subcultivation, and prolongs their differentiation potential [4]. When mADSCs were cultured on a hyaluronan precoated surface, cell aggregates formed with a much more gradual growth profile. Hyaluronan-containing matrix seemed to be a poor attachment substratum for cell growth, as previously observed [59]. At later passages, aggregation limited propagation and contact inhibition, resulting in a slight increase of p16INK4a expression, consistent with previous reports [60]. Our study provided evidence that placenta-derived mesenchymal stem cells (PDMSCs) grown on a hyaluronan-coated surface are maintained in slow-cycling mode and that a prolonged G_1 phase occurs through elevated levels of $p27^{kip}$ and p130, which are responsible for suppressing cell entry into S-phase [6]. This in turn may be the main cause of reduced proliferation observed in stem cells cultured on hyaluronan-coated surfaces [4]. This line of evidence was confirmed by another research group who found that a high percentage of primary rat calvarial osteoblasts, in the presence of sulfated hyaluronan (HAS) derivatives, remained in G_1 phase with a concomitant decrease in the number of cells in S and G_2/M phase, eventually leading to a decrease in cell proliferation [61]. Recent studies have shown that prolonged G_1 transit is associated with an increase in $p27^{Kip1}$ [62]. Because p27 phosphorylation by cyclin E/Cdk2 is a prerequisite for its ubiquitination and degradation, it is interesting that in CD44-treated cells cyclin E/Cdk2 kinase activity is decreased, suggesting that CD44 might inhibit the ubiquitin-dependent proteolytic pathway of p27, leaving this molecule in an active form [62]. The specific signaling cascade that inhibits p27

degradation via CD44 remains to be characterized. However, it was speculated that blockage of the Ras pathway, which represents a principal force in driving the cell cycle [63], is involved in this process [64–66].

Hyaluronan thus appears to be effective in maintaining mADSCs in a proliferative state, delays senescence, and, more strikingly, directly influences cell proliferation. Different forms of hyaluronan supplementation may cause distinct proliferative behaviors in stem cells. Although the mechanism by which hyaluronan promotes or slows proliferation and preserves the differentiation potential of mADSCs merits further investigation, the addition of hyaluronan to a culture system may be a useful approach for expanding adult stem cells in vitro without losing their replicative and differentiation capabilities.

4.4. Cell Differentiation. In native tissue, MSCs reside in a defined microenvironment that regulates stem cell survival, self-renewal, and differentiation through growth factors, cell-cell contact, and cell-matrix adhesion. This occurs due to the direct influence of cell adhesion to their underlying biomaterials. Although soluble factors are potent regulators of stem cell differentiation, recent discoveries have underscored the importance of the physical and chemical characteristics of the matrices in determining stem cell fate [67]. Long-term culture of undifferentiated ESCs on a hyaluronan-coated surface instead of feeder layers yields pluripotency and differentiation characteristics similar to those of cells cultured on mouse embryonic fibroblasts (mEF) after 1 month of culture [68]. It seems that hyaluronan matrices act as a unique microenvironment for propagation of hESCs, likely due to the regulatory role of hyaluronan in the maintenance of hESCs in their undifferentiated state in vitro and in vivo. Indeed, in humans, the hyaluronan content is greatest in undifferentiated cells during early embryogenesis and decreases at the onset of differentiation [39]. It has been suggested that hESCs are able to take up and degrade hyaluronan through CD44 and thereby remodel hyaluronan matrices, a feature necessary for cell survival and migration [42]. After activation of several environmental cues that dictate the need for fully differentiated cells, hyaluronan supplementation may in turn induce faster cell attachment and enhance cell differentiation, possibly through improved cell-cell communication [33]. How hyaluronan may mediate this event is still unknown but has been confirmed by a new, engineered class of hyaluronan-based hydrogels that provide a natural extracellular matrix environment with a complex mechanical and biochemical interplay. The hydrogel induced osteoblast differentiation of MSCs without the use of osteogenic media. This most likely occurred through the enhancement of cell adhesion [33].

4.5. Multidrug Resistance. "Classic multidrug" resistance (MDR) is caused by increased drug export through ATP-dependent efflux pumps, such as multidrug-resistance proteins (MRPs), and other members of the ATP-binding cassette (ABC) transporter families [69]. The finding that hyaluronan stimulates cell survival signaling and that treatment of tumor cells with hyaluronidase increased the activities of various chemotherapeutic agents [70] led to the further investigation of the possible role of hyaluronan in drug resistance. Increased hyaluronan production stimulates drug resistance in drug-sensitive cancer cells, whereas disruption of endogenous hyaluronan-induced signaling suppressed resistance to doxorubicin, taxol, vincristine, and methotrexate [71]. Therefore, the effects of hyaluronan on cell-survival signaling might alter drug resistance. Although the antiapoptotic effect of hyaluronan probably contributes to these phenomena, it is also known that lipid products of PI_3K, such as phosphatidylinositol-3,4-diphosphate, and phosphatidylinositol-3,4,5-triphosphate, directly mediate the function of ABC transporters that are involved in bile transport [72]. Because hyaluronan stimulates PI_3K activity, it might also influence drug resistance by stimulating drug transport [2]. Thus, transmembrane pumps may induce MDR by decreasing the intracellular accumulation and retention of drugs. Of particular interest is a recent work [73] indicating that inhibitors of MDR block hyaluronan synthesis and secretion and that hyaluronan might be secreted through multidrug transporters. Another possible regulator of hyaluronan-mediated multidrug resistance is the extracellular-matrix metalloproteinase inducer (EMMPRIN). EMMPRIN also stimulates the production of hyaluronan in mammary carcinoma cells [74]. Consequently, EMMPRIN promotes cell-survival signaling and induces multidrug resistance in a hyaluronan-dependent manner. In previous studies, we provided evidence that PDMSCs grown on a concentrated hyaluronan-coated surface become doxorubicin-resistant and that the interaction between CD44 and hyaluronan is crucial for this drug resistance [5]. Hyaluronan-CD44 interactions upregulate expression of drug transporters, including MDR1 [75], MRP2 [75], and BCRP [76]. In addition, small fragments of hyaluronan may mediate multidrug resistance by binding to CD44 and promoting translocation of a specific transcriptional regulator (YB-1) of the multidrug resistance MDR1 gene. This event simultaneously induces P-glycoprotein, the product of the MDR1 gene, and a broad-spectrum multidrug efflux protein present in both cancerous and healthy cells. Previous studies have reported that MDR is an important regulator of stem cell commitment [77]. Thus, we suggest that P-glycoprotein induced by the hyaluronan-coated surface holds PDMSCs in a primitive state by enabling them to extrude molecules required for differentiation. To the best of our knowledge, no reports have been published on MDR acquisition in mesenchymal stem cells; therefore, our study was the first to show that a hyaluronan substratum induces PDMSCs to acquire MDR as a result of increased P-glycoprotein expression through CD44 signaling. These and previous observations [4, 6] lead us to suggest that hyaluronan may cause PDMSCs to become dormant, the natural state of stem cells, which is consistent with slow cycling and drug resistance [5].

5. Hyaluronan Regulates Tissue Morphogenesis

Hyaluronan plays an important role in many morphogenetic processes during vertebrate development. Interaction

of hyaluronan with surrounding stem cells regulates cell differentiation or the fate of cells. After hyaluronan comes in contact with surrounding cells through receptors, hyaluronan degradation by proteolysis releases cell-associated factors which affect cell motility.

5.1. HA in Cardiogenesis. High-molecular-weight hyaluronan (HMW-HA) stimulated epicardial cells, during formation of coronary vasculature in embryonic development, promoted association of hyaluronan receptor CD44 with MEKK1 protein, induced MEKK1 phosphorylation, and activated the ERK-dependent and NFκB-dependent pathways. Two methods of CD44 blockage decreased the HMW-HA-induced invasive response in epicardial cells [40]. Recent development of hyaluronan mixed esters of butyric and retinoic acids to improve the yield of cardiovascular stem cells revealed an enhancement in smad1, 3, and 4 gene expression and further upregulation of the cardiogenic gene Nkx-2.5 expression, which led to high cardiogenesis from stem cells [78].

5.2. HA in Osteogenesis and Chondrogenesis. The application of hyaluronan for bone regeneration is well known. In fact, the importance of hyaluronan in spine development was found in a hyaluronan synthase-2 (Has2) knockout mouse model [79]. Previous reports and our unpublished data have demonstrated the potential of hyaluronan in the enhanced chondrogenic and osteogenic differentiation potentials of mesenchymal stem cells via stimulated expression of specific target genes. These genes include chondrogenic markers such as sulfated glycosaminoglycans, SOX-9, aggrecan, and collagen type II; osteogenic markers alkaline phosphatase (AKP), osterix, runx2, and collagen type I [80–82].

5.3. HA in Neurogenesis. There is substantial evidence of hyaluronan participation in morphogenesis and neural cell differentiation in the central nervous system. Sulfated hyaluronan can increase proliferation of normal human astrocytes and differentiation through enhanced expression of connexin-26, -32, and -43 [83]. Hyaluronan-containing hydrogels provide a suitable environment for neural stem cell growth and differentiation [84, 85].

5.4. HA in Angiogenesis. Previous reports have shown that tumor growth and metastasis are dependent on neovascularization, and oligo-hyaluronan induces angiogenesis in vivo [3]. Oligo hyaluronan-induced proliferation of endothelial cells and tube formation during angiogenesis is mediated by CD44 signaling, followed by phosphorylation of Src, FAK, and ERK1/2 proteins, leading to upregulation of *c-jun* and *c-fos*. This phenomenon is reversed after silencing of CD44 with siRNA [86]. Hyaluronan has been tested for cell therapy in a limb ischemic mouse model and was shown to enhance angiogenesis and improve revascularization [55]. Our unpublished data demonstrated that in vitro culture of PDMSCs with high-molecular-weight hyaluronan slightly increased the expression of angiogenic markers, including KDR, CD31, and vWF.

6. Role of Hyaluronan and Stem Cells on Tissue Regeneration

Hyaluronan plays an important role in preservation of the normal extracellular matrix structure and induces neodermis at the wound bed [87]. It is extensively used as an important component of assorted categories of biomaterials. Hyaluronan, which supports stem cell interaction with extracellular matrix molecules [88], is capable of regulating the inflammatory chemokines, receptors, metalloproteinases, and tissue inhibitors that are necessary to form an efficient scaffold. Modulation of the expression of inflammatory factors (CXCL12, CXCL13, and CXCR5) in mesenchymal stem cells by hyaluronan contributes to regenerative processes [89]. In addition, fragmented hyaluronan stimulates inflammatory gene expression in various immune cells at injury sites via TLR4, TLR2, and CD44 [90]. This occurs through downregulation of the anti-inflammatory A2a receptor [91]. During leukocyte recruitment, hyaluronan interaction with CD44 activates various inflammatory cells, such as macrophages, through CD44-dependent signaling [92]. In contrast, low-molecular-weight hyaluronan induces dendritic cell maturation and promotes dendritic and endothelial cell release of proinflammatory cytokines such as TNFα, IL-1β, and IL-12 through TLR4 [93, 94]

The migratory, proliferative, and differentiation influences of hyaluronan in stem cells provide insights for the development of potent biomaterials for regeneration of full-thickness wounds (e.g., diabetic foot ulcer and burn wounds) through initial restoration of the dermal layer and a later application of differentiated local cells that may lead to faster and better regeneration [95]. For instance, regeneration of bone defects can be achieved by combining cells and osteogenic signals in a suitable scaffold. Combination of human bone-marrow-derived mesenchymal stromal cells and TGFβ growth factor involved in chondrogenesis, on a commercially available hyaluronan biomaterial scaffold showed that cells were capable of proliferating and differentiating, forming a cartilage-like construct in vitro with increased expression of typical chondrogenic markers [88]. Combination of stem cells with bone morphogenetic protein-2 (BMP-2) in hyaluronan-based hydrogel has also been widely used for the same purpose [96].

Neural stem cell (NSC) therapy can be used for nerve and brain regeneration. Hyaluronan is the major extracellular matrix component of the adult central nervous system. Its presence alone is necessary for the differentiation of NSCs to neuronal cells in vitro [84]. Nevertheless, other reports have shown that using a 3D scaffold composed of HA, collagen, and the nerve growth factor neurotrophin-3 provided perfect nerve regeneration in vivo through differentiation of NSCs [97]. The efficacy of hyaluronan in several aspects of tissue regeneration is becoming better understood. This knowledge provides important insights into the therapeutic utility of stem cells in human disease.

7. Conclusion

The physiological role of hyaluronan, especially in regulation of cellular activities, may provide a potent therapeutic

alternative with profound advantages. However, this hypothesis cannot be applied to clinical practice without a more complete biological understanding of its mechanisms. Different mechanisms of hyaluronan regulation in stem cells are now emerging but at a very slow pace. The possibility of maintaining stemness or even inducing differentiation by manipulation of hyaluronan concentration/molecular weight, or by targeting genes activated by hyaluronan, may be a start point in the race to find new therapies for fatal diseases. Still, many knowledge gaps need to be filled, opening a window for future research efforts.

Acknowledgments

This work was supported by Research Grants NSC 98∼99-3111-B-006-002, NSC 99-2321-B-006-017, and NSC 100-2325-B-006-013 from the National Science Council of Taiwan. The authors also appreciate Dr. C. M. Liu for providing the figure. Mairim Alexandra Solis and Ying-Hui Chen contributed equally to this work.

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Hindawi Publishing Corporation
Biochemistry Research International
Volume 2012, Article ID 893947, 9 pages
doi:10.1155/2012/893947

Review Article

Chain Gangs: New Aspects of Hyaluronan Metabolism

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Received 25 August 2011; Revised 10 November 2011; Accepted 12 November 2011

Academic Editor: Timothy Douglas

Hyaluronan is a matrix polymer prominent in tissues undergoing rapid growth, development, and repair, in embryology and during malignant progression. It reaches 10^7 Daltons in size but also exists in fragmented forms with size-specific actions. It has intracellular forms whose functions are less well known. Hyaluronan occurs in all vertebrate tissues with 50% present in skin. Hyaluronan provides a scaffold on which sulfated proteoglycans and matrix proteins are organized. These supramolecular structures are able to entrap water and ions to provide tissues with hydration and turgor. Hyaluronan is recognized by membrane receptors that trigger intracellular signaling pathways regulating proliferation, migration, and differentiation. Cell responses are often dependent on polymer size. Catabolic turnover occurs by hyaluronidases and by free radicals, though proportions between these have not been determined. New aspects of hyaluronan biology have recently become realized: involvement in autophagy, in the pathology of diabetes., the ability to modulate immune responses through effects on T regulatory cells and, in its fragmented forms, by being able to engage several toll-like receptors. It is also apparent that hyaluronan synthases and hyaluronidases are regulated at many more levels than previously realized, and that the several hyaluronidases have functions in addition to their enzymatic activities.

1. Introduction

Hyaluronan (HA, hyaluronic acid) is a glycosaminoglycan (GAG) polymer that is a major component of the extracellular matrix (ECM) [1, 2]. It can occur as a high-molecular-weight (HMW) polymer, reaching $>10^7$ Da, but also exists in much smaller forms. The myriad of shorter HA chains occurs with activities that appear to be size specific. HA is major component of the ECM, particularly prominent during embryogenesis, in tissues undergoing rapid growth and development, during repair and regeneration, and in association with aggressive malignancies. Recent evidence indicates that HA also exists in an intracellular form. However, its functions therein are not as well established. Here, we attempt to summarize recent findings of this widely distributed GAG and to correlate specific functions with size and location of this intriguing carbohydrate chain.

2. Hyaluronan, a Highly Ironic Acid

2.1. Overview. HA is a strictly alternating disaccharide of N-acetylglucosamine and glucuronic acid connected by β1-4 and β1-3 glycosidic bonds, respectively. It is the only GAG that is not sulfated and without a covalent attachment to a proteoglycan core protein. It participates in many disparate processes, such as cell motility, tissue proliferation, embryonic development, malignant progression and metastasis, wound healing, and angiogenesis. HA is extruded into the extracellular space through the plasma membrane as it is being synthesized. Otherwise, the cell would become engorged with this huge space-occupying polymer.

HA has a relatively small volume of solvent water, but because of its great negative charge at neutral pH, it has an immense volume of aqueous domain that accompanies the molecule. This forces open tissue spaces through which cells can travel. The HA molecule, by binding to cell

surface receptors that interact with the cytoskeleton and that stimulate signal transduction pathways, endow cells with motility [3].

2.2. Hyaluronan of the Extracellular Matrix. Hyaluronan, in addition to occurring in a great number of sizes, also exists in a number of different forms in the vertebrate ECM. It can be firmly intercalated within a proteoglycan complex such as in cartilage, where it is held by electrostatic interactions by aggrecan and link protein with an avidity that approaches that of the avidin: biotin complex. HA can loosely be associated with an array of proteoglycans in loose connective tissues and in tissues undergoing rapid proliferation, development repair, and regeneration. It has a key role in organization of the entire ECM. HA can occur bound to the surface of cells by a number of membrane receptors.

The argument can be made that there are actually two forms of ECM, a widespread general intercellular matrix, and a more delicate pericellular matrix, often referred to as a glycocalyx. HA is a major component of both of these matrices, but is the greater portion of the total matrix of the glycocalyx. The glycocalyx of endothelial cells extends into the vascular lumen. The HA of the endothelial glycocalyx controls permeability and the diffusion of small solutes [4–6]. The functional lumen of the vascular system is far smaller than reflected in formalin-fixed tissue sections, occupying less that 25% of the apparent volume. There are proteoglycan- and HA-rich fronds that extend into the lumen, referred to by physiologists as "Duling's Lawn." This "lawn" disappears in a murine model, albeit transiently, following an *in vivo* injection of hyaluronidase (B.R. Duling, personal communication).

One of the several nonenzymatic functions of the cell-surface-bound hyaluronidase, Hyal2, is formation of the glycocalyx [7]. In structures such as bone, tendon, cartilage, and loose connective tissues, the ECM constitutes a major proportion of total tissue mass. In the intimate pericellular matrix that envelops individual cells, HA remains consistently the major ECM component and is critically involved in mechanotransduction, in rounding of cells during mitosis, in adhesion and deadhesion of cells, and in cell locomotion [8].

How HA becomes distributed into the two ECM components following synthesis is probably managed by the binding proteins that interact and decorate the polymer, including cell-bound receptors, and proteins with a wide range of avidity for HA. Such binding occurs in response to specific tissue needs and stresses, particularly in inflammation.

The polymer also exists in a freely circuiting form in lymphatics and in the cardiovascular system where it can be decorated by a number of binding proteins termed the hyaladherens, attached by both electrostatic and covalent interactions. These include cross-linking proteins such as tenascin, TSG-6 (tumor-necrosis-factor-stimulated gene 6), inter-alpha-trypsin inhibitor, pentraxin, and thrombospondin. Even in these circulating forms, there is probably some structural organization conferred by these associated proteins [9, 10].

