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Serum hyaluronidase aberrations in metabolic and morphogenetic disorders

Berta Fiszer-Szafarz[1], Barbara Czartoryska[1,2] and Anna Tylki-Szymanska[3]

[1] *Institut Curie-Biologie, Centre Universitaire, 91405 Orsay, France,* [2] *Institute of Psychiatry and Neurology, Department of Genetics, 02-957 Warsaw, Poland,* [3] *Children's Memorial Health Institute, Department of Metabolic Diseases, 04-736 Warsaw, Poland*

Hyaluronidases are endo-glycosidases that degrade both hyaluronan (hyaluronic acid) (HA) and chondroitin sulfates. Deficiency of hyaluronidase activity has been predicted to result in a phenotype similar to that observed in mucopolysaccharidosis (MPS). In the present study, we surveyed a variety of patients with phenotypes similar to those observed in MPS, but without significant mucopolysaccharidurie to determine if some are based on aberrations in serum hyaluronidase (Hyal-1) activity. The study included patients with well-characterized dysmorphic disorders occurring on genetic basis, as well as those of unkown etiology. The purpose of the study was to establish how wide spread were abnormalities in levels of circulating Hyal-1 activity. A simple and sensitive semi-quantitative zymographic procedure was used for the determination of activity. Levels of both ß-*N*-acetylglucosaminidase and ß-glucuronidase whose activities contribute to the total breakdown of hyaluronan (HA) were also measured, as well as the concentration of circulating HA. Among 48 patients with bone or connective tissue abnormalities, low levels of Hyal-1 activity were found in six patients compared to levels in 100 healthy donors (2.0–3.2 units/μL vs 6($\pm$1 SE) units/μL). These six patients exhibited a wide spectrum of clinical abnormalities, in particular shortened extremities: they included three patients with unknown causes of clinical symptoms, one patient with Sanfilippo disease, one of the seven patients with achondroplasia, and one with hypophosphotemic rickets. Normal levels of serum Hyal-1 activities were found in patients with Morquio disease, GM1 gangliosidosis, I cell-disease, 6 of the 7 patients with achondroplasia, Marfan's-syndrome and Ehlers-Danlos syndrome. No patient totally lacked serum Hyal-1 activity. Serum HA concentration was elevated in patients with Sanfilippo A and I-cell disease. Determination of serum and leukocyte Hyal-1 and serum HA may be useful to evaluate patients with metabolic and morphogenetic disorders. *Published in 2005.*

Keywords: **human, serum hyaluronidase, leukocyte hyaluronidase, serum hyaluronan, dysostosis-multiplex, Sanfilippo-A disease, achondroplasia, hypophosphotemic rickets, I-cell disease**

Introduction

Glycosaminoglycans, formerly termed mucopolysaccharides, are catabolized by a combination of acid hydrolases, predominantly glycosidases and sulfatases. Deficiency in any these enzymes are the basis of inborn errors of metabolism referred to as mucopolysaccharidosis (MPS). Enzyme loss prevents complete breakdown of their substrates, causing an accumulation within lysosomes of undegraded material accompanied by wide-spread tissue and organ dysfunction. There are currently 10 such inborn enzyme deficiences, giving rise to 10 distinct MPS. They are chronic and progressive disorders, and are associated with skeletal and joint abnormalities, multiple dysostosis, and abnormal faces [1].

To whom correspondence should be addressed: Berta Fiszer-Szafarz Institut Curie, Centre Universitaire, 91405 Orsay, France. E-mail: bfiszer-szafarz@wanadoo.fr

The hyaluronidases are a family of enzymes that degrade hyaluronan (hyaluronic acid) HA as well as chondroitin -4 and -6 sulfates. Hyaluronidases are glycoproteins widely expressed in a number of human tissues and body fluids [2–8]. Six genes were identified within the human genome, for review see [9], and the proteins corresponding to each gene may exist in several forms due to various sialic acid modifications [5]. The circulating and synovial fluid hyaluronidases (Hyal-1) (serum hyaluronidase) are identical enzymes [5,10]. *In vitro* the enzymes have optimal pH activity between 3.3 and 4.0. Their mechanisms of action *in vivo* are unknown. A number of vertebrate species are lacking serum hyaluronidase activity [3] that could be attributed to the existence of hyaluronidase inhibitors. Several hyaluronidase inhibitors are present in human serum [11–15].

Hyaluronan synthases, HA, and hyaluronidases are involved in the regulation of many biological processes such as embryogenesis [16–18], maintenance and integrity of the extracellular

matrix, cellular proliferation and migration, and wound healing [9]. The expression of hyaluronidase by tumor cells induces angiogenesis *in vivo* [19]. Partially degraded HA present in human cancerous serum, when injected into chick embryos, induces cerebral or generalized proliferation of capillary blood vessels [20].