A universal transitional ECM has recently become identified that enwraps muscle bundles, consisting of HA, tenascin, and fibronectin. This complex, synthesized by the deep fascia, supports limb regeneration in the salamander [11]. In higher vertebrates, this same complex may support muscle regeneration and repair. In surgery, it is customary in procedures involving limbs, muscles, and nerves, to provide a "fascial flap." This improves recovery, but no mechanism has ever been formulated as to how this occurs. The fascial "transitional matrix," rich in HA, may provide such a mechanism.

2.3. Hyaluronan Cables. Hyaluronan can also exist in cable forms, as has recently been documented [12], reflecting the remarkable diversity of this simple molecule. Incorporation of the heavy chain of IαI (inter-alpha-trypsin inhibitor) into bundles of HA facilitates formation of these cables, becoming simultaneously adhesion depots for inflammatory cells. These HA cables may be able to modulate the intensity of the inflammatory response. In marked contrast, the HA of the pericellular glycocalyx is not able to bind inflammatory cells, and actively excludes them. Of interest is that IαI is not associated with the HA of the glycocalyx and may therefore be the key mechanism. Other HA-binding proteins, such as versican, are found in both cables and in the glycocalyx.

2.4. Intracellular Hyaluronan. Intracellular HA has been well documented since 1999 [13–15], but its precise functions remain elusive. Many obstacles hinder precise localization, the most important being masking of HA by HA-binding macromolecules termed hyaladherens. These include proteoglycans, glycoproteins, and HA receptors. Despite that problem, many studies indicate that HA is indeed present intracellularly. Such HA is more prominent in proliferating cells where it is associated with microtubules, RHAMM (receptor for HA-mediated motility), and the mitotic spindle, suggesting functions associated with some aspects of mitosis and motility [14]. Intracellular HA is also more prominent in association with the stem cell niche, and with cellular stress responses such as inflammation. However, precise molecular mechanisms of such HA actions remain elusive.

Other conundrums associated with the distinction between intracellular and extracellular HA are the mode of synthesis, and distribution. Is all HA that is synthesized, secreted by cells through the plasma membrane and intracellular HA is then taken up by cells secondarily? Or are there two pathways of HA synthesis, one for export and another for maintenance within the intracellular compartment. Intracellular HA is found intracellularly in the cytoplasm, nucleus, and nucleolus. What are the mechanisms for the distribution to HA to each specific location?

3. The Hyaluronan Synthases

Three isoforms of a single enzyme synthesize HA, dual-headed transferases that utilize as substrates alternately UDP-glucuronic acid, and UDP-N-acetylglucosamine. These are

membrane proteins, located within and on the inner surface of the plasma membrane. The HA synthases are themselves synthesized in the endoplasmic reticulum in an inactive form, transported through the Golgi in an inactive form, and are then inserted into the plasma membrane. The enzymes must be activated, and they then begin to add alternatively the glucuronic acid and N-acetylglucosamine units to the reducing end of the growing polymer from their respective UDP precursors.

The active sites of the HA synthase enzymes protrude from the inner surface of the cell membrane. The newly made HA is extruded through the plasma membrane into the extracellular space, either into the ECM or on to the cell surface as it is being synthesized, permitting unconstrained polymer growth without destruction of the cell.

The three synthase genes in the mammalian genome code for HAS1, 2, and 3. These homologous isoenzymes each contain seven membrane-associated regions, and a central cytoplasmic domain possessing consensus sequences that are substrates for phosphorylation by protein kinase C. Even though they have 50–71% amino acid sequence identity, they are located on three separate chromosomes, human chromosomes 19, 8, and 16, respectively. This suggests gene duplications with divergent evolution. They are differentially regulated, each producing a different size polymer [16, 17], with independent gene regulation and kinetics.

A number of biologicals are directly able to regulate expression of HA synthase mRNA, including upregulation by bFGF [18, 19], and several proinflammatory cytokines [20] as well as suppression, by glucocorticoids [21, 22]. The latter may account for the atrophic changes observed following long-term glucocorticoid administration. Enhanced expression occurs also through lactoferrin, which also upregulates transcription of a type-1 collagen gene, COL1A1 [23]. These together suggest a mechanism by which wound healing is stimulated.

The activity of the HAS proteins can also be modulated by posttranslational modifications, such as ubiquitination and dimerization [24]. Aberrant splice variants of HAS1 are able to modulate expression of the normally spliced variant [25]. Abnormalities of the HA synthase enzymes have been associated with transformation and tumor metastases [2, 26]. Inherited and acquired variations in the HAS1 gene can contribute to disease progression in multiple myeloma and in Waldenstrom's macroglobulinemia [27]. Stimulation of the HA synthases in cultured cells in the presence of increased glucose promotes the deposition of an HA-rich matrix [28, 29]. This matrix is highly adhesive for monocytes and promises to provide new insights into diabetes. The HAS genes and their gene products are obviously tightly controlled at a vast number of levels, reflecting the extreme importance of HA in so many biological systems and the involvement in so many disease states [30].

4. Hyaluronan Catabolism

The breakdown of HA in tissues can occur by two entirely separate mechanisms, either enzymatically, by a class of enzymes known as hyaluronidases, or by cleavages that are nonenzymatic oxidation reactions.

4.1. The Hyaluronidases. Hyaluronan metabolism is extraordinarily active in vertebrates. The average 70 Kg individual has approximately 15 g of HA, 5 g of which turns over daily. HA has a half-life of 3–5 minutes in the circulation and just days in most tissues [31, 32]. Why the body eliminates HA so rapidly is not known. Hyaluronan is a scavenger of free radicals and reactive oxygen species. Removing these hazardous molecules and other toxic materials quickly may explain the rapid rate of HA elimination. The function of HA as a mechanism for the removal of toxins has not been explored but deserves attention.

There are two separate mechanisms for removing HA from the body, local cleavage reactions, catalyzed by free radicals and enzymatic activity, and by whole body elimination through the action of liver and kidneys. Ligation of the hepatic or renal arteries causes an instant rise in circulating HA levels [33], demonstrating the efficiency of such mechanisms. Clinically, this explains also the high levels of HA in patients in renal or hepatic failure, as well as in patients following liver or kidney transplants experiencing transplant rejection.

There are several classes of enzymes referred to as hyaluronidases: (1) prokaryotic enzymes that are eliminases, yielding disaccharides as the predominant products, (2) the eukaryotic enzymes that use a hydrolytic cleave reaction, adding water across the bond to be cleaved. Both of these classes of endoglycosidases are enzymes that cleave β-endo-N-acetylglucosamine bonds. The prokaryotic enzymes have specificity for HA, while the eukaryotic enzymes are also able to cleave chondroitin sulfate, albeit at a much slower rate, and (3) a curious class of hyaluronidases that are β-endoglucuronidases used by leeches and other invertebrates. Much less is known about this class of enzyme.

Eukaryotic hyaluronidases were until recently relatively neglected enzymes, largely because of the difficult in purifying them. Hyaluronidases are extremely unstable in the absence of detergents and protease inhibitors. They occur in very low concentrations, with activities one log greater than other globular enzyme proteins. The concentration of hyaluronidase in human plasma is 60 ng/mL [34].

There are six hyaluronidase-like sequences in the human genome, while rodents have seven such sequences. All are transcriptionally active with unique tissue distributions. In the human, three genes (*HYAL1, HYAL2*, and *HYAL3*) are found tightly clustered on chromosome 3p21.3. Another three genes (*HYAL4, PHYAL1* (a pseudogene), and PH20, sperm adhesion molecule1 (*SPAM1*)) are clustered similarly on chromosome 7q31.3 [35].

The enzymes HYAL1 and 2 constitute the major hyaluronidases in somatic tissues; HYAL1, an acid-active lysosomal enzyme, was the first somatic hyaluronidase to be isolated and characterized. Why an acid-active hyaluronidase should occur in plasma is not clear. HYAL1 is able to utilize HA of any size as a substrate and generates predominantly tetrasaccharides. HYAL2 is also acid active, anchored to

plasma membranes by a GPI (glycosylphosphatidylinositol) link.

The schema for HA catabolism [36, 37] indicates that HMW HA is taken up by cells by the concerted effort of the receptor CD44, the enzyme HYAL2, and the proton pump Na^+H^+ exchanger-1 (NHE1). An acidic microenvironment is created on the surface of the cell in membrane invaginations termed "lipid rafts" [38]. HYAL2 cleaves the HMW HA to a limit product of approximately 20 kDa, or about 50 disaccharide units. The resulting HA fragment is delivered to early endosomes and to lysosomes wherein the lysosomal enzyme HYAL1 cleaves the HA further to predominantly tetrasaccharide fragments. HYAL1, in the artificial test tube environment, can accept any sized HA polymer as a substrate and degrade it to the limit tetrasaccharide. The additional activities of the lysosomal exoglycosidases, β-glucuronidase, and β-hexosaminidase cleave fragments to single hexose sugars that then exit lysosomes to enter cytosolic pathways.

Not all tissues that contain HYAL1 activity synthesize that enzyme. Active endocytosis of the protein from the circulation occurs [39]. Monocytes contain no mRNA for HYAL1, yet have very high levels of enzyme activity (unpublished observations). Megakaryocytes and platelets contain no HYAL1 [40], perhaps because they lack the receptors for endocytosis of circulating HYAL1.

Evidence from an unexpected quarter sheds light on this anomalous situation. In the lysosomal storage disease termed X-disease, the mannitol-6-P receptors for lysosomal enzymes are defective. In such cultured fibroblasts, most lysosomal enzymes are absent, but levels of HYAL1 are normal. This suggests that the HYAL1 is taken by an unknown and, as yet unidentified receptor, one that is not affected by the lesion in the mannose-6-P receptor pathway. From this, it can be deduced that the unknown receptor is absent or blocked in platelets and megakaryocytes.

These hyaluronidases are endoglycolytic activities with specificity for the β1-4 glycosidic bond. These enzymes have a hydrolytic mechanism of action, compared to the eliminase mechanism of prokaryotes. Isolation and characterization of the vertebrate enzymes indicate that they are ubiquitous. Rapid progress in genome analyses has provided much information. There are six hyaluronidases in the human genome [41, 42] with seven being present in the murine genome. Two hyaluronidases, HYAL1 and HYAL2, are the predominant activities in somatic tissues, working in concert to degrade HMW polymers to tetrasaccharides as the predominant end product.

HYAL1 is an acid-active lysosomal enzyme and the first to be isolated and characterized [34, 43, 44]. HYAL1 is a 57 kDa polypeptide that also occurs in a processed 45 kDa form, the result of two endoprotease reactions, disulfide bonds binding the resulting two chains together. These two forms do not have a zymogen-active enzyme relationship, since they have similar specific activities. The fragment generated by the cleavage reactions may also have an important function in the overall economy of the niche.

An inactive 70-kDa-precursor form of HYAL1 is present in some mammalian sera, including calf serum, as can be observed on electrophoretic gels by Western blot. The inactive unprocessed form occurs in sera from many species in which it is claimed that no acid-active enzyme exists [45].

It is not clear why an acid-active enzyme is present in the circulation. Some animals have no apparent activity in serum or plasma. At one time, it was assumed that the serum enzyme, now known to be HYAL1, could not be an important activity if it was entirely absent in some mammalian species. However, it has been demonstrated that an inactive high molecular form occurs, as shown by Western blot in gels of such sera (R. Stern, unpublished observations).

HYAL2 has several forms, one being anchored to the external surface of plasma membranes by a GPI-link. HYAL2 also occurs in a processed soluble form, and a portion can be detected in early lysosomes. The several forms are thought to shuttle between various cellular compartments in the process of HA internalization and degradation, as it is delivered from the extracellular space in steps to the final destination in acid lysosomes. HYAL2 cleaves the large HA polymer to a limit product of 20 kDa, or about 50 disaccharide units, while HYAL-1 *in vitro* is able to digest any HA chain including the high-molecular-weight polymer to the tetrasaccharide fragment.

While only two hyaluronidases participate in HA catabolism in normal somatic tissues, aberrant expression of other hyaluronidases can be detected in cancerous tissues. One of the products of the six hyaluronidase-like sequences in the human genome, PH-20, a neutral-active hyaluronidase, was originally assumed to be sperm specific. Using more sensitive techniques, the enzyme can be detected in other tissues, in the epididymis, seminal vesicles, and prostate gland [46]. Additionally, PH20 can be aberrantly expressed in a number of human malignancies [47–50].

4.2. Nonenzymatic Cleavage. A portion of the breakdown of HA takes place by oxidation reactions. The cleavage is caused by unstable molecules, by reactive oxygen species (ROS) and by free radicals [51, 52]. The proportions of HA catabolism between enzymatic and oxidative cleavage reactions have not been established, though it is assumed that the former predominates. These proportions may vary considerably, depending on the physiological situation. Early in acute inflammation, the myeloperoxidase enzyme reaction associated with neutrophils may be an important catalytic mechanism for HA breakdown.

There are major differences between the products of enzymatic and free radical catabolism. While enzymatic cleavage results in polymers that are identical in structure to the parent polymer, oxidative cleave generates polymers with oxidized termini. Such structural differences may provide metabolic cues. It is possible to determine the proportion of HA breakdown enzymatic and nonenzymatic mechanisms, based on such differences between their respective reaction products. However, to date, there has been no research performed to address this issue, despite its intrinsic importance in biology. ROS are constantly being produced in the body and are tightly regulated to maintain a redox balance or homeostasis, together with a series of antioxidants and attendant reactions, for example, glutathione, superoxide dismutase and the hexose monophosphate shunt.

HA, hyaluronidases, and these redox mechanisms constitute interactions between a number of physiological situations as well as the pathophysiology of infection, inflammation, and the immune responses. This is an important area of biology also that has been relatively neglected, although inroads are currently being undertaken [53].

5. Hyaluronan Receptors

There are an ever-growing number of HA receptors. A conundrum to be dealt with is the indistinct boundary between membrane-bound receptors and HA-binding proteins, known as hyaladherens. The prominent receptor, CD44, also occurs as a soluble circulating protein, perhaps functioning as a decoy for the cell-bound receptor.

5.1. CD44. The CD (clusters of differentiation) system is commonly used as cell markers in immunophenotyping. CD44 is considered the most prominent receptor for HA. It is a transmembrane glycoprotein that occurs in a wide variety of isoforms, products of a single gene with expression of 10 variant exons [54]. The CD44 isoform can occur with the total absence of variant exon insertions, referred to as CD44S or the standard form. The variant exons are all inserted in various combinations into a single extracellular position near the membrane insertion site. Additional variations in CD44 structure occur as a result of posttranslational glycosylation, addition of various GAGs, including chondroitin sulfate and heparan sulfate. CD44 is widely distributed, being found on virtually all cells except red blood cells.

CD44 plays a major role in myriads of physiological processes [55, 56] and is particularly important in malignancy, in tumor cell homing and metastasis. There is a vast literature on the combinations of variant exons that occur on tissue-specific cancers.

5.2. RHAMM. RHAMM is also given the designation CD168. This protein exists in the nucleus and cytoplasm, as well as on the cell surface. This receptor binds to the mitotic spindle, participates in cell locomotion, focal adhesion, and contact inhibition. It also has many functions that support the transformed state [57], levels being over-expressed in many human cancers. To designate it as a receptor only is obviously a simplification. Many of the proteins associated with HA metabolism have multiple functions. The names scientists apply to these proteins can be misleading, as the assumption is often made that their names designate their only function.

RHAMM can partner with CD44 on cell surfaces where it enhances CD44-mediated signaling. It thus contributes to tumor progression by enhancing tumor-promoting properties of CD44. The close coordination of the RHAMM and CD44 receptors is further supported by the observation that mice with a genetic deletion of CD44 upregulate expression of RHAMM, the latter being able to substitute for many of the functions usually carried out by CD44 [58].

5.3. HARE. HARE (HA receptor for endocytosis), also known as stabilin-2, binds and internalizes not only HA and chondroitin, but also sulfated GAGs such as chondroitin sulfate, dermatan sulfate, and heparin [59].

5.4. LYVE-1. LYVE-1 stands for (lymphatic vessel endothelial HA receptor)-1. This cell surface receptor on lymphatic endothelial cells for HA is the subject of debate, but its conservation in evolution indicates that it is an important molecule [60]. LYVE-1 is not restricted to lymphatic vessels, but is also expressed in liver sinusoids, suggesting that it is involved in HA drainage in lymphatics and liver, and therefore in overall body turnover.

An unexpected complexity is the regulation of LYVE-1 HA-binding activity by glycosylation and sialylation. This receptor, like CD44, may become active only after appropriate unmasking *in vivo* [61].

5.5. TLR-2 and -4. TLR (toll-like receptors) -2, and -4, CDs 282 and 284 respectively, are membrane receptors responsible for initiating action from cells of the innate immune system by stimulation of pathogen-associated molecular patterns (PAMPs) [62, 63]. TLR-2 is present on macrophages where, when stimulated by gram-positive bacteria, it induces transcription factor NF-KappaB and thereby the release of proinflammatory cytokines. TLR-4 is also found on macrophages where the stimulation is induced by lipopolysaccharide on gram-negative bacteria. These in turn initiate signal transduction pathways within macrophages. Since the cellular coat of both *Streptococcus* A and C contain HA components, data suggests that short chain HA may function as a PAMP in the inflammatory environment. This signal transduction has been demonstrated in lung and epithelial tissue in both infectious and traumatic situations [60–64].

6. Chain Length as an Information-Rich System

Despite its simple repeating structure, HA has a wide range and occasionally contradictory functions, even though it is without branch points and without sulfation or other secondary modifications. The multiple functions can be attributed to differences in chain length. The variation in size is an extraordinarily rich informational system [65, 66]. In general, high-molecular-weight HA occurs in normal healthy tissues. Fragmented HA, on the other hand, is highly inflammatory, angiogenic, and immune stimulatory, a reflection of tissues under stress. The large HA polymers are, in marked contrast, anti-inflammatory, anti-angiogenic, and immunesuppressive.

Hyaluronan is critical in edema, inflammation, and during wound healing. Polymer size is critical throughout these processes. Tissue edema is one of the five cardinal signs of inflammation. Such swelling is comprised predominantly of HA, a concept that is not sufficiently appreciated. Indeed, many of the inflammatory cytokines induce HA production, and, conversely, HA fragments induce such cytokine synthesis in a self-stimulatory cycle [55, 67]. Hyaluronan, in a size-specific manner, also modulates a great number of immune responses [68].

7. The Malignant Connection

Hyaluronan plays a critical role in malignancy [69–71], levels of HA often correlating with tumor aggressiveness and poor prognosis. Such correlations have been validated for both epithelial tumors (carcinomas), as well as for malignancies of mesenchymal origin (sarcomas). Of particular interest is the observation that the HA content of the nonmalignant stromal component of tumors also correlates with malignant aggressiveness, suggesting that the stromal component is not entirely normal. Tumors may commandeer stromal populations from bone marrow or induce expansion of stem cell-like or populations of fibroblasts that resemble embryonic fibroblasts.

The balance of synthesis and degradation of HA is regulatory for cancer cell proliferation and motility *in vitro*, and in tumor invasion and progression *in vivo* [72–76]. Of particular importance is the essential role of HA in the microenvironment during the process of tumor initiation and early progression [76]. As mentioned, the importance of local stroma in supporting such events, and peculiarities of the attendant HA metabolism is critical for success of the cancer. A recent volume profiles the many functions of HA in cancer biology and in the transformed state [77].

8. Hyaluronan in Skin

More than 50% of total body HA is contained in skin and thus requires "special handling." It is responsible for skin hydration, and protects skin against free radical damage, particularly against UV light, both UVA and UVB.

There are many reports that document decreasing levels of skin HA with aging, with the suggestion that this is responsible for the deteriorating appearance of skin with aging. Such observations are based on histochemical staining criteria. However, biochemical extraction indicates that HA levels remain constant with age. The HA becomes increasingly tissue associated, becoming progressively more resistant to extraction as a function of age [78].

The apparent results of the histochemical investigations can be explained by increasing competition between tissue proteins for HA binding sites and the HA-binding peptide as a function of age. The HA, encased within tissue proteins may be restricted from functioning as a hydrating molecule. This proviso also indicates that the HA-staining procedure, as normally performed with an HA-binding peptide, is not a quantitative procedure.

Hyaluronan in skin occurs in both dermis and epidermis, with dermis containing the greater proportion. Epidermal HA is more loosely associated and more easily extracted from tissue. Formalin, an aqueous fixative, easily removes most HA from epidermis and is less able to extract HA from dermis. Alcoholic acid formalin enhances histolocalization of epidermal HA, and indicates that considerable levels are contained therein [79].

Skin HA has a very rapid turnover, with a half-life of 1-2 days in the epidermis [80]. The turnover rate in the dermis is similar, with catabolism occurring in liver and lymph nodes, following lymphatic drainage [31]. The turnover mechanism in the epidermis is not clear and may be a combination of free radical fragmentation stimulated by UV light, and enzymatic degradation.

Hyaluronan is most prominent in the upper spinous and granular, where much of it is extracellular. In the basal layer, HA is predominantly intracellular and is not easily eluted out during aqueous fixation. Basal keratinocyte HA is involved in mitotic events, presumably, while extracellular HA in the upper layers of the epidermis is involved in barrier disassociation and sloughing of cells.

Hyaluronan of the epidermal ECM forms two different structures; a pericellular coat close to the plasma membrane, forming the pericellular matrix, and HA chains that coalesce into large cables. Such cables, with induced expression under inflammatory events, bind leukocytes, whereas the pericellular HA does not [28, 81].

The HA content of the dermis is far greater than that of the epidermis. The papillary dermis has more prominent levels of HA than the reticular dermis. Exogenous HA is cleared from the dermis and rapidly degraded [31]. The dermal fibroblast provides the synthetic machinery for dermal HA. What makes the papillary dermal fibroblast different from other fibroblasts is not known. However, these cells have an HA synthetic capacity similar to that of the fibroblasts that line joint synovium, responsible for the HA-rich synovial fluid, or the hyalocytes of the eye that provide the vitreous of the ocular chambers.

9. Recent Additions to the Many Functions of HA

Despite the fact that HA does not give up its secrets easily, new functions for HA continue to emerge. An interesting connection has been described between HA and autophagy. Hyperglycemia is one of many factors that induces autophagy. Cells cultured in hyperglycemic medium initiate a stress response that includes HA synthesis. The HA is extruded into the ECM in a structural form recognized by inflammatory cells [29]. Cyclin D3 is central to such events. Abnormal deposits of HA and cyclin D3 occur in diabetic rat glomeruli, indicating that a similar process occurs *in vivo* [82]. More importantly, the HA stress response and autophagy may be major contributors to the multiple pathologies associated with diabetes [28].

Matrix HA also has a dramatic effect in immune regulation, on the phenotype of regulatory T cells [83]. The HA-containing ECM functions as a biosensor for the inflammatory microenvironment important for immune tolerance. HA that is HMW promotes induction of IL-10-producing regulatory T cells from T-cell precursors, while fragmented HA does not. This has been demonstrated in an IL-10-dependent mouse colitis system, in which HMW HA suppresses disease through such T-cell induction. The ability of HMW HA to cross-link CD44 on the cell surface may be an underlying mechanism for this phenomenon [84], thereby maintaining immunological tolerance.

10. Conclusion

There remain many questions regarding this highly ionic as well as highly ironic polymer. How do cell response mechanisms distinguish between the different sizes of linear HA polymers, given the identity of their chemical structures? One intuitive answer is that various HA chain sizes take on different physical-chemical configurations and that the sensing mechanisms respond to such differences. Another possibility is that different-sized HA fragments can associate with different profiles of hyaladherens or that there are major changes in affinity, depending on HA size. It is probably a combination of all such factors that determine the differences in responses.

Abbreviation

CD:	Clusters of differentiation
ECM:	Extracellular matrix
GAG:	Glycosaminoglycan
GPI-linked:	Glycosylphosphatidylinositol-linked
HA:	Hyaluronan, hyaluronic acid
HARE:	HA receptor for endocytosis
HAS:	HA synthase
HAS:	The gene that codes for a HAS
HMW:	High molecular weight
HYAL:	Hyaluronidase
HYAL:	The gene that codes for a hyaluronidase
LYVE-1:	Lymphatic vessel endothelial HA receptor
NHE1:	Na^+H^+ exchanger-1
NF-KappaB:	Nuclear factor kappa-light-chain-enhancer of activated B cells
PAMP:	Pathogen-associated molecular pattern
PHYAL:	A pseudogene for a hyaluronidase
RHAMM:	Receptor for HA-mediated motility
ROS:	Reactive oxygen species
TLR:	Toll-like receptor
TSG-6:	Tumor-necrosis-factor-stimulated gene 6
UV:	Ultraviolet.

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Hindawi Publishing Corporation
Biochemistry Research International
Volume 2012, Article ID 281284, 6 pages
doi:10.1155/2012/281284

Research Article

Association between Human Plasma Chondroitin Sulfate Isomers and Carotid Atherosclerotic Plaques

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Received 24 June 2011; Revised 21 October 2011; Accepted 9 November 2011

Academic Editor: Timothy Douglas

Several studies have evidenced variations in plasma glycosaminoglycans content in physiological and pathological conditions. In normal human plasma GAGs are present mainly as undersulfated chondroitin sulfate (CS). The aim of the present study was to evaluate possible correlations between plasma CS level/structure and the presence/typology of carotid atherosclerotic lesion. Plasma CS was purified from 46 control subjects and 47 patients undergoing carotid endarterectomy showing either a soft or a hard plaque. The concentration and structural characteristics of plasma CS were assessed by capillary electrophoresis of constituent unsaturated fluorophore-labeled disaccharides. Results showed that the concentration of total CS isomers was increased by 21.4% ($P < 0.01$) in plasma of patients, due to a significant increase of undersulfated CS. Consequently, in patients the plasma CS charge density was significantly reduced with respect to that of controls. After sorting for plaque typology, we found that patients with soft plaques and those with hard ones differently contribute to the observed changes. In plasma from patients with soft plaques, the increase in CS content was not associated with modifications of its sulfation pattern. On the contrary, the presence of hard plaques was associated with CS sulfation pattern modifications in presence of quite normal total CS isomers levels. These results suggest that the plasma CS content and structure could be related to the presence and the typology of atherosclerotic plaque and could provide a useful diagnostic tool, as well as information on the molecular mechanisms responsible for plaque instability.

1. Introduction

Atherosclerosis is a progressive disease characterized by the accumulation of lipids and fibrous elements in medium and large arteries. Plaque rupture and thrombosis are the most important clinical complication in the pathogenesis of acute coronary syndromes and peripheral vascular disease [1, 2]. Although numerous risk factors such as hypertension, diabetes, and hyperlipidemia are thought to play a role in the development and progression of this pathology [3], the mechanisms underlying plaque formation and progression are still largely unknown. Abnormal expression and structural modifications of arterial chondroitin sulfate proteoglycans (CS-PGs) have been implicated in atherosclerosis progression [4–6]. Arterial CS-PGs are markedly increased in early atherosclerotic lesions, participating in lipid retention, modification, and accumulation. Furthermore, CS-PGs play a key role in inflammation processes associated with atherosclerosis [6]. Numerous *in situ* lines of evidence indicate that plaque instability is caused by a substantial increase of both proteolytic activity and inflammatory state [7]. However, nowadays specific systemic markers for early diagnosis and prognosis of atheromatous lesions have not yet been identified.

Several studies have evidenced variations in plasma glycosaminoglycan (GAG) levels associated with both physiological and pathological conditions, such as strong physical

training [8], chronic lymphocytic leukaemia and essential thrombocythaemia [9], lupus erythematosus [10], and mucopolysaccharidosis [11, 12].

Chondroitin sulfate (CS) is the main GAG in normal human plasma. It consists of 12–18 repeating disaccharide units, each containing a hexuronic acid linked by β $(1 \rightarrow 3)$ bond to a *N*-acetyl-D-galactosamine residue, of which about 30% is sulfated at C-4 hydroxyl group of the hexosamine. It circulates covalently linked to the proteoglycan bikunin [13], a light subunit carrying the antiproteinase activity of plasma serine-proteinase inter-α-inhibitor (IαI). Several data suggest that bikunin plays an important role in inflammation. The IαI family molecules are synthesized in hepatocytes where one or two polypeptides, called the heavy chains (HCs), are linked to the CS chain of bikunin. After a stimulus, the IαI molecules leave the circulation and, in extravascular sites, the HCs are transferred from CS chain to the locally synthesized hyaluronic acid (HA) to form the serum-derived hyaluronan-associated-protein- (SHAP-) HA complex, which plays important roles in stabilizing extracellular matrices and it is often associated with inflammatory conditions [14]. Moreover, it has been described that bikunin also acts as a growth factor for endothelial cells, regulates the intracellular calcium levels, and inhibits kidney stone formation and smooth muscle cell contraction [15].

The aim of the present study was to evaluate possible correlations between plasma chondroitin sulfate (CS) level/structure and the presence/typology of carotid atherosclerotic lesion.

2. Materials and Methods

2.1. Sample Collection. CS isomers analyses were conducted on preoperative plasma samples from 47 patients (stenosis > 70%, 70.4 ± 7.9 years old) undergoing carotid endarterectomy, showing either a soft (26 patients) or a hard (21 patients) plaque, and from 46 healthy normolipidemic volunteers (control group), aged from 20 to 75 (47.8 ± 14.4 years old). The main clinical parameters of the patients under study are reported in Table 1. Lipid profiles and homocysteine levels in patients and controls are reported in Table 2. Plaque typology was assessed by ultrasonography using a Mylab 70 Xvision ecocolordoppler equipped with a LA332 AppleProbe 11–3 MHz (Esaote). Plaques were classified according to the Gray-Weale classification [16] in soft, with hypoechoic features (types 1 and 2), and hard, with hyperechoic features (types 3, 4, and 5). Informed consent was obtained before enrolment. The study was approved by the local ethical committees in accordance with institution guidelines.

2.2. Plasma CS Isomers Analysis. GAG purification was performed by a microanalytic preparative method, as previously described [17]. Briefly, 500 μL of plasma samples were subjected to proteolytic treatment with papain. Plasma CS was purified by anion exchange chromatography and precipitated with 5 volumes of ethanol at −20°C for 24 h. Subsequently, purified plasma CS isomers were subjected to depolymerization by using chondroitin ABC lyase (0.1 U per 100 μg hexuronic acid) and the unsaturated disaccharides were derivatized with 0.1 mol/L 2-aminoacridone (AMAC) [18].

Table 1: Main clinical parameters of the 47 patients undergoing carotid endarterectomy according to plaque typology.

	Hard (21)	Soft (26)	All (47)
Age	70.9 ± 5.1	70.2 ± 9.3	70.4 ± 7.9
Sex ratio (m/f)	2/1	1.3/1	1.6/1
Body mass index (kg/m^2)	26.9 ± 3.4	27.9 ± 3.8	27.5 ± 3.6
Symptomatic (%)	44.4	56.2	51
Transient ischemic attack (%)	33.3	25	28
Ictus (%)	11.1	31.2	22
Diabetes (%)	44.4	25	34
On therapy (%)	75	75	75
HbA_{1C} (%)	4.5 ± 2.6	4.5 ± 3.0	4.5 ± 2.8
Hypertension (%)	100	93.75	97
On therapy (%)	100	100	100
Dyslipidemic (%)	77.8	75	76
On therapy (%)	100	100	100
Smokers (%)	77.8	68.7	73.0
CRP (mg/dL)	1.16 ± 1.05	1.34 ± 0.84	1.26 ± 0.92

Separation and quantitation of chondroitin sulfate-derived disaccharides was obtained by capillary electrophoresis (CE) analysis by using a P/ACE capillary electrophoresis system (Beckman) equipped with a 75 mm id and 47 cm length uncoated fused-silica capillary using a 60 mmol/L sodium acetate buffer, containing 0.05% methylcellulose [17]. Separations were carried out at 25°C and monitored with a laser-induced fluorescence (LIF) detector at 488 nm excitation and 520 nm emission wavelengths. For quantitative analyses a homemade standard CS was purified from human plasma pools by a preparative approach. Briefly, standard CS isomers were obtained from papain-treated plasma by anion exchange chromatography, assayed for hexuronic acid content [19], lyophilized into aliquots, and stored at −20°C. For Δ-disaccharide analyses, a calibration curve was determined by submitting plasma-purified CS isomers to chondroitin ABC lyase treatment and derivatization procedure.

CS levels were expressed as μg of hexuronic acid per mL of plasma (μg_{UA}/mL), and CS charge density was evaluated as ratio of 4-sulfated Δ-disaccharides (ΔDi-4S) and total unsaturated disaccharides (ΔDi-4S + ΔDi-nonS).

2.3. Statistical Analyses. Statistical analyses were conducted by using SigmaStat software (Systat Software, Inc.). Student's *t*-test was used for evaluating differences between normally distributed data, while the Mann-Whitney Rank Sum test was applied to nonparametric ones. Values of $P < 0.05$ were considered to be significant. Correlations between CS content, CS charge density, and age were determined by the Pearson Product Moment Correlation test with 95% confidence intervals.

Table 2: Lipid profiles and homocysteine levels in patients and controls.

	Hard (21)	Soft (26)	All patients (47)	Controls (46)
Age	70.9 ± 5.1	70.2 ± 9.3	70.4 ± 7.9	47.8 ± 14.4
Sex ratio (m/f)	2/1	1.3/1	1.6/1	1/1.5
Triglycerides (mg/dL)	119.9 ± 55.1	112.7 ± 67.1	115.4 ± 61.7	86.6 ± 28.7
Total cholesterol (mg/dL)	187.9 ± 43.3	164.5 ± 38.3	173.2 ± 41.0	175.7 ± 17.5
LDL cholesterol (mg/dL)	102.4 ± 35.9	90.9 ± 35.6	95.2 ± 35.4	108.3 ± 12.6
HDL cholesterol (mg/dL)	61.2 ± 17.0	51.5 ± 10.9	55.1 ± 14.0	50.1 ± 10.9
Homocysteine (μmol/L)	12.5 ± 4.7	11.5 ± 4.1	11.8 ± 4.3	10.32 ± 3.27

Table 3: Total plasma CS, ΔDi-4S, and ΔDi-nonS levels and CS charge density in control subjects, in the totality of patients and according to plaque typology.

	Total CS (μg_{UA}/mL)	ΔDi-4S (μg_{UA}/mL)	ΔDi-nonS (μg_{UA}/mL)	#CS charge density (%)
Controls ($n = 46$)	5.17 ± 1.48	1.61 ± 0.56	3.56 ± 0.99	30.8 ± 4.5
Patients ($n = 47$)	6.28 ± 2.28	1.82 ± 0.77	4.46 ± 1.59	28.6 ± 4.6
Soft ($n = 26$)	6.76 ± 2.16	2.03 ± 0.75	4.73 ± 1.48	29.7 ± 4.0
Hard ($n = 21$)	5.69 ± 2.34	1.57 ± 0.74	4.12 ± 1.69	27.1 ± 4.8
Patient versus control	**0.009***	0.132	**0.002**	**0.022**
Soft versus control	**<0.001**	**0.017**	**0.001**	0.337
Hard versus control	0.321*	0.81	0.169	**0.004**
Hard versus soft	0.114	**0.04**	0.202	0.055

P values obtained by Student's t-tests for normally distributed parameters or by *the Mann-Whitney Rank Sum tests for nonparametric ones are reported. #CS charge density was evaluated as ratio between ΔDi-4S and the sum of ΔDi-nonS and ΔDi-4S. Significant differences are reported in bold ($P < 0.05$).

3. Results

Plasma samples from atherosclerotic patients and from healthy volunteers were analysed. Patients were sorted in two groups according to the typology of their carotid lesion (either soft or hard) as evaluated by ecocolordoppler ultrasonography. The two groups of patients did not differ for the main clinical (Table 1) and biochemical parameters (Table 2), neither as a whole nor subsorting for gender. Lipid profiles and homocysteine levels (Table 2) were similar in patient and control groups ($P > 0.05$).

CE was used to analyze the quantity and fine structure of plasma CS isomers. The adopted method allows the simultaneous determination of hyaluronan- and CS-derived disaccharides with a good reproducibility of both the migration times (CV%, 0.25) and the peak areas (CV%, 1.4). Intra- and interassay CVs were 5.37 and 7.23%, respectively, and analytical recovery was about 86% [17]. This method has been validated by a comparison with a reference assay (fluorophore-assisted carbohydrate gel electrophoresis) using the Passing and Bablock regression analysis and the Bland-Altman test as specific statistical methods for measurement comparison [17].

CE analyses of plasma CS isomers showed the presence of two main unsaturated disaccharides, the nonsulfated (ΔDi-nonS) and the 4-monosulfated (ΔDi-4S).

To rule out any influence of age on our analytical and structural results, we evaluated its association with both content and charge density of plasma CS in control group. In this respect, no correlation was found even after sorting controls for gender. The age did not correlate neither with ΔDi-nonS ($r = -0.219$; $P = 0.143$) nor with ΔDi-4S ($r = -0.002$; $P = 0.143$) levels.