Perturbations in hyaluronidases activities are observed in a variety of diseases, such as rheumatoid arthritis and osteoarthritis [21], cancer [9,22–24], psoriasis [25], pseudoxanthoma elasticum [26] and scleroderma [27]. Until recently no specific diseases had been demonstrated to be caused by Hyal-1 dysfunction, although earlier studies suggest that some syndromes of unknown etiology are associated with low levels of hyaluronidase activity in serum [4]. To date, there has been only one patient reported with the complete absence of detectable circulating Hyal-1 activity [28]. A mutation in the amino acid sequence of the patient's Hyal-1 was shown [29].

In the present study we surveyed a variety of patients with phenotypes similar to those observed in MPS to determine if some may be on the basis of aberrations in serum Hyal-1 activity. The study included patients with well-characterized dysmorphic disorders occurring on a genetic basis, as well as those of unknown etiology. The purpose of the study was to establish how wide spread were abnormalities in levels of circulating Hyal-1 activity. A simple and sensitive semi-quantitative zymographic procedure was used for the determination of Hyal-1activity. Levels of ß-*N*-acetylglucosaminidase and ß-glucuronidase activities, that participate in the total breakdown of HA, were also measured as well as the concentration of circulating HA.

Materials and methods

Selection of patients and clinical observations

Forty-eight children and adolescents were selected from the Children's Memorial Health Institute, Warsaw, Poland. All patients presented with various bone and connective tissue abnormalities such as dysplasias, deformations, and changes of bone structure. Among the forty-eight patients, eighteen patients with clinically or biochemically well-characterized dysmorphic disorders were also examined, as shown in Table 1.

Laboratory data: control sera were provided by 100 healthy donors from the Employee Health Survey of the Curie Institute (Orsay, France). The six control sera used for ß-*N*-acetyl-glucosaminidase and ß-glucuronidase determinations were from the Institute of Psychiatry and Neurology (Warsaw, Poland).

The patients with decreased levels of serum hyaluronidase activity were monitored, when possible, for several years.

Table 1. Different pathologies of patients

Disorders	*No of patients*
Skeleton deformities[a]	11
Possible mucopolysaccharidosis[a]	6
Dysostosis multiplex[a]	2
Dysplasia[a]	4
Osteoporosis[a]	4
Subcutaneous accumulation of MPS[a]	1
Osteochondrodysplasia[a]	1
Osteogenesis imperfecta[a]	1
Sanfilippo disease A	1
Morquio disease	2
GM1 Gangliosidosis	1
I-cell disease	2
Achondroplasia	7
Marfan's syndrome	2
Ehlers-Danlos syndrome	2
Hypophosphotemic rickets	1

[a]Patients with dysmorphic features that resembled the mucopolysaccharidosis phenotype, but without significant mucopolysaccharidurie.

Chemicals

Human umbilical cord HA (H 1751), hyaluronidase EC 3.2.1.35 type VI from bovine testes (H 3631), and acrylamide/bis-acrylamide were from Sigma (St Louis, MO, USA), and 4-methylumbelliferyl-2-acetamido-2-deoxy-ß-D-glucopyranoside and 4-methylumbelliferyl-ß-D-glucuronide came from Koch-Light, England. Stains-all®, the cationic carbocyanine dye 1-ethyl-2-[3-(1-ethyl-naphtho[1,2d]-thiazolin-2-ylidene)-2-methylpropenyl]naphtho[1,2d]-thiazolium bromide no 2718, was obtained from Eastman-Kodak (Rochester, NY, USA). All other chemicals were from Merck (Darmstadt, Germany).

Serum and leukocyte hyaluronidases

Leukocytes from five healthy donors and three patients were isolated [30] and treated with 0.1% Triton X-100, kept for 1 h at room temperature, homogenized, and centrifuged for 15 min at $3000 \times g$. The supernatant was used for enzyme assay.

Activities were studied using HA-polyacrylamide gel electrophoresis (20 μg/ml HA-5% polyacrylamide gels). Sera were diluted with 50% sucrose-0.1% bromphenol blue, and a final volume equivalent to 0.5 μl of serum was applied per slot. For leukocytes an equivalent amount of protein was applied per slot. Electrophoresis was performed in a buffer containing 0.09 M Tris, 0.09 M boric acid, and 2 mM EDTA (pH 8.3) at 4°C for 2.5 h. Under these conditions Hyal-1 was inactive. The gel was then incubated at 37°C in 0.1 M formic acid, 0.15 M NaCl buffer (pH 3.6) overnight to allow the enzyme to degrade the substrate and then stained with "Stains-all", a very sensitive staining agent. After being thoroughly washed with water the gel appeared blue with pink spots. The blue color is due to the staining of hyaluronan, and the pink spots indicate the enzyme localization [5,31]. As the "Stains-all" dye stains a variety of acidic macromolecules, some extra colored spots may appear with certain complex biological materials [32]. These spots are

opaque and display all kinds of colors