Statistical analyses allowed us to evidence significant differences between the whole group of patients and the group of controls (Table 3) consisting in an increase of undersulfated CS levels (4.46 ± 1.59 versus 3.56 ± 0.99 μg_{UA}/mL, $P = 0.002$), with consequent increase of total CS content (6.28 ± 2.28 versus 5.17 ± 1.48 μg_{UA}/mL, $P = 0.009$) and reduction in its charge density (28.6 ± 4.6 versus 30.8 ± 4.5%, $P = 0.022$). Interestingly, after sorting for plaque typology, we evidenced significant differences in total CS concentration only in patients with a soft plaque with respect to healthy subjects (6.76 ± 2.16 versus 5.17 ± 1.48 μg_{UA}/mL, $P < 0.001$), due to significantly higher levels of both ΔDi-nonS (4.73 ± 1.48 versus 3.56 ± 0.99 μg_{UA}/mL, $P = 0.001$) and ΔDi-4S (2.03 ± 0.75 versus 1.61 ± 0.56, $P = 0.017$), whereas patients with hard plaques showed quite normal levels. In plasma from patients with a soft plaque both normosulfated and undersulfated CS isomers were significantly increased and their relative proportions were unchanged. So, no significant changes in CS charge density were detected. On the contrary, in plasma from patients with hard plaques, although the levels of both CS isomers were quite normal, a significant difference in their relative proportions was found with respect to controls, producing a significantly lower CS charge density (−12%).

No differences by Student's t-test in both total CS content and CS charge density emerged after subsorting both patients and controls for gender.

4. Discussion

Human normal plasma contains principally an undersulfated form of chondroitin at a concentration of about 4 μg_{UA}/mL [20], whose origin and physiological roles have not yet been fully elucidated. It circulates covalently linked to bikunin [13, 14], or as a main product of tissue catabolism or produced by blood cells, such as lymphocytes, associated with a variety of plasma proteins [21, 22]. It is known that the plasma GAG association with low-density lipoproteins could affect some of their physicochemical properties [23] and that some physiological and pathological conditions could lead to an increase in plasma GAG levels [8–12]. Moreover, mediators of inflammation such as some cytokines and growth factors are able to modulate the size, the degree, and the pattern of sulfation, as well as the degree of epimerization of the GAG chains [24–26].

In this work we studied possible correlations between plasma CS level/structure and presence/typology of carotid atherosclerotic lesion. In a previous paper [17], we developed a sensitive and reproducible analytical method for the quantitative and structural evaluation of human plasma CS isomers using capillary electrophoresis (CE). Herein, this analytical approach was adopted to evaluate CS concentrations and structural characteristics in 46 healthy human subjects and in 47 atherosclerotic patients having either a soft or a hard plaque.

Plaque typology was assessed by duplex ultrasonography which represents a noninvasive method for the carotid plaque characterization. This technique allows to detect areas with different shades of grey that provide information on plaque consistency. In particular, hypoechoic features are associated with lipid-rich carotid plaques, while hyperechoic features are with fibrous or fibrocalcific ones [16]. In this respect, several recent ultrasound studies have demonstrated that hypoechoic plaques, with a low GSM (gray-scale median) value, were associated with an increased risk of cerebrovascular ischemic events [27–29]. So, the soft plaque shows characteristics of instability and propension to rupture. It is generally held that plaque instability is caused by a substantial increase in proteolytic activity and inflammatory status.

To rule out any influence of age on our results, we evaluated its association with plasma CS content and sulfation pattern by means of Pearson's correlation. In this respect, no correlation was found. Few conflicting data regarding the relationship between plasma GAG content and ageing are present in the literature. Some investigations found that total plasma GAG content does not vary with age [30, 31]. Conversely, a positive correlation between total plasma GAG levels and age has been reported in males [32]. Qualitative analysis of intact plasma GAGs by using cellulose acetate electrophoresis has shown a decline of CS with proceeding age [33], while the structural analysis of plasma CS after depolymerization with specific chondro-/dermatolyases has revealed a significant increase of CS amount and its charge density depending on age [34]. On the basis of our results, both plasma CS levels and its charge density seem to be unaffected by age. Moreover, no correlation was found with gender.

With regard to the influence of atherosclerotic lesion presence, we found significant differences in total plasma CS content (+21.4%), between the group of patients and the controls. These differences were ascribable to significantly higher levels of undersulfated CS in these patients (+25.3%).

The statistical analyses of the influence of atherosclerotic lesion typology on plasma CS content and sulfation pattern showed that in presence of soft plaques CS content was significantly increased, without significant changes in its charge density, whereas in presence of hard plaques its charge density was reduced, without changes in its content.

The significance of the observed modifications in plasma CS isomers of patients undergoing endarterectomy could be related to different PG/GAG metabolism in vascular tissue in presence of soft/hard atherosclerotic plaques. Atherosclerosis has been associated with a biosynthetic imbalance of chondroitin sulfate proteoglycans [4–6]. It has been described that the ratio of 6-sulfated to 4-sulfated disaccharides is increased in atherosclerotic type II aortas and significantly decreased in atherosclerotic type V, indicating that vascular concentration of GAGs is differently affected during the progression of the disease [4]. Increased levels of both undersulfated and normosulfated CS have been described in postoperative serum samples of patients submitted to coronary artery bypass surgery and proposed as indicative of an inflammatory state of the patient [35].

In this regard, it has been reported that in inflammatory diseases the CS chains carried by bikunin increase in size proportionally to the severity of the inflammatory response [36], while their sulfation degree decreases [37].

Moreover, several studies reported that sulfation pattern of CS isomers may be important for their protective role from oxidative damage [38–43]. The antioxidant properties of GAGs could be explained by both GAGs chelating properties on divalent cations (such as Cu^{2+} and Fe^{2+}, responsible for the initiation of hydroxyl radical reactions) and their improving effects on endogenous antioxidant defences. In particular, the anti-oxidant properties of CS isomers seem to be related to the sulfation at position 4 of galactosamine residue of disaccharide units.

On a whole, our data show that plasma CS level and structure are significantly different in atherosclerotic patients with respect to controls and that these differences do not depend on age. Moreover, the obtained results suggest that both content and distribution of the circulating CS isomers are associated with the presence as well as with the typology of carotid plaque. If plasma CS levels and structure reflect, almost partly, the CS-PGs metabolism in affected vascular tissues, the determination of plasma CS isomers may provide information on molecular mechanisms of atherosclerosis progression.

Therefore, evaluating content and distribution of plasma CS isomers could be useful in diagnosis of carotid atherosclerosis. Further studies on subjects with preclinical atherosclerosis would be advisable. Indeed, finding an association between these molecules and early atherosclerosis stages

could provide an important tool for the follow-up of patients in attempting to prevent inauspicious cerebrovascular events.

Acknowledgment

This study was supported by grants from "Fondazione Banco di Sardegna," Sassari, Italy.

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Hindawi Publishing Corporation
Biochemistry Research International
Volume 2012, Article ID 859231, 10 pages
doi:10.1155/2012/859231

Review Article

No-Reflow Phenomenon and Endothelial Glycocalyx of Microcirculation

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Received 7 June 2011; Accepted 20 September 2011

Academic Editor: Barbara Bartolini

The progress in reperfusion therapy dictated the necessity for developing new tools and procedures for adjacent/additional therapy of acute cardiovascular disorders. The adjacent therapy is targeted on the damage of the microcirculation, leading to the unfavorable prognosis for the patients. The no-reflow phenomenon holds special place in the multifactorial etiology of the microcirculation disorders, offering a new challenge in treating the patients associated with ST-segment elevation on ECG at myocardial infarction. One of the numerous causes of no-reflow, the influence of the endothelial glycocalyx of the microcirculation, is analyzed. The results obtained in the studies of the endothelial glycocalyx ultrastructure are generalized, the effect that the fragments of the glycocalyx glycosaminoglycans have on the function of the vascular wall is demonstrated. The trends in searching for correlations between the thickness of the capillary glycocalyx and the cardiovascular disease risk are noted.

1. Introduction

The continuous improvement of the methods, tools, and medications for reperfusion therapy is motivated by the necessity to reduce the high morbidity and mortality rates for cardiovascular disorders. Acute coronary syndrome is a critical manifestation of ischemic heart disease. The onset of this syndrome can lead to unstable angina pectoris attacks, to the acute myocardial infarction (AMI), and to the sudden cardiac death. The size of myocardial infarction serves as the primary determinant of the prognosis for such patients. The mortality rate over the first six months in patients with infarct size over 20% left ventricle was 2%, and increased to 4.5%, when the lesion size was higher than 35% [1]. An understandable significance of reduction in AMI size, however, did not find a reliable clinical confirmation in the studies [2–4]. The reason is that the reduction of AMI size (although it has a significant effect on the prognosis in the patients with the lesion size of 20% left ventricle and more) is achieved more often in the patients who have smaller lesions (<20% left ventricle), that is, in the patients who already have a favorable prognosis [5]. Therefore, a successful treatment of severe cases is of especial importance for reducing mortality rates in the patients with AMI. The contemporary reperfusion therapy limits AMI sizes approximately by 50% of ischemized area in half of the patients with AMI, leaving out a quarter of the patients with more than 75% of the lesion in the risk zone. This sets an objective of reducing the infarct size from 75% of the lesion or higher down to less than 40% [5]. In a quarter of the patients with AMI this goal could not be accomplished by using the required reperfusion therapy alone, and so an adjunctive (additional) therapy is necessary [6]; the purpose of this adjacent (adjunctive) therapy is to reduce the infarction damage of the left ventricle down to 20% or less [5].

2. Necessity for Adjacent Therapy in Treating Disorders of Microcirculation/ Tissue Perfusion

One of the reasons for severe progression of AMI is the damage of the tissue perfusion/microcirculation [7]. The positron emission tomography shows that some of the

patients with AMI have reduced tissue perfusion caused by the damage of the capillaries induced by ischemia and reperfusion, even after the successful thrombolysis in the infarct-related artery (patency rates reaching TIMI grade 3). As a result, the function of the left ventricle is not restored, which leads to the unfavorable prognosis for these patients. The functional recovery of the affected myocardium was observed only when the patency of the obstructed vessel and an adequate restoration of tissue permeability were ensured [7]. An adjunctive therapy is required for the functional restoration of the microvasculature [6]. The necessity of additional therapeutic intervention for these disorders in the case of complicated AMI was demonstrated by using echocardiography, nuclear magnetic resonance, Doppler scanning [8], angiography (employing semiquantitative classification of microcirculatory perfusion levels) [9], electrocardiography, by measuring plasma levels of the myocardial proteins (creatine kinase MB, troponin T or I, myoglobin) [10, 11], and using positron emission tomography [12]. These data helped to move from the concept of open artery to the achievement of the "optimal reperfusion," designed, aside from the reperfusion of an occluded artery, to eliminate the dysfunction of microcirculation, improve tissue perfusion, and reduce the "reperfusion" damage [13]. The current situation shows that the use of adjunctive therapy in patients with acute cardiovascular disorders may have various purposes [6, 13]. This review is primarily focused on the problems associated with disorders of microcirculation.

3. Disturbances of Microcirculation: No-Reflow Phenomenon

Microcirculation is a part of blood circulation system. Oxygen, nutrients, hormones, and metabolites are exchanged between the circulating blood and the parenchymal cells in the microcirculation bed. The microvessels are divided by their anatomic characters and the direction of blood flow into arterioles, capillaries, and venules, the diameter of which is thought to be less than 100 [14] or 200 μm [15]. Arterioles, the thin branches of arteries, are the principal resistance component of microcirculation. The venules serve as a large reservoir of low pressure (capable to hold up to 75% total blood volume), through which the blood returns into the heart. The main function of capillaries is to facilitate the exchange of molecules between blood and tissues. An adequate capillary circulation is a necessary prerequisite for normal perfusion and organ functioning. Capillary patency is the principal determinant of capillary tissue perfusion and is characterized by the functional capillary density (FCD). The latter is defined as the number of functioning capillaries (i.e., the capillaries with circulating erythrocytes) per tissue area. The major distinctive feature of microcirculation is its heterogeneity (in terms of blood flow distribution, FCD, expression of vasoactive products, etc.). The disorders of the microcirculation are sepsis, hypovolemic and cardiogenic shock, and no-reflow phenomenon (the failure to restore the microflow) [14, 16]. It is believed that in the case of sepsis (associated with local hypoxia and organ dysfunction) and cardiogenic shock (i.e., a response to the generalized inflammation with compensatory redistribution of blood volume/due to active venoconstriction/with reduction of the peripheral vascular capacity), the disturbances of microcirculation occur irrespective of the systemic hemodynamic changes, although in the latter case this conclusion is disputed [14]. In the case of hypovolemic shock (caused, e.g., by a significant blood loss, and accompanied by pronounced hypotension), the changes in microcirculation are, at least, not completely independent from the hemodynamic parameters. The hemodynamic parameters are correlated with the "no-reflow" phenomenon [17, 18]. One of the definitions of the "no-reflow" phenomenon is incomplete and nonunified reperfusion at the microcirculatory level, despite an adequate reopening of the proximal artery after a period of transient ischemia [19]. The cases of vascular damage of the myocardium allow us to generalize the concept of no-reflow as the state of myocardial tissue hypoperfusion with the patency of epicardial coronary arteries [15, 18, 20]. Two types of no-reflow are distinguished: "structural" no-reflow, characterized by irreversible damage to the cells of the microcirculation, and "functional" no-reflow, characterized by the disturbance of microcirculation caused by vasoconstriction and/or microembolization [21]. The latter can be caused by the fragments of a lysed thrombus, destroyed atherosclerotic plaque, aggregates of erythrocytes or leukocytes with platelets, or by large platelets [15, 20, 22]. The high volumetric content of necrotic material in the plaque correlates with the development of microembolization, determined by the levels of the released seromarkers—creatine kinase and troponin I [23]. The stenting causes less prominent release of cardiac markers than does the atheroablative technology. These interventions can be associated with "reperfusion" no-reflow (which occurs after ischemia/reperfusion during the treatment of AMI) and with "interventional" no-reflow (associated with angioplasty without myocardial infarction and without a prolonged ischemia prior to the procedure) [15]. The duration of ischemia can have a significant effect on specific manifestations of no-reflow. "Interventional" no-reflow following a short period of ischemia (seconds or minutes) is caused primarily by microvascular obstruction, inflammatory response, or secondary ischemia. A prolonged (hours) ischemia causes no-reflow due to "reperfusion" (ischemia/reperfusion) injury, myocardial edema, endothelial swelling, capillary embolism, vasospasm, or inflammatory response [15]. The first 2-3 hours of ischemia in the case of AMI lead to myofibrillar edema, followed (6-7 hours of ischemia) by interstitial edema [24]. A more prolonged ischemia leads to the reduction of edema, to the destruction of microcirculation bed, and to the thinning of the myocardial walls. The inhibition of δ-isoform of protein kinase C by a fragment of its inhibitor, protein δV1-1, performed on isolated hearts of transgenic mice expressing δV1-1 and on AMI model in pigs reduces the microcirculatory dysfunction, decreasing the no-reflow phenomenon [25, 26]. The elevation in the levels of plasma thromboxane A2 was observed in the patients with AMI (with ST-segment elevation on ECG), following the primary stenting [20, 22]. The data of the multivariate analysis shows

that the level of thromboxane A2 is an independent predictor of the no-reflow phenomenon after the angioplasty, which sets new goals for reducing of this complication. However, the efforts of pharmacologists and clinicians to achieve the same results using adenosine, sodium nitroprusside, nitroglycerine, nicorandil, verapamil, glycoprotein inhibitor IIb/IIIa, and papaverine have not as yet produced any tangible results [15, 26]. The multifactorial nature of the no-reflow development is emphasized by the assumption that the formation of fibrin clots with reduced permeability and increased resistance to fibrinolysis can be affected in these conditions by the genetic data of the patient [27]. The diversity of the available data and the lack of an effective approach to the therapy of no-reflow phenomenon make this goal the next challenge in treating the patients with AMI [20, 26]. The personalized management of no-reflow has been proposed for clinical application on the basis of the assessment of the prevailing mechanisms of no-reflow operating in each patient [28]. Variable combinations of human no-reflow causes include four pathogenetic components: distal atherothrombotic embolization, ischemic and/or reperfusion injury, susceptibility of coronary microcirculation to damage. In order to understand the mechanism of this pathology, we should know its components. The review presented above shows that the investigators tend to neglect the role of the carbohydrate coating of the microvessels, which (due to the reduction of the effective size of the vascular lumen and due to the vessel walls coming closer together and to the occlusion material) cannot be completely uninvolved in the development of the microcirculation disorders [29].

4. Carbohydrate Coating of the Vascular Wall

The endothelial glycocalyx (EG) is an uneven "fluffy" polysaccharide coating over the vascular wall toward the vessel lumen. This polysaccharide "fur" on the cell surface is composed of proteoglycans and glycoproteins, attached to the cell membrane and capable to bind blood constituents, which can be important for the vascular function. The size of the glycocalyx in the microvessels is 0.4-0.5 μm, which is 10–20% of the vascular volume [30]. The diameter of the cell-free layer in these vessels is over 20 μm [31]. It is believed that the glycocalyx permeability is mediated by the hyaluronan, and the glycocalyx volume is regulated by the proteoglycans [32, 33]. The composition of glycosaminoglycans implies the content of hyaluronan more than 40%, heparansulfate more than 50%, and chondroitinsulfate/dermatansulfate around 10% [34]. These glycosaminoglycans (besides hyaluronan) are linked covalently to core proteins [34–36]. They together with glycosaminoglycans form the proteoglycans. Core proteins are syndecans (number of subtypes is four) and glypicans (number of subtypes is six). Syndecans are linked firm to cell membrane via a membrane-spanning domain, but glypicans are linked via a glycosylphosphatidylinositol anchor. There are membrane proteoglycans (syndecans of subtypes 1–4 and glypicans of subtypes 1–6), soluble proteoglycans (perlecan, biglycan, and others), and five types of glycosaminoglycan chains (heparin sulfate, chondroitin sulfate, dermatan sulfate, keratan sulfate, hyaluronic acid). These components consist of membrane-bound and associated layers of glycocalyx. Composition and function of endothelial glycocalyx are detailed in [34, 36]. It should be noted that glycocalyx composition cannot be viewed as a static object. There is a dynamic equilibrium between glycocalyx layer of soluble components and the flowing blood. It is possible to say the glycocalyx is an intricate self-renewing 3D mesh of various polysaccharide derivatives [34–36]. EG is therefore considered to be a protective layer on the vascular wall providing defense against pathogens, a traffic meshwork barrier for transendothelium transportation of molecules, and a porous hydrodynamic partner in interaction with red and white blood cells in the microcirculation.

The resistance of arterioles to the protein flux through their walls was found to be associated with the glycocalyx influence [37]. The inflammation and the influence of ischemia/reperfusion facilitate the breakdown of glycocalyx in the venules and capillaries [38–40]. When microthromboses in mice were induced by an infective stimulus (lipopolysaccharide), the levels of von Willebrand factor expression were increased in the venules, but not in the arterioles [41]. The degradation of glycocalyx in the myocardial capillaries results in a rapid development of myocardial tissue edema [42]. The treatment with hyaluronidase of hamster capillary glycocalyx has shown that it is the pronounced hyaluronandependent [30]. Hyaluronidase treatment was associated with the reduction of the glycocalyx volume, the increase of the volume of circulating plasma, the decrease of FCD, and the elevation of hematocrit. These changes in the glycocalyx lead to the alteration of the transendothelial permeability, thereby causing the swelling of the endothelial cells, which is one of the regulatory factors for FCD [30]. The reduction of FCD shows how the worsening of the condition of capillary glycocalyx can lead to the onset of the disease. The properties of the microcirculation glycocalyx can mediate the regulation of the blood transport function; these properties have recently been reviewed [29]. In humans, the evidence for shedding of EG during ischemia/reperfusion procedures was demonstrated already also [43].

5. Glycocalyx Components for Drug Targeting

The possibility of using glycocalyx components in the targeted drug delivery systems appears to be both interesting and promising [44]. The exposure of chondroitinsulfate proteoglycan in the subendothelial layer of the rabbit arteries after its stenting causes binding of the positively charged liposomes to this substance (along with cationic lipid TRX-20 and prednisolon serving as the drug) and the accumulation of such nanoparticles in the lesion area [45]. This has resulted in a prominent reduction of the neointima growth in the area of the stent. The treatment of the affected area with chondroitinase ABC prior to the injection of liposomes prevents their binding in this area. The experiment conducted on subendothelial cells (from human aorta) has confirmed a noticeable binding of the cationic

TRX-20 liposomes to these cells, but not to the endothelial cells [46]. The treatment of subendothelial cells (smooth muscles and mesangial cells) with chondroitinase (but not with heparinase) reduces their binding to the liposomes studied, which proves the specificity of this interaction. It should be noted that liposomes can measurably bind to the endothelium, depending on the type of cationic lipids and their concentration in the liposomes [47]. The effects of binding of various ligands (lipoproteins, proteins, peptides, saccharide derivatives, etc.) to the components of EG have recently been reviewed [29]. Erythrocyte-associated tissue-type plasminogen activator (tPA) was proved to have a potential of a very effective agent of cerebrovascular thromboprophylaxis [48]. Biotinilated tPA and erythrocytes are associated via streptavidin [49]. The use of this adduct in mice with cerebral thrombosis results in thrombolysis, generating a rapid and prolonged reperfusion, whereas no effect is observed when tPA itself is administered even in ten-fold higher doses. Because of the coupling with erythrocytes, tPA has a prolonged half-life period in the blood, the ability to lyse fresh thrombi (rather than old hemostatic plugs), and a reduced efficiency of inhibition by plasminogen activator inhibitor of type I (PAI-1) [49]. The latter observation was explained by the protection provided by the erythrocyte glycocalyx preventing tPA from interacting with PAI-1, which disappears when the erythrocytes are treated with a mixture of neuraminidase, hyaluronidase, and heparinase [50]. The erythrocyte glycocalyx does not prevent the associated tPA from interacting with fibrin and plasminogen, but protects it from glycation by glucose. Of course, the similarity and difference of glycocalyx on leucocytes, erythrocytes, subendothelial cells to that on the endothelium has to determine else. A protective effect of glycocalyx is thought to be associated with screening the interaction centers on tPA provided by glycocalyx and with changes in electrostatic interactions. In our opinion, such effects (binding of the cationic liposomes to chondroitinsulfate-, but not to heparansulfate-proteoglycan [45], inhibition of the associated tPA interaction with PAI-1, but not with fibrin and plasminogen [49], that occur in the space of specifically positioned electrostatic charges) imply the presence of a suitable charge distribution network in glycocalyx. The orienting effect of this network on the binding counter-partner enables the detection of its suitability/unsuitability for a productive interaction. The presence of this charge distribution network can signify a specific ultrastructure of the glycocalyx. The study of this network is currently underway [29, 50].

6. The Study of Glycocalyx Ultrastructure

The samples of microvascular (capillaries and venules) glycocalyx from the frog endothelium prepared for electron microscopy using various techniques (freezing and chemical fixation) have yielded similar results [51]. The size of glycocalyx in the normal microvessels was less than 0.2 μm; it was, however, observed that its *in vivo* size could be much higher and could be reduced, when the samples are prepared for electron microscopy. In the inflamed microvessels, where inflammation was induced using "temperature jump" technique, the glycocalyx changed its shape to form protuberances on the cell surface (extensions, depressions, and other irregularities) and increased in thickness (0.3-0.4 μm). Computerized autocorrection functions and Fourier transformations reveal the ultrastructure of EG in the microvessels. A structural quasiperiodicity of the glycocalyx meshwork was detected along the horizontal and vertical axes (the periodicity intervals are ~20 nm, the diameter of fibers is 10–12 nm, and the distance between the bases of quasihexagonal meshwork elements (Figure 1) is approximately 100 nm [51]). This structure is consistent with the fiber matrix model that views the glycocalyx as an extracellular molecular filter. A systematic variation in the length of the side proteoglycan chains on the cell surface (axial periodicity along the proteoglycan molecule), and/or regulatory binding of plasma proteins (e.g., such ones albumin and orosomucoid [36]) by highly charged side chains of glycoproteins regularly positioned in the nodes of the glycocalyx network can serve as regulatory forces that mediate the transport through the glycocalyx (Figure 2) [51]. A specific profile of the electrostatic surface potential formed by this network is likely to trigger the initiation of recognition/binding of the interaction counter-partner (with a sufficiently complementary electrostatic surface potential) or to ignore the unsuitable counter-partner. This could have explained a targeted binding of the cationic liposomes to chondroitinsulfate-, but not to heparansulfate-proteoglycan [45, 46], as well as the inhibition by the erythrocyte glycocalyx of tPA interaction with PAI-1, but not with fibrin and plasminogen [49, 50]. This question definitely warrants further study.

The treatment of the blood cells, intended for scanning electron microscopy, with 2.56 M (15%) NaCl solution [52] provides a very informative approach for studying this problem. During this procedure, gel microenvironment is formed around the cells (taken from the patient blood with chronic lymphatic leukemia or myeloleukemia); this microenvironment has a regular meshwork structure. The cell diameter, including the swollen glycocalyx-gel around the cell, is approximately 10–20 μm. The meshwork periodic structure (period of 100–150 nm) of the tumor cells was clearly revealed by atomic force microscopy [53]. A reversible increase in size of the cellular glycocalyx reaches three orders of magnitude [52, 53], which makes this approach a very convenient technique for studying the structure of glycocalyx.

At the same time, it is assumed [50] that the organization of the glycocalyx ultrastructure (100 nm topographic distances between the glycocalyx fibers on the cellular surface, Figure 1) is determined by the quasiregular architecture of the submembrane cytoskeleton. It is possible that the inflammation alters the glycocalyx by changing the structure of the cytoskeleton, which in turn triggers the over-production of the glycocalyx [51]. This change can be initiated as a result of the effect that the high ionic strength of the medium has on the cytoskeleton of the tumor cells and/or as a result of the increased swelling of the glycocalyx [52, 53].

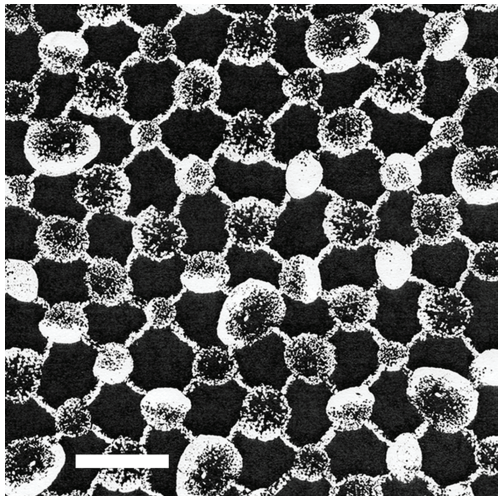

Figure 1: Reconstructed high-periodicity meshwork of the blood leukocyte glycocalyx composed of individual natural nanoparticles. The meshwork composition is projected onto a plane. Electron microscopy: the bar corresponds to the minimum size of the nanoparticle, which is 50 nm under 15% NaCl electrolyte concentration (hypertonic solution) or 3 nm under 0.9% NaCl electrolyte concentration (isotonic solution) (Golovanov MV, Bauer J, unpublished data personal report, 2009).

The quantitative study of the proposed structural glycocalyx model [51] has shown that a high flexural rigidity of the fibers on the cell surface is provided by the support of the rigid actin filaments of the cortical cytoskeleton in the transmembrane complexes of the core glycocalyx proteins [54]. As a result, the submembrane "roots" of the glycocalyx "bushes" are holding firmly the "branches" of their fiber clusters (Figure 2). The long arm of the lever formed by the core glycoproteins on the outside of the cell provides significant mechanical advantages inside the cell in terms of increasing the flux forces, exerted on the glycocalyx, in transferring the interaction to the cortical cytoskeleton. Small flux forces acting on the glycocalyx are amplified by a sufficient intracellular lever, capable of deforming the cortical cytoskeleton. It is assumed that such mechanotransduction is the first step in the activation chain of intracellular signaling [54]. Hyaluronan, a component of the glycocalyx, can interact with the cytoskeleton through CD44 and RHAMM, the hyaladherins associated with the glycocalyx [55]. RHAMM is associated with the microtubules (of the mitotic cells), around which the internalized hyaluronan is distributed [56, 57]. The microtubules are an element in the development of the cytoskeleton pressure for regulating cellular junctions and cell shapes [58]. In this connection, it should be mentioned that the appearance of cilia and flagella on the endothelial surface, formed by the centrosomal centrioles and assembled from the microtubules, results in the development of endothelial dysfunction in the areas of high-risk circulation (with an oscillating shear stress) [59]. Moreover, endothelial cilia can serve as regulators of the calcium signaling and NO production via polycystin-1 [60, 61]. Enzyme destruction of EG (treatment with heparinase, hyaluronidase, neuraminidase, excluding chondroitinase) completely blocked shear-induced NO production by endothelial cells [62, 63]. None of these enzymes affected bradykinin- or histamine-induced NO production, indicating the multeity of NO production machinery. Meanwhile, it conjectures the glycocalyx may be organized into two layers: an inner region of several tens of nm near the apical membrane surface and an outer layer up to 0.5 μm thick, which contains the extended core proteins [64]. Between these layers may be located hyaluronan. Heparansulfate seems to locate in outer layer due to its prevailing content among glycocalyx glycosaminoglycans [34] and observable role in mechanotransduction of shear stress into endothelial cells for NO production (Figure 2) [65]. Under physiological conditions, EG has several well defined functions aimed at preserving the integrity of the vessel wall: inert barrier, molecular sieve, reservoir for biologically active compounds, mechanotransductor transferring shear stress into shear-dependent endothelial responses [64]. As a whole, according to the "double barrier concept," vascular barrier function is provided by two important components, the endothelial glycocalyx and the endothelial cells themselves [40, 43]. It can be hypothesized that extracellular stimuli are conveyed to the cytoskeleton through the structure of the glycocalyx, and then the endothelial cell uses the cytoskeleton to develop a response, which can result, for instance, in a change of cell shape or an initiation of new events that alter the cellular functions.

7. Glycocalyx Degradation and Its Fragments in Blood Circulation

The degradation of EG due to the development of vascular lesions leads to the release of its fragments into the circulation. These fragments are currently believed to be glycosaminoglycans, previously attached to the proteins on the endothelial surface. The molecular size of hyaluronan far exceeds the sizes of other glycocalyx glycosaminoglycans. The hyaluronan molecule can be as long as 2.0–2.5 μm [55]. The effects associated with the formation of the hyaluronan fragments have already been discussed in the literature [55, 66, 67]. There is a mounting body of data showing different types of biological activity of the hyaluronan fragments, depending on their molecular weight [29]. The full-size forms of hyaluronan have proved to have anti-inflammatory and anti-angiogenic properties, the oligomers of 4–50 disaccharides have angiogenic and pro-inflammatory properties and stimulate tumor invasions, whereas the fragments of 3–12 and 6-7 hyalurononic polymeric moieties were found to suppress tumor growth [66, 67]. The degradation of hyaluronan is important for the functioning of the capillaries because hyaluronan regulates their permeability and the junctions between endothelial cells [30, 68]. Hyaluronan fragments are released in the circulation due to biochemical degradation of the glycocalyx, *de novo* synthesis, and the effect of the oxidative stress [29, 67]. Inflammatory processes caused by the oxidative fragmentation of hyaluronan, were inhibited by the extracellular superoxide dismutase [69]. The exogenous hyaluronan fragments, from decasaccharides upward, displace hyaluronan from the cell surface, whereas

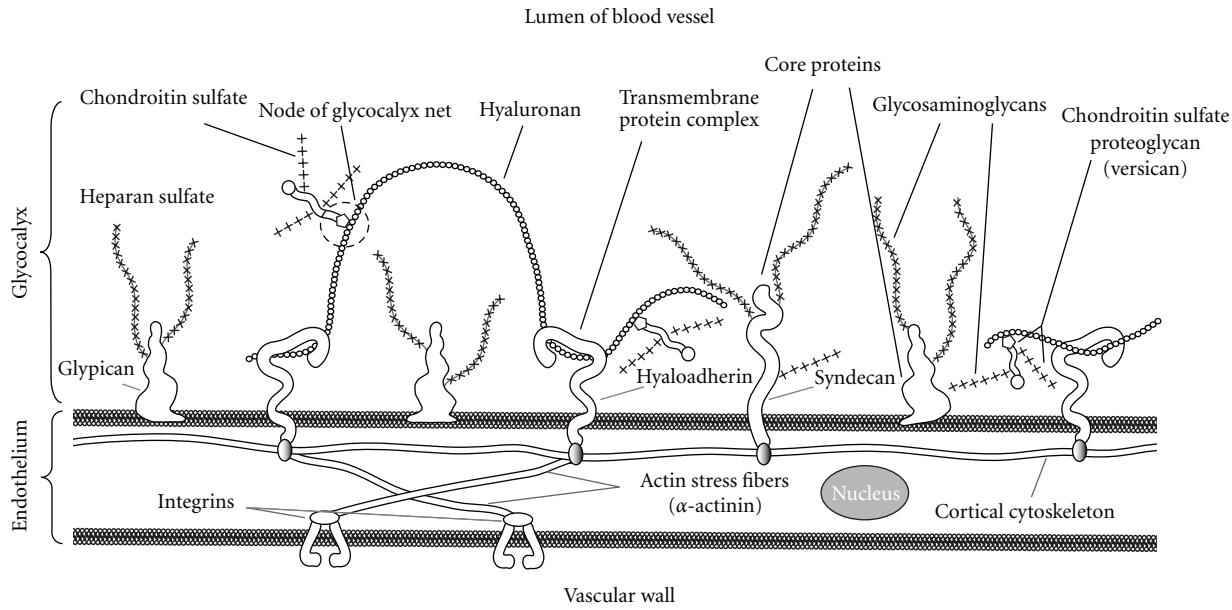

Figure 2: Schematic representation of the glycocalyx meshwork associated with cytoskeleton. Hyaluronan weaves into glycocalyx and binds with hyaladherins (CD-44 (hyalreceptor), RHAMM (receptor for hyaluronan-mediated motility), other proteins, or hyaluronansynthases). Chondroitin sulfate proteoglycan (versican) interacts with hyaluronan to form high molecular mass stable aggregates. Enhanced formation of hyaluronan-versican pericellular coat is observed under pathophysiological conditions (inflammation, early atherosclerosis, restenosis, plaque thrombosis, and others).

the chondroitinsulfate does not have such effect [70]. The splitting of CD44 was caused by the small fragments of hyaluronan (6.9 kDa and other fragments with molecular weight less than 36 kDa), which in turn led to the increase of tumor cell mobility due to detachment/dissociation of these cells from the tumor [71]. Hyaluronan oligosaccharides (composed of 4–16 saccharides) inhibit neointima growth in the area of the rat aorta affected by balloon catheterization [72]. This makes such hyaluronan fragments potential agents for preventing restenoses following the angioplasty, because their formation is caused predominantly by the growth of the extracellular matrix, rather than by cell proliferation [55, 73]. Hyaluronic acid was proposed to be used as thromboresistant coating of the stents and endovascular devices [74].

Heparin, known for its anticoagulative properties, can interact with fibronectin, a protein of the extracellular matrix, thereby triggering the transformation of its molecule on the cell surface from closed to open conformation [75]. The binding of heparin to the open conformation is weakened, heparin dissociates from the protein, and the binding centers of vascular endothelium growth factor become exposed on the open conformation. Heparin, for instance, can regulate the functions of extracellular matrix.

The enzymatic destruction of glycocalyx is accomplished by neuraminidase, chondroitinase, heparinase, hyaluronidase, and other biocatalysts [26, 46, 50, 55, 67]. The treatment with mammal hyaluronidase results in the formation of oligosaccharides with an even number of polymeric moieties that have *N*-acetylglucosamine at the reducing end of the fragment [67, 76]. Such derivatives give rise to glycating agents [77]. The glycation of biomacromolecules, especially in carbohydrate disorders, results in formation of Amadori end-products, whose accumulation in the body tissues facilitates the development of metabolic disorders, the increase in rigidity of the vascular wall, and production of the reactive oxygen species [77–80]. The interaction of Amadori end-products (advanced glycation end-products) with their corresponding receptors can facilitate these processes, by affecting the energetics of myocardial metabolism, its function, and by contributing to the myocardial damage after ischemia/reperfusion [81]. In order to achieve therapeutic effect, the formation of Amadori end-products should be blocked and a soluble form of the receptor should be used [82].

The glycosaminoglycan biosynthesis comprises several steps. The glycosaminoglycan chain initiation is catalyzed by xylosyltransferase-1/2 that transfers xylose residues to a certain serine in the core protein. This is the first step in the assembly of the linkage tetrasaccharide GlcAβ(1–3)Galβ(1–3)Galβ(1–4)Xylβ(1-O-Ser) linking the protein and the future glycosaminoglycan chain. Galactosyl transferase-1 and -2, and glucuronyl transferase-1 complete the assembly of the linkage tetrasaccharide by a consecutive transfer of two galactose residues and one residue of glucuronic acid. The subsequent elongation of the chain is accomplished by an alternate addition of the transferred units of D-glucuronic acid and *N*-acetyl-D-glucosamine to the tetrasaccharide. The final step in the synthesis of proteoglycans is associated with highly coordinated multiple sulfation and epimerization of the glycosaminoglycan. The biological significance of glycosaminoglycans is studied by exposing them to various agents in the course of the glycosaminoglycan biosynthesis. The following substances can serve as agents that change the glycosaminoglycan biosynthesis: sodium chlorate, a bleaching

agent, and brefedlin A, a fungal metabolite. Unfortunately, these agents turned out to be highly lethal for the animal models. The modified analogues of xylose were proved to be more suitable for studying [83]. These studies of xylose analogues are currently underway and they can help to examine the biological role of glycosaminoglycan derivates, synthesized *de novo* in the organism.

8. Present Methods for Glycocalyx Study

In order to evaluate glycocalyx state, various methods employing reporter agents (isotopes, labeled erythrocytes, dextran) and novel research equipment (laser doppler flowmetry, positron emission, single-photon computerized, magnetic resonance (using gadolinium, iron oxide) tomography or their combinations) are used [14, 84, 85]. There are ongoing studies looking for correlations between the endothelial glycocalyx thickness in the microcirculation and the risk factors for cardiovascular disorders. The novel microcirculation imaging methods that proved effective during the studies with human volunteers are now being developed: orthogonal polarization spectral imaging (OPS, measured in the sublingual area) [86] and sidestream dark field imaging (SDF, measured on the nail fold) [14]. Cardiovascular magnetic resonance imaging of myocardial edema detects acute ischemic myocyte injury before the onset of irreversible damage [87]. This imaging may be a useful diagnostic marker in clinical settings (unstable angina or evolving infarction). The integration of the imaging techniques with glycocalyx degradation products in plasma will conduce in elicitation of EG role for cardiovascular risk stratification. EG is present in macro- as well as microvasculature and its thickness progresses with increasing vascular diameter [34]. In larger vessel, the two-photon laser scanning microscopy is suitable technique to visualize the delicate EG [88, 89]. The glycocalyx is thought to be a similarity in capillaries and large vessel throughout the whole body [42]. It is not strange therefore that arterial glycocalyx dysfunction could consider the first step in atherothrombosis process [90]. Glycocalyx is implicated also in development of diabetes, atherosclerosis, and ischemia/reperfusion injury [36]. The predicting the future is ungrateful job but further research of EG looks like the hopeful and breakthrough investigation in order to decrease the spread of cardiovascular diseases.

9. Conclusion and Perspective

The progress in reperfusion therapy leads to the necessity of developing new tools for adjacent treatment of cardiovascular disorders. One of the purposes of this treatment is the damages of the microcirculation in acute coronary syndrome. The unfavorable prognosis for the patients without "optimal" reperfusion places them in the high risk groups that have the no-reflow phenomenon in microcirculation. The clinical significance of successful treatment of this disorder offers a new challenge in treating the patients with AMI associated with ST-segment elevation. The studies of the no-reflow phenomenon reveal various causes for its development, paying, however, clearly insufficient attention to the role of EG. The study of the glycocalyx demonstrates that this spatial meshwork structure provides a protective function for the cell surface and that glycocalyx is capable to serve as a molecular filter and hydrodynamic partner in the interaction with cells and blood components. The action of chemical (enzymes, reactive oxygen species, changes of the medium pH value) and physical (shear stress, temperature, ultrasound, photo- and radio- emission, etc.) factors changes the structure of the glycocalyx, which is currently intensively studied and can influence the extent of the tissue perfusion. A diverse biological activity of EG fragments is also clearly evident and can substantially differ, depending on the molecular weight of these fragments. It is assumed that the glycocalyx, being connected to the cytoskeleton, serves as a mechanochemical transducer of the effect that blood circulation has on the processes of cell signaling. This may suggest a predictive role for the state of glycocalyx in the microcirculation. The path leading to our understanding of the mechanism for these effects is a very difficult way. However, an intention of following this path is justified by the prospective of successful treatment in the patients with complicated cardiovascular disorders.

Abbreviations

AMI: Acute myocardial infarction
CD44: Hyalreceptor
ECG: Electrocardiogram
EG: Endothelial glycocalyx
FCD: Functional capillary density
OPS: Orthogonal polarization spectral imaging
PAI-1: Plasminogen activator inhibitor of type 1
RHAMM: Receptor for hyaluronan-mediated motility
SDF: Sidestream dark field imaging
tPA: Tissue-type plasminogen activator.

Acknowledgments

The authors are grateful to Dr. V. M. Golovanov, a Researcher of Russian Cancer Research Center, Russia, and to Dr. Johann Bauer (SiHaTho GmbH, Germany) for their kind assistance in collecting the material and for fruitful discussions. This work is partly funded by Russian Foundation of Basic Research (Grants 09-04-00023 and 07-04-12057-ofi) and by Rosmedtechnology Agency.

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Hindawi Publishing Corporation
Biochemistry Research International
Volume 2012, Article ID 471325, 16 pages
doi:10.1155/2012/471325

Review Article

Glycosaminoglycan Storage Disorders: A Review

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Received 2 June 2011; Accepted 9 August 2011

Academic Editor: Laura Alaniz

Impaired degradation of glycosaminoglycans (GAGs) with consequent intralysosomal accumulation of undegraded products causes a group of lysosomal storage disorders known as mucopolysaccharidoses (MPSs). Characteristically, MPSs are recognized by increased excretion in urine of partially degraded GAGs which ultimately result in progressive cell, tissue, and organ dysfunction. There are eleven different enzymes involved in the stepwise degradation of GAGs. Deficiencies in each of those enzymes result in seven different MPSs, all sharing a series of clinical features, though in variable degrees. Usually MPS are characterized by a chronic and progressive course, with different degrees of severity. Typical symptoms include organomegaly, dysostosis multiplex, and coarse facies. Central nervous system, hearing, vision, and cardiovascular function may also be affected. Here, we provide an overview of the molecular basis, enzymatic defects, clinical manifestations, and diagnosis of each MPS, focusing also on the available animal models and describing potential perspectives of therapy for each one.

1. Introduction

The mucopolysaccharidoses (MPSs) are a group of lysosomal storage disorders caused by deficiency of enzymes catalyzing the stepwise degradation of glycosaminoglycans (GAGs) and characterized by intralysosomal accumulation and increased excretion in urine of partially degraded GAGs, which ultimately results in cell, tissue, and organ dysfunction [1].

Glycosaminoglycans (previously called mucopolysaccharides), with the exception of hyaluronic acid, are the degradation products of proteoglycans that exist in the extracellular matrix and are proteolytic cleaved, giving origin to GAGs, which enter the lysosome for intracellular digestion. There are four different pathways of lysosomal degradation of GAGs, depending on the molecule to be degraded: dermatan sulfate, heparan sulfate, keratan sulfate, and chondroitin sulfate. The stepwise degradation of glycosaminoglycans requires 10 different enzymes: four glycosidases, five sulfatases, and one nonhydrolytic transferase, whose structure, biosynthesis, processing, and cDNA sequence have already been extensively documented. Deficiencies of each one of these enzymes have already been reported and result in seven different MPSs, all of them sharing a series of clinical features, even though in variable degrees (summarized in Table 1) [1, 2].

Usually, MPSs are characterized by a chronic and progressive course, with different velocities of progression depending on the severity of each one. The typical symptoms include organomegaly, dysostosis multiplex, and a characteristic abnormal facies. Hearing, vision, and cardiovascular function may also be affected. Additionally, joint mobility may also be compromised. The majority of symptoms may be explained by abnormal accumulation of undegraded substrates within the lysosomes. In fact, the continued presentation of GAGs to cell for degradation results in storage, which gives rise to an enlargement of lysosomes. As substrates accumulate, the lysosomes swell and occupy more and more of the cytoplasm. As a consequence of this increased number and size of lysosomes, other cellular organelles may be obscured, and the nuclear outline may be deformed. As the process continues, the enlarged cells lead to organomegaly. Abnormalities observed in heart cells and function may also be explained by GAGs accumulation. The increase of storage material within the cells of the heart valves causes an alteration of the cell's outline, changing

Table 1: Summary table of mucopolysaccharidoses.

Pathology	Subtype	Enzyme deficiency	Gene (localization)	Affected GAG	Clinical manifestations	Animal model
MPS I	Hurler (H)	α-L-iduronidase	*IDUA* 4p16.3	Dermatan and heparan sulfate	Corneal clouding; dysostosis multiplex; organomegaly; heart disease; mental retardation; death in childhood.	Feline [5]; canine [6]; knock-out mouse [7]
	Hurler -Scheie (H/S)	α-L-iduronidase		Dermatan and heparan sulfate	Intermediate phenotype, between MPS IH and MPS IS.	
	Scheie (S)	α-L-iduronidase		Dermatan and heparan sulfate	Corneal clouding; stiff joints; normal intelligence and life span.	
MPS II	Hunter	Irudonate sulfatase	*IDS* Xq28	Dermatan and heparan sulfate	Dysostosis multiplex; organomegaly; no corneal clouding; mental retardation; death before 15 years (severe); Short stature; normal intelligence; survival to 20s to 60s (mild)	Canine [8]; knock-out mouse [9]
MPS III	Sanfilippo A	Heparan-N-sulfatase	*SGSH* 17q25.3	Heparan sulfate	Relatively mild somatic manifestations; hyperactivity; profound mental deterioration.	Canine [10]; spontaneous mouse mutant [11]
	Sanfilippo B	α-N-Acetylglucosaminidase	*NAGLU* 17q21	Heparan sulfate	Phenotype similar to MPS IIIA.	Canine [12]
	Sanfilippo C	Heparan acetyl-CoA:α-glucosaminide N-acetyltransferase	*HGSNAT* 8p11.1	Heparan sulfate	Phenotype similar to MPS IIIA.	
	Sanfilippo D	N-Acetylglucosamine 6-sulfatase	*GNS* 12q14	Heparan sulfate	Phenotype similar to MPS IIIA.	Caprine [13]
MPS IV	Morquio A	Galactose 6-sulfatase	*GALNS* 16q24.3	Keratan and chondroitin sulfate	Distinctive skeletal abnormalities; corneal clouding; odontoid hypoplasia; milder forms known to exist.	Mouse [14]
	Morquio B	β-galactosidase	*GLB1* 3p21.33	Keratan sulfate	Phenotype similar to MPS IVA, with the same spectrum of severity.	
MPS V	** This designation is no longer used; the phenotype, which was first classified as MPS V, was found to be the milder form of MPS I (Scheie syndrome)**					
MPS VI (Maroteaux-Lamy)		Arylsulfatase B (N-acetylglucosamine 4-sulfatase)	*ARSB* 5q11-q13	dermatan sulfate	Dysostosis multiplex; corneal clouding; normal intelligence; survival to teens in severe form; milder forms also documented.	Feline [15]
MPS VII (Sly)		β-glucuronidase	*GUSB* 7q21.11	dermatan, keratan and chondroitin sulfate	Dysostosis multiplex; hepatomegaly; wide spectrum of severity including fetal hydrops and neonatal form.	Canine [16]; spontaneous mouse mutant [17]; mouse [18]
MPS VIII	*∗The designation MPS VIII was based on incorrect data and is no longer used.∗*					
MPS IX		Hyaluronidase 1	*HYAL* 3p21.3			Mouse [19]

them from fusiform to round. As a consequence, the valve leaflet and cordae tendinea become thickener and interfere with normal cardiac function, producing valvular stenosis. At corneal level, also, storage of undegraded GAGs results in reflection and refraction of light, leading to the cloudiness which is so typical of these pathologies. Also at the CNS level, swollen neurons and lysosomes may produce lesions that include the development of meganeurites and neurite sprouting (reviewed in [3, 4]).

Traditionally, MPSs are recognized through analysis of urinary GAGs. Several methods have been devised, to precise qualitative identification and quantitative measurements. These analyses of urinary GAGs allow discrimination between broad classes of MPSs but cannot distinguish subgroups. Definitive diagnosis is usually accessed through enzymatic assays of the defective enzyme in cultured fibroblasts, leukocytes, and serum or plasma (reviewed in [1]). During the last decade; however, dried blood spot technology was also introduced for enzymatic assays, allowing cheaper, easier, feasible diagnosis and opening the possibility for large population screenings (see Section 11 for more details).

In general, MPSs are transmitted in an autosomal recessive fashion, except for MPS II, which is X-linked.

This paper provides an overview of the molecular basis, enzymatic defects, clinical manifestations, and diagnosis of each glycosaminoglycan storage disease, focusing also on the respective animal models and describing potential perspectives of therapy which are being tested as well as the ones which are already available (summarized in Table 2).

2. Mucopolysaccharidosis I

Mucopolysaccharidosis I is caused by a deficiency of α-L-iduronidase (IDUA; EC 3.2.1.76) and can result in a wide range of phenotypic involvement with three major recognized clinical entities: Hurler (MPS IH; MIM#607014), Hurler-Scheie (MPS IH/S; MIM#607015), and Scheie (MPS IS) syndromes. Hurler and Scheie syndromes represent phenotypes at the severe and mild ends of the MPS I clinical spectrum; respectively, and the Hurler-Scheie syndrome is intermediate in phenotypic expression [20]. It is important to stress that, although MPS I may be subdivided into these three clinically diverse entities, the underlying enzymatic defect is common to all of them, being all caused by mutation in the gene encoding α-L-iduronidase (*IDUA*).

Functionally, α-L-iduronidase is essential to the correct metabolism of both dermatan sulfate and of heparan sulfate, hydrolyzing the terminal α-L-iduronic acid residues of the above-referred glycosaminoglycans [1].

In 1992, Scott and colleagues [21] were able to clone and purify the gene that encodes this enzyme, *IDUA*, demonstrating that it spans approximately 19 kb and contains 14 exons. The first 2 exons are separated by an intron of 566 bp, a large intron of approximately 13 kb follows, and the last 12 exons are clustered within 4.5 kb. Previously, this gene was mapped to 4p16.3, through unequivocal in situ hybridization and southern blot analysis of mouse-human cell hybrids [22].

There are, presently, several animal models known for MPS I.

In 1979, Haskins and colleagues [5] described α-L-iduronidase deficiency in a cat, and, few years later, Shull et al. [6] and Spellacy et al. [23] reported a similar deficiency in the dog. Subsequent studies lead to cloning and characterization of the canine IDUA gene as well as the mutation causing the observed phenotype [24, 25] and proved it to be a good model for study of human MPS I. So, in 1994, Shull and collaborators [26] published the first results of enzyme replacement therapy in the canine model. Through intravenous administration of recombinant human α-L-iduronidase, these authors managed to obtain a remarkable resolution of lysosomal storage in both hepatocytes and Kupffer's cells. In the same year, Grosson et al. [27] mapped the homologous IDUA locus in the mouse to chromosome 5. That knowledge was later used to create a knock-out mouse presenting the characteristic MPS I features [7, 28].

Currently, both hematopoietic stem cell transplantation (HSCT) and enzyme replacement therapy (ERT) using laronidase (recombinant human α-L-iduronidase, Aldurazyme) are available for MPS I. HSCT is the recommended treatment for patients with severe MPS I, before 2 years of age [29–32]. ERT is recommended for the other cases, and it has been shown to be effective in ameliorating some of the clinical manifestations of MPS disease. Among positive effects are decreased hepatosplenomegaly, improved respiratory and myocardial function and physical capacity [33–35] as well as improvement in active movement followed by enhanced self-care [36]. Recently, several reports have been published trying to evaluate long-term effect of ERT on the natural history of treated patients. From those studies, several conclusions have been reached. Concerning treated patients' growth pattern, it became clear that children with MPS I grow considerably slower than healthy individuals, and differences between healthy and affected children increase with age [37]. Other relevant evidences show that early treatment of attenuated MPS I may significantly delay or prevent the onset of the major clinical signs, substantially modifying the natural history of the disease [38]. Additional investigation is needed to clarify the mechanisms by which improvements are achieved in laronidase-treated patients. Such knowledge may support the development of ERT directly targeting the brain.

2.1. Hurler's Syndrome (MPS IH). Hurler's syndrome is the most severe form of MPS I and has been, over the last decades, the prototype description of MPS. Nevertheless, this may be misleading, since not all MPSs share the same features, and this pathology in particular is not representative of all of them, but only of the most severe end of a broad clinical spectrum (reviewed in [1]). Like all other MPSs, the clinical course of this disease is progressive, with multiple organ and tissue involvement. Hallmark clinical features of Hurler syndrome include coarse facies, corneal clouding, mental retardation, hernias, dysostosis multiplex, and hepatosplenomegaly. Children with Hurler's syndrome appear normal at birth and develop the characteristic appearance over the first years of life [39]. Length is often normal until about 2 years of age when growth stops; by age of 3 years, height is under the third percentile [40]. Cardiac

Table 2: Available therapeutic approaches for mucopolysaccharidoses.

Pathology	Subtype	Available therapeutic approaches
MPS I	Hurler (H)	HSCT (recommended before 2 years of age)
	Hurler -Scheie (H/S)	ERT with Aldurazyme (laronidase; recombinant human α-L-iduronidase)
	Scheie (S)	ERT with Aldurazyme (laronidase; recombinant human α-L-iduronidase)
MPS II	Hunter	ERT with Elaprase (idursulfase; recombinant human iduronate sulfatase)
MPS III	Sanfilippo A	*Not available*
	Sanfilippo B	*Not available*
	Sanfilippo C	*Not available*
	Sanfilippo D	*Not available*
MPS IV	Morquio A	*ERT: ongoing clinical trial (with recombinant human GALNS)*
	Morquio B	*Not available*
MPS VI (Maroteaux-Lamy)		ERT with Naglazyme (galsulfase; recombinant human arylsulfatase B)
MPS VII (Sly)		*Not available*
MPS IX		*Not available*

disease and respiratory complications are common. Acute cardiomyopathy associated with endocardial fibroelastosis has been a presenting condition in some infants with MPS I less than 1 year of age [41]. Upper and lower respiratory tract infections are also frequent [42]. Developmental delay is often apparent by 12 to 24 months of age, with a maximum functional age of 2 to 4 years followed by progressive deterioration. Most children develop limited language as a consequence of developmental delay, chronic hearing loss, and enlarged tongue [1]. Dermal melanocytosis may also be found in Hurler patients [43], as well as in patients suffering from other LSDs, such as GM1 gangliosidosis. Nevertheless, Hurler's syndrome is the most common lysosomal storage disease associated with dermal melanocytosis, as revealed by a literature analysis.

2.2. Hurler-Scheie's Syndrome (MPS IH/S). MPS IH/S corresponds to a clinical phenotype which is intermediate between the Hurler and the Scheie syndromes. It is characterized by progressive somatic involvement with dysostosis multiplex but little or no mental retardation. First symptoms usually occur between 3 and 8 years. Characteristic features of Hurler's syndrome, such as corneal clouding, joint stiffness, deafness, and valvular heart disease, can also appear in MPS IH/S patients. Nevertheless, the onset of these symptoms occurs much later than that in the severe MPS I type, beginning in the midteens and leading to significant impairment and loss of function. Other clinical features, such as micrognathism, pachymeningitis cervicalis, and compression of the cervical cord due to GAG accumulation in the dura, may also occur. Cardiac and respiratory complications may explain the high clinical mortality (reviewed in [1]).

2.3. Scheie's Syndrome (MPS IS). Scheie's syndrome was earlier thought to be a separate entity designated MPS V, instead of a phenotypical subtype of MPS I [44]. This pathology is characterized by a mild phenotype in which dysostosis multiplex can be present. Joint involvement is marked in the hand with a claw-hand deformity. Patients also have genu valgum, stiff, painful feet, and *pes cavus* [1]. Cardiac and respiratory complications are much milder than in the Hurler syndrome, with aortic and mitral valvular disease being a common feature [45]. At a respiratory level, Perks et al. [46] have reported two brothers with Scheie's syndrome suffering from sleep apnea, but no other complications are known. Intelligence is normal [1]. Pachymeningitis cervicalis (compression of the cervical cord secondary to glycosaminoglycan accumulation in the dura) may also occur.

3. Mucopolysaccharidosis II (Hunter's Syndrome)

Mucopolysaccharidosis II is the sole MPS transmitted in an X-linked manner and is caused by deficiency of the lysosomal enzyme iduronate sulfatase, which is crucial to the correct degradation of heparan and dermatan sulfate, by cleaving their O-linked sulfate. As a result, there is a progressive accumulation of glycosaminoglycans in nearly all cell types, tissues, and organs. Patients with MPS II excrete excessive amounts of dermatan sulfate and heparan sulfate in the urine [20, 47]. Hunter syndrome is caused by mutation in the gene encoding iduronate-2-sulfatase (*IDS*).

Although the disease is known since the early 1970s, being the first MPS to be defined clinically in humans, it was not until the 1990s that the IDS was cloned. In 1991, Wilson et al. [48] localized the gene to Xq28. Two years later, Flomen and coworkers [49] described the gene's structure as containing 9 exons and characterized the intron sequences surrounding them. In the same year, Wilson et al. [50] reported the complete sequence of the *IDS* gene, which spans approximately 24 kb. The potential promoter for *IDS* lacks a TATA box but contains GC box consensus sequences, which are consistent with its role as a housekeeping gene.

Curiously, a second *IDS* gene (*IDS2*) was identified by Bondeson et al. [51]. It is a pseudogene and is located within 90 kb telomeric region of the *IDS* gene and involved in a recombination event with the primary *IDS* gene in about 13% of patients with the Hunter syndrome.

Traditionally, the Hunter syndrome comprises 2 recognized clinical entities, according to the severity of symptoms: mild and severe. Although largely used, this nomenclature does have its difficulties, since the mild and severe forms represent the two ends of a wide and continuous spectrum of clinical severity. Also, in terms of iduronate deficiency, these forms cannot be distinguished since the enzyme's activity is equally deficient in both (reviewed in [1]). They are, though, separated almost exclusively on clinical grounds, although nowadays mutation analysis may help distinguish them.

This classification of MPS goes back to 1972, when McKusick distinguished between the severe form (which he called MPS IIA), with progressive mental retardation and physical disability and death before age 15 years in most cases, and the mild form (called MPS IIB) compatible with survival to adulthood and in which intellect is impaired minimally, if at all. He also pointed out the lack of corneal clouding in the X-linked form of MPS as opposed to the autosomal forms.

Presently, this classification has become obsolete since, in 2008, Wraith et al. [47] stated that MPS II should be regarded as a continuum between the two extremes (severe and attenuated). They noted that, although the clinical course for the more severely affected patients is relatively predictable, there is considerable variability in the clinical phenotype and progression of the more attenuated form of the disease and, so, it would not be correct to consider the milder form as a separate entity but, instead, look at Hunter's disease as a phenotypical continuum, with several possible degrees of severity.

In 1998, Wilkerson et al. [8] described Hunter's syndrome in a Labrador retriever, with the typical clinical features observed in humans: coarse facies, macrodactyly, corneal dystrophy, progressive CNS deterioration, and positive biochemical diagnosis for MPS through urine analysis.

After the successful results obtained in improving certain disease manifestations in patients with MPS I, including visceral manifestations and attenuation of neurologic disease progression [29, 52], hematopoietic stem cells transplantation (HSCT) has also been performed in several patients with MPS II. Unfortunately, although the transplantation of hematopoietic stem cells provides some enzymatic reconstruction in many target tissues with decreased excretion of GAGs in urine, decreased liver and spleen volumes, diminished facial coarsening, and improved respiratory function and joint mobility [53, 54], the results at neurological level were disappointing (reviewed in [55]). The additional risk of morbidity and mortality associated to this procedure led investigators to focus their attention in ERT for this pathology, with much better results, as discussed below.

A knock-out mouse model for MPS II was developed by replacing exon 4 and a portion of exon 5 of *IDS* with the neomycin-resistance gene [9, 56]. Affected mice exhibit a phenotype with notorious similarities to human disease, both at the biochemical and the clinical levels [9]. Several studies with this knock-out mouse model were done to assess the effect of ERT [56] as well as dose and various dosing regimens of idursulfase in urine and tissue GAG levels [57]. The results of these studies were quite promising, with a marked decrease in urinary GAGs as well as decreased GAG accumulation in several tissues [56] verified for several idursulfase doses and several dosing frequencies [57]. These studies have been used to support the first clinical trial of recombinant *IDS* in Hunter's syndrome patients. At the moment, both phase I/II [58] and phase II/III [59] clinical studies have proven not only the efficacy but also the safety of idursulfase replacement therapy. Consequently, ERT with recombinant human iduronate sulfatase (Elaprase, idursulfase, Shire Human Genetic Therapies Inc.) was approved in the US (July, 2006) and the European Union (January, 2007) for the treatment and the management of MPS II. The recommended dose is 0.5 mg/kg administered once weekly as an intravenous infusion (reviewed in [55]). As time goes by, additional evidence on the efficacy of ERT for MPS II patients is being published, as long-term treatments are successful. This is the case of a recently published report on the improvements observed in a 7 years and 10 months old child who began a 36 months' treatment with Elaprase at 4 years and 10 months. At the end of the treatment, the child presented normal excretion of GAGs in urine, normal-sized liver and spleen, and significant bone remodeling. Cardiac and neurological development, however, still progressively deteriorated [60]. This year, protective effects of ERT in MPS II patients were also reported for DNA damaging in leukocytes [61] and oxidative stress [62].

4. Mucopolysaccharidosis III (Sanfilippo's Syndrome)

The Sanfilippo syndrome, or mucopolysaccharidosis III, is caused by impaired degradation of heparan sulfate [1] and includes 4 subtypes, each due to the deficiency of a different enzyme: heparan N-sulfatase (type A; MIM no. 252900), α-N-acetylglucosaminidase (type B; MIM no. 252920), acetyl CoA: α-glucosaminide acetyltransferase (type C; MIM no. 252930), and N-acetylglucosamine-6-sulfatase (type D; MIM#252940). At a clinical level, the four subtypes are quite similar, with a characteristic severe central nervous system degeneration associated with mild somatic disease. Onset of clinical features usually occurs between 2 and 6 years, severe neurologic degeneration occurs in most patients between 6 and 10 years of age, and death occurs typically during the second or third decade of life. Type A has been reported to be the most severe, with earlier onset and rapid progression of symptoms and shorter survival [63].

4.1. Mucopolysaccharidosis IIIA (Sanfilippo A). General MPS IIIA clinical features include severe mental retardation with relatively mild somatic features (moderately severe claw hand and visceromegaly, little or no corneal clouding, little or no vertebral change). Usually, this pathology is characterized by marked overactivity, destructive tendencies, and other behavioral aberrations.

MPS IIIA phenotype is caused by mutations in the gene encoding N-sulfoglucosamine sulfohydrolase, also named heparan sulfate sulfatase (*SGSH*; 605270). This enzyme is specific for sulfate groups linked to the amino group of glucosamine.

In 1995, the gene encoding N-sulfoglucosamine sulfohydrolase, *SGSH*, was isolated, sequenced, and cloned [64]. Later, it was shown to contain 8 exons spanning approximately 11 kb [65].

There are two animal models known for MPS IIIA. The first to be discovered was the canine model when Fischer et al. [10] identified sulfaminidase deficiency in two adult wire-haired dachshund littermates. Subsequently, Aronovich et al. [66] determined the normal sequence of the canine heparan sulfate sulfatase gene and cDNA, through PCR-based approaches. Another model was described in 2001, when Bhattacharyya and collaborators [11] found a spontaneous mouse mutant of MPS IIIA resulting from a missense mutation (D31N) in the murine sulfatase gene. Affected mice die at about 10 months of age, exhibiting notorious visceromegaly, distended lysosomes and heparan sulfate accumulation in urine. Hemsley and Hopwood [67] found that these mice had severe brain involvement, with impaired open field locomotor activity and behavioral changes, suggesting axonal degeneration. Later, Settembre et al. [68] observed increased autophagosomes resulting from autophagosome-lysosome function in these mice. Similar findings were observed in another mouse model of another lysosomal storage disorder (multiple sulfatase deficiency; MSD; MIM no. 272200), reinforcing the recent idea that these diseases are disorders of autophagy, which may be a common mechanism for neurodegenerative lysosomal storage disorders.

MPS IIIA mice were recently tested for substrate deprivation therapy with both genistein and rhodamine B, two chemicals that inhibit GAG synthesis ([4, 69], reviewed in [70]). Encouraging results were obtained with both compounds, and this therapeutic approach started to be considered for several MPSs (see Section 11 for more details). Other interesting results were also obtained when siRNAs were used to reduce GAG synthesis in MPS IIIA mice. Last year, this approach was tested by Dziedzic et al. [71], who managed to reduce mRNA levels of four genes, *XYLT1, XYLT2, GALTI*, and *GALTII*, whose products are involved in GAG synthesis. This decrease of levels of transcripts corresponded to a decrease in levels of proteins encoded by them. Moreover, efficiency of GAG production in these fibroblasts was considerably reduced after treatment of the cells with siRNA. Either way, substrate deprivation therapy seems to be a promising approach for Sanfilippo's syndrome type A.

Gene therapy approaches are also being tested in MPS IIIA mice. Recently, promising results have been reported by Fraldi et al. [72], who performed experiments with intracerebral adeno-associated-virus- (AAV-) mediated delivery of *SGSH* gene, together with *SMUF1* gene, which exhibits an enhancing effect on sulfatase activity when coexpressed with sulfatases. They observed a visible reduction in lysosomal storage and inflammatory markers in transduced brain regions, together with an improvement in both motor and cognitive functions.

4.2. Mucopolysaccharidosis IIIB (Sanfilippo B). With a phenotype quite similar to MPS IIIA, the Sanfilippo syndrome B is characterized by deficiencies of α-N-acetylglucosaminidase, caused by mutations in the *NAGLU* gene that encodes this enzyme. α-N-Acetylglucosaminidase is required for the removal of the N-acetylglucosamine residues that exist in heparan sulfate or are generated during lysosomal degradation of this polymer by the action of heparan acetyl-CoA: α-glucosaminide N-acetyltransferase (reviewed in [1]).

The *NAGLU* gene was cloned in 1995 by Zhao and colleagues [73]. The deduced 743-amino acid protein has a 20- to 23-residue leader sequence, consistent with a signal peptide, and 6 potential N-glycosylation sites. It contains 6 exons and spans 8.3 kb on chromosome 17q21 [74].

Similarly to the above-referred MPS III syndrome, there is also a natural occurring mutant for Sanfilippo B. It was described by Ellinwood and coworkers, in 2003, in Schipperke's dogs [12].

During the last decade, Li et al. [75] created a laboratorial murine MPS IIIB was also constructed trough targeted disruption of the *NAGLU* gene [76]. With a phenotype quite similar to that of patients with MPS IIIB, this model began immediately to be used for therapeutic approaches as well as for pathogenesis studies. The first studies were done to evaluate the potential of ERT for this pathology [76]. The results, however, were quite disappointing since the recombinant NAGLU produced in Chinese hamster ovary (CHO) cells was not efficiently captured by MPS IIIB cells, either *in vitro* [77, 78] or *in vivo* [76]. This difficulty has turned the search for a treatment for MPS IIIB even more challenging. Presently, several therapies are under evaluation for this disease, including cell-mediated therapy, enzyme enhancement therapy, substrate deprivation therapy, and viral gene therapy (reviewed in [79]).

Promising results are being achieved through gene therapy approaches in MPS IIIB mice, namely, through direct microinjection into the brain of adeno-associated virus (AAV) vectors coding for NAGLU [80–82] and intravenous injections and intracranial gene delivery of lentiviral (LV) vector of NAGLU [83–85].

4.3. Mucopolysaccharidosis IIIC (Sanfilippo C). Sanfilippo syndrome C is, in general, characterized by the same clinical features described to MPS IIIA. Nevertheless, the enzyme deficiency in this pathology is different from the one causing the latter. Type C disease is caused by mutations in the gene encoding heparan acetyl-CoA: α-glucosaminide N-acetyltransferase (*HGSNAT*; 610453). This is the only known lysosomal enzyme that is not a hydrolase. It catalyzes the acetylation of the glucosamine amino groups that have become exposed by the action of heparan-N-sulfatase (reviewed in [1]).

The *HGSNAT* gene was cloned in parallel by two different groups, during the last decade: Fan et al. [86] and Hřebíček et al. [87]. The molecular defects underlying MPS IIIC remained unknown for almost three decades due to the low tissue content and the instability of *HGSNAT* [88].

To date, 54 *HGSNAT* sequence variants have been identified including 13 splice-site mutations, 11 insertions and deletions with consequent frameshifts and premature termination of translation, 8 nonsense, and 18 missense (reviewed in [89]).

Recently, two independent studies from Feldhammer et al. [88] and Fedele and Hopwood [90] have performed exhaustive functional analysis of the majority of the missense mutations already reported for the *HGSNAT* gene. Attention was focused in this particular type of mutations since there are several MPS IIIC patients carrying only missense mutations, either homozygous or heterozygous, who present an unexpected severe phenotype. In fact, although splicing and frameshift mutations are usually associated to that type of phenotype, since they give rise to premature termination codons and trigger nonsense-mediated mRNA decay (NMD); missense mutations are traditionally associated to milder disease. Nevertheless, this typical/general pattern is not observed for MPS IIIC. That is why these alterations were specifically cloned, expressed, and analyzed for their folding, targeting, and enzymatic activities. As a result, Fedele and Hopwood [90] have observed that the expression levels and enzymatic activity of most mutants were extremely low or even negligible. Feldhammer and colleagues [88], on the other hand, have observed that those mutations cause a misfolding of the enzyme, which is not correctly glycosylated. As a consequence, HGSNAT is not targeted to the lysosome but, instead, stays in the endoplasmic reticulum (ER). Thus, enzyme folding defects due to missense mutations, together with NMD seem to be the major molecular mechanisms underlying MPS IIIC. This makes MPS IIIC a good candidate for enzyme enhancement therapy, where active site-specific inhibitors are used as pharmacological chaperones to modify the conformation of the mutant lysosomal enzymes usually retained and degraded in the ER, in order to increase the level of the residual activity to a point which is sufficient to reverse the clinical phenotypes [88]. Together with inhibitors of heparan sulphate synthesis, pharmacological chaperones are currently being tested to reduce storage of this polymer in the CNS to levels sufficient to stop neuronal death and reverse inflammation.

4.4. Mucopolysaccharidosis IIID (Sanfilippo D). Like the previous MPS III subtypes, Sanfilippo's syndrome D presents a phenotype similar to MPS IIIA, with a singular enzyme deficiency underlying it: mutation in the gene encoding N-acetylglucosamine-6-sulfatase (GNS; 607664). The enzyme was originally described as specific for the 6-sulphated N-acetylglucosamine residues of heparan sulphate. However, the early data have been reinterpreted, and given that this sulfatase is in fact able to desulphate the 6-sulphated N-acetylglucosamine present in α- or in β-linkage or even as a free monosaccharide (reviewed in [1]).

N-Acetylglucosamine-6-sulfatase (EC 3.1.6.14) was purified and characterized by Freeman et al. [91], who identified 4 different forms of the enzyme in liver. Its catalytic properties were studied by Freeman and Hopwood [92]. Afterwards, Robertson et al. [93] assigned the glucosamine-6-sulfatase gene, which they symbolized *G6S*, to chromosome 12q14 by *in situ* hybridization of a tritium-labeled *G6S* cDNA probe. The localization was confirmed by using the cDNA clone in analyses of DNA from human/mouse hybrid cell lines. More recently, that information was completed by the work of Mok et al. [94], who amplified and sequenced the promoter and 14 exons of the *GNS* gene from a patient with MPS IIID. By analyzing that patient, it was also possible to identify a homozygous nonsense mutation in exon 9, predicted to result in premature termination at codon 355, as well as two common synonymous coding SNPs. At the same time, another group identified a 1-bp deletion in the *GNS* gene in another affected individual [95].

A naturally occurring large animal model was described by Thompson et al. [13], who reported type D Sanfilippo's syndrome in a Nubian goat. Later, caprine MPS IIID was used to evaluate the efficacy of ERT in this pathology. Recombinant caprine N-acetylglucosamine-6-sulfatase was administered intravenously to one MPS IIID goat at 2, 3, and 4 weeks of age. As a result, a marked reduction of lysosomal storage vacuoles was observed in hepatic cells, but no amelioration was noticed concerning the CNS lesions. No residual enzyme activity was observed either in brain or liver. Taking this preliminary results into account, it was considered that other treatment regimens will be necessary for MPS IIID [96].

5. Mucopolysaccharidosis IV

Mucopolysaccharidosis IV, or Morquio's syndrome, is caused by impaired degradation of keratan sulphate. Presently, there are two known enzyme deficiencies causing 2 different subtypes of Morquio's syndrome: deficiency in N-acetylglucosamine-6-sulfatase (causing Morquio's disease type A; MIM no. 253000) and deficiency in β-galactosidase (causing Morquio's disease type B; MIM no. 253010). Both MPS IV subtypes present a wide spectrum of clinical manifestations, but there are some characteristic common features: short trunk dwarfism, fine corneal deposits, spondyloepiphyseal dysplasia. Actually, the predominant clinical features of Morquio's syndrome are the ones related to the skeleton. Most of the times, this severe somatic disease is accompanied by a normal intelligence [1]. Patients with the severe phenotype do not normally survive past the second or third decade of life [97].

5.1. Morquio's Syndrome Type A. Morquio's syndrome A is caused by mutations in the gene encoding galactosamine-6-sulphate sulfatase (*GALNS*), which plays a crucial role on the degradation of both keratan sulphate and chondroitin sulphate.

The gene coding for human galactosamine-6-sulphate sulfatase (*GALNS*), was mapped to chromosome 16q24.3 trough fluorescence *in situ* hybridization assays [98]. Its structure was described at the same time by independent groups as comprising 14 exons and spanning approximately 40–50 kb [99, 100]. Curiously, the *GALNS* gene contains an *Alu* repeat in intron 5 and a VNTR-like sequence in intron 6 [100].

No natural occurring model is known for either type A or type B Morquio's syndrome. Nevertheless, a laboratorial murine model for type A syndrome was created from an induced disruption in exon 2 of the *GALNS* gene. Mutants presented no detectable enzyme activity and showed

increased GAG levels in urine. GAGs accumulation was also detected in several tissues including liver, kidney, spleen, heart, brain, and bone marrow [14]. These mice were later tested for enzyme replacement therapy and, after a 12-week long treatment with native GALNS or SUMF1-modified GALNS, showed manifest clinical improvement, demonstrated by a marked reduction of storage material in visceral organs, bone marrow, heart valves, ligaments, and connective tissue. The clearance of stored material in brain was dose dependent, and the keratan sulphate blood levels were reduced to normal [101].

Presently, there is no effective therapy for MPS IVA and care has been palliative, as in the majority of LSDs. Enzyme replacement therapy (ERT) and hematopoietic stem cells therapy (HSCT) have been considered as potential therapeutic approaches for MPS IVA (reviewed in [102]), ERT being, though, the most attractive candidate, since affected patients lack CNS involvement.

Recently, Rodríguez et al. [103] have produced a recombinant GALNS enzyme in *Escherichia coli* BL21. To produce sufficient amounts of purified GALNS enzyme, high level expression of GALNS in Chinese hamster ovary (CHO) cells has been established as a source of selectively secreted human recombinant enzyme. This recombinant enzyme has already been tested in the murine knock-out model, with consequent clearance of tissue and blood keratan sulphate [101]. These results provided important preclinical data for the design of GALNS ERT trials, which are now in course.

5.2. Morquio's Syndrome Type B. Although presenting overlapping clinical features, Morquio's syndrome B is genetically distinct from Morquio's syndrome A, being caused by impairments in another enzyme involved in the stepwise degradation of keratan sulphate: β-galactosidase, which is coded by the *GLB1* gene. Beta galactosidase hydrolases terminal β-linked galactose residues found in GM1 ganglioside, glycoproteins, and oligosaccharides, as well as in keratan sulphate (reviewed in [1]).

The *GLB1* gene spans 62.5 kb and contains 16 exons [104, 105] and maps to chromosome 3p21.33 [106]. The deduced 677-residue protein has a calculated molecular mass of 75 kD and contains a putative 23-residue signal sequence and 7 potential asparagine-linked glycosylation sites. It may be interesting to refer that the *GLB1* gene gives rise to 2 alternatively spliced mRNAs: a major 2.5-kb transcript that encodes the classic lysosomal form of the enzyme of 677 amino acids, and a minor 2.0-kb transcript that encodes a β-galactosidase-related protein (elastin-binding protein, EBP) of 546 amino acids with no enzymatic activity and a different subcellular localization. Exons 3, 4, and 6 are absent in the 2.0-kb mRNA as a consequence of alternative splicing of the pre-mRNA [107–109].

Presently, there are no known animal models for MPS IVB, either natural or engineered.

6. Mucopolysaccharidosis V

The designation MPS V is no longer used. In fact, the phenotype which was first classified as MPS V, was later found to be the milder form of MPS I (Scheie's syndrome), caused by deficiencies in α-L-iduronidase, with the typical stiff joints, clouding of the cornea most dense peripherally, survival to a late age with little if any impairment of intellect and aortic regurgitation [44].

7. Mucopolysaccharidosis VI (Maroteaux-Lamy Syndrome)

Mucopolysaccharidosis type VI is an autosomal recessive lysosomal storage disorder resulting from a deficiency of arylsulfatase B (N-acetylgalactosamine-4-sulfatase). Clinical features and severity are variable but usually include short stature, hepatosplenomegaly, dysostosis multiplex, stiff joints, corneal clouding, cardiac abnormalities, and facial dysmorphism. Intelligence is usually normal [110].

Arylsulfatase B is a lysosomal enzyme that removes the C4 sulphate ester group from the N-acetylgalactosamine sugar residue at the nonreducing terminus of dermatan sulphate and chondroitin sulphate, during lysosomal degradation [111]. The gene that codes for this enzyme was first mapped to chromosome 5q11-q13 [112] and is now known to contain 8 exons and span about 206 kb [111].

In 2002, a 3-year-old Siamese/short-haired European cat was referred for clinical disease characterized by dwarfism, facial dysmorphia, paralysis, small and curled ears, corneal clouding, and large areas of alopecia. X-ray examination showed multiple bone dysplasias. These features lead to suspect from a mucopolysaccharide storage disorder. Subsequent analysis proved it to be a natural occurring form of the Maroteaux-Lamy syndrome [15].

This MPS VI model has been extensively used over the last years to test ERT for this specific pathology. In 2003, Auclair and colleagues [113] have evaluated the cats' response to infusions of recombinant human N-acetylgalactosamine-4-sulfatase (rhASB) and observed an overall improvement in the disease condition at physical, neurological, and skeletal levels. Later, the same team has demonstrated that a high rate of immunotolerance towards rhASB can be achieved in MPS VI cats with a short-course tolerisation regimen [114], which may help the implementation of such procedures. Another interesting approach was designed, specifically to ameliorate joint disease in MPS IVA, through long-term articular administration of rhASB, leading to a notorious improvement in feline joint disease [115]. These successful results lead to the development of clinical trials in MPS VI patients, and three clinical studies including 56 patients have evaluated the efficacy and safety. As a consequence, enzyme replacement therapy (ERT) became available. The specific ERT for MPS VI, galsulfase (Naglazyme, Biomarin Pharmaceutical) was approved in 2005 by FDA and in 2006 by EMA. Long-term follow-up data with patients treated up to 5 years showed that ERT is well tolerated and associated with sustained improvements in the patients' clinical condition [2, 116].

Even though presently there is ERT available for these patients, other therapeutic approaches are being tested in animal models for MPS VI. In 2009, the first attempt of

successful gene therapy was performed through lentiviral-mediated gene transfer to joint tissues of the rat, with consequent correction of MPS VI cells [117]. This year, another study, involving intravascular administration of adeno-associated viral vectors in MPS VI cats, was published. After gene transfer the authors observed clearance of GAG storage, improvement of long bone length, reduction of heart valve thickness, and improvement in spontaneous mobility [118]. Either way, promising therapeutic strategies for MPS VI patients may be arising.

8. Mucopolysaccharidosis VII (Sly's Syndrome)

MPS VII, also known as Sly's syndrome, is characterized by the impossibility to degrade glucuronic acid-containing GAGs, due to impaired function of β-glucuronidase, which removes the glucuronic acid residues present in dermatan sulphate as well as in heparan and chonroitin sulphates (reviewed in [1]). Clinical features are highly variable, with phenotypes ranging from severe fetal hydrops to mild forms allowing survival into adulthood. Typical features include hepatomegaly, skeletal abnormalities, coarse facial features, and variable degrees of mental impairment [119].

MPS VII was first reported by Sly and collaborators in 1973, in a boy with skeletal changes consistent with MPS, hepatosplenomegaly, and granular inclusions in granulocytes. Additional features included hernias, unusual facies, protruding sternum, thoracolumbar gibbus, vertebral deformities, and mental deficiency. When β-glucuronidase activity was measured in fibroblasts, obtained values were less than 2% of control values. Both parents and several sibs of the mother showed an intermediate level of the enzyme [120].

In 1990, Miller et al. [121] reported that the gene encoding β-glucuronidase (*GUSB)* is 21 kb long, contains 12 exons, and gives rise to two different types of cDNAs, through an alternate splicing mechanism. Speleman et al. [122] used fluorescence in situ hybridization to map the *GUSB* gene to 7q11.21-q11.22. This map position was confirmed by dual-color hybridization of β-glucuronidase and another gene which had been mapped proximal to it: elastin (7q11.23).

Several pseudogenes, located on chromosomes 5, 6, 7, 20, 22, and Y, were also detected by Shipley et al. [123], when amplifying exons 2–4, 3, 6-7, and 11.

In 2009, Tomatsu et al. [124] provided a review of mutations in the *GUSB* gene that cause MPS type VII. Forty-nine different pathogenic mutations have been reported in the literature, with approximately 90% of them being missense mutations. Approximately 40% of the known *GUSB* mutations occur at CpG sites within the gene. The most common mutation is L176F, which has been found in several populations: American (Caucasian), Brazilian, British, Chilean, French, Mexican, Polish, Spanish, and Turkish ([125–127], reviewed in [124]). Genotype/phenotype analysis indicated that the most severe phenotype was associated with truncating mutations and with mutations affecting either the hydrophobic core or the modification of packing.

In 1984, mucopolysaccharidosis type VII (Sly syndrome) was described in a mixed-breed dog [16]. Since then, several other affected dogs have been studied, in the animal colony established at the University of Pennsylvania, the School of Veterinary Medicine [128] and, later, in a 12-week-old male German Shepherd dog studied in the same school [129]. All dogs shared the same missense mutation and developed similar phenotypes with skeletal deformities, corneal cloudiness, cytoplasmic granules in the neutrophils and lymphocytes of blood and CSF, and glycosaminoglycans in urine [129]. Another animal model was described as naturally occurring: the $gus^{mps/mps}$ mouse, which has a 1 bp deletion in exon 10 resulting in a progressive degenerative disease that reduces lifespan and causes facial dysmorphism, growth retardation, deafness, and behavioral defects [17]. Nevertheless, opportunities for experimental therapies were greatly expanded by the work of Tomatsu et al., in 2006 [18], who developed a new MPS VII mouse model, which is tolerant to both human and murine GUS, without the characteristic immune responses that complicated evaluation of the long-term benefits of enzyme replacement or gene therapy when the naturally occurring mice were used. Ever since, several therapeutic approaches have been attempted in MPS VII mice, and the results have been encouraging. That is the case of the works by Bosch and collaborators, who have been working on gene therapy for this pathology, in order to correct brain lesions. They have used both adeno-associated virus (AAV) [130] and lentivirus-mediated gene transfer [131] and observed that there was a significant correction of pathology in the brain of affected mice.

Other therapeutic approaches had already been attempted, but their results were not as promising. In fact, in 1998, allogenic bone marrow transplantation was reported in a 12-year-old Japanese girl with consequent improvement of motor function and daily life activities, decrease of upper respiratory and ear infections, but no improvement at all in cognitive function [132].

9. Mucopolysaccharidosis VIII

The clinical entity once known as MPS VIII was described in a single patient, in the late 1970s. The patient, a 5-year-old child, presented short stature, coarse hair, hepatomegaly, mild dysostosis multiplex, mental retardation, and no signs of corneal clouding. Biochemical analysis of the urine revealed increased excretion of keratan and heparan sulphate [133, 134]. The biochemical findings described by this group lead to suspect the existence of two hexosamine sulfatases and propose the existence of this novel MPS, caused by glucosamine-6-sulfatase [133].

Nevertheless, subsequent analysis on Diferrante's laboratory brought this idea down, and the designation MPS VIII was abandoned [135].

10. Mucopolysaccharidosis IX

Mucopolysaccharidosis IX, also known as hyaluronidase deficiency, is caused by mutations in the *HYAL1* gene.

This disease was first discovered by Natowicz et al. [136] in a 14-year-old girl with short stature and multiple periarticular soft-tissue masses. Radiographic analysis showed nodular synovia, acetabular erosions, and a popliteal cyst. Lysosomal storage of hyaluronan (HA) was evident within the macrophages and fibroblasts of biopsied soft-tissue masses, and serum concentrations were elevated 38–90-fold. She was proven to have a storage disease of hyaluronan (hyaluronic acid) due to a genetic deficiency of hyaluronidase. The descriptions of hyaluronidase deficiency in this family are consistent with autosomal recessive inheritance.

In order to determine the molecular basis of MPS IX, Triggs-Raine et al. [137] analyzed 2 different candidate genes tandemly distributed on chromosome 3p21.3, both encoding proteins with homology to a sperm enzyme with hyaluronidase activity. These genes, *HYAL1* and *HYAL2*, encode 2 distinct lysosomal hyaluronidases with different substrate specificities. When characterizing the patient with hyaluronidase deficiency originally reported in [136], they verified that he was a compound heterozygote for 2 mutations in the *HYAL1* gene: a missense mutation (c.1412G>A), which introduced a nonconservative amino acid substitution in a putative active site residue (p.Glu268Lys) and a complex intragenic rearrangement, 1361del37ins14, which resulted in a premature termination codon. Through this work, they have also showed that these 2 hyaluronidase genes, together with a third adjacent HYAL3 gene, had markedly different tissue expression patterns, consistent with differing roles in the metabolism of hyaluronan. These findings allowed this team not only to explain the unexpectedly mild phenotype of MPS IX but also to predict the existence of other hyaluronidase-deficiency disorders.

Presently, three other hyaluronidase-related genes (*HYAL4*, *HYALP1*, *SPAM1*) have been identified at 7q31.3 [138]. These genes are predicted to encode hyaluronidases, endoglycosidases that initiate the degradation of HA, a large negatively charged GAG found in the extracellular matrix (ECM) of all vertebrate cells [19].

Since there is only one patient reported to date, the development and characterization of a model of Hyal1 deficiency was the first logical step in understanding the main phenotypic symptoms associated with MPS IX. During this decade, a mouse model for MPS IX has become available and was fully characterized [19]. Overall, it was observed that the murine MPS IX model displays the key features of the human disease. Nevertheless, during the same year, another mutant mouse suffering from a hyaluronidase deficiency was described, this one deficient in HYAL2 [139]. Skeletal and hematological anomalies were described in this model, raising the possibility that a similar defect, defining a new MPS disorder, exists in humans [139].

11. Conclusion

The elucidation of enzyme deficiencies underlying mucopolysaccharidoses was crucial to unveil the normal pathways of glycosaminoglycan catabolism. In fact, only through the consequences of their absence became the role of several enzymes evident. The majority of these enzyme deficiencies were discovered during the 1970s. Over the last decades the enzyme deficiencies underlying each disease, and the molecular defects causing them have been identified and extensively analyzed and characterized. As a result, six different MPS are known, caused by deficiencies in one of the ten different enzymes necessary to intralysosomal degradation of GAGs through one of the four different degradation pathways.

Each disease has its own hallmark features. Nevertheless, a common pattern arouses: MPS are usually chronic, with a progressive course and different severity degrees. Organomegaly, dysostosis multiplex, and CNS involvement are common but not necessary features.

Over the years, several MPS have been recognized in animals as naturally occurring diseases, and others were created by knock-out technology. Most animal colonies have been established from single related heterozygous animals, in such a way that the affected offspring is homozygous for the same mutant allele. All these models present disease pathology similar to that seen in humans, making the animals extremely valuable for both investigation of disease pathogenesis and testing of therapies. Large animal homologues are similar to humans in natural genetic diversity, approaches to therapy and care, and possibility of evaluating long-term effects of treatment. Presently, therapeutic strategies for MPS include enzyme replacement therapy, heterologous bone marrow transplantation, and somatic cell gene transfer, all of which have been tested in animals with some success. During the 80s, transplantation of hematopoietic stem cells was tested for several MPSs. Theoretically, haematopoietic stem cells taken from a normal compatible donor and transplanted into an enzyme-deficient recipient can provide a safe, permanent, and self-replicating source of bone marrow-derived cells. By secreting active lysosomal enzymes, these cells cross-connect nonbone marrow-derived cells. Several animal models for GAGs storage diseases have already been subjected to/undergone BMT. From those experiments, along with human clinical trials already tried, it was possible to verify that there are important variations in therapeutic response among different pathologies with some diseases with CNS pathology which can be successfully treated by BMT (the severe form of MPS I, being the example for the GAG storage disorders; [140]) whereas others cannot (MPS II and III in which BMT was tested with few success; [3, 141]). These variations are usually attributed to the different capacities of secretion, stability, and uptake of each specific enzyme. Nevertheless, important conclusions could be drawn from the collective experience of postnatal transplantation including the idea that the earlier the transplants are performed, the better the clinical response. In the 90s; however, a novel approach started to be tested: ERT. Nowadays, it has been the most tested approach in animal models of GAG storage disorders. Until now, the obtained results have been highlighting the potential of administered recombinant enzyme to reduce GAG accumulation. ERTs are presently available for MPS I (since 2003), II (since 2005), and VI (since 2006). Clinical trials are also in course for MPS IVA treatment through ERT. Nevertheless, this approach is

ineffective for the brain since recombinant enzymes are not able to cross the blood-brain barrier (BBB). This is one of the reasons why other therapies are being tested for MPS with CNS involvement. ERT with direct administration of the recombinant enzyme into the brain (intrathecal injections) is also being considered in order to overcome that difficulty. Presently, such approaches are being considered for MPS IIIA and to overcome the cognitive deficit of MPS II and MPS I (reviewed in [142]).

Somatic cell gene transfer is another possible approach, but a long way needs yet to be travelled towards such a therapy is applicable to patients.

Finally, substrate deprivation therapy (or substrate reduction therapy) is also being considered for some MPSs. This approach is being tested with both genistein and rhodamine B (reviewed in [70]). Genistein, a chemical from the group of isoflavones, has been shown to inhibit the synthesis of GAGs in fibroblasts of patients with various forms of MPSs, namely, types I, II, IIIA, and IIIB [4, 143]. Similar results were obtained with rhodamine B, an inhibitor with an unknown mechanism of action. Remarkably, in MPS IIIA mice treated with rhodamine B, GAG storage decreased not only in somatic tissues, but also in brain, with improved behaviours of the animals [144, 145]. These encouraging results lead to the development of open-label pilot clinical studies with children suffering from Sanfilippo's syndrome types A and B in which a genistein-rich isoflavone extract (SE-2000, Biofarm, Poland) orally administered for 12 months. After one year of treatment, statistically significant improvement in all tested parameters was demonstrated (reviewed in [70]).

In order to better quantify and assess the efficacy of these therapeutic approaches, investigators have been trying to indentify suitable biomarkers for MPS, which allow the evaluation of short- and long-term treatment effects. This is also assuming particular importance since early detection of MPS is an important factor in treatment success. Recently discovered biomarkers include heparin cofactor II-thrombin complex and dermatan sulphate:chondroitin sulphate ratio [146]. Other biomarkers and/or therapeutic targets for MPS joint and bone disease recently identified through animal studies include several proinflammatory cytokines, nitric oxide, and matrix metalloproteinases (MMPs; [147]).

Another hot topic which is recently being discussed refers to the possibility of including some MPSs (particularly type I, IIIA, IIIB, and VI) in neonatal screening programs [148]. The ongoing development of enzyme replacement therapy and other treatments for several LSDs, including MPSs combined with the growing evidence that early commencement of therapy improves outcomes, has increased the pressure for the introduction of newborn screening programs, and a number of pilot studies are ongoing [148–152]. This is only possible thanks to the significant advances that were made in last decade since dried blood spot technology was introduced for enzymatic assays and lysosomal protein profile was developed.

Overall, there are encouraging results, with some therapeutic approaches already approved and others under development. Either way, it is important to stress that the management of MPS requires lifelong attention to the multisystemic involvement by a team of specialists experienced in dealing with these diseases, since none of the therapeutic options currently available result in complete resolution of morbidity.

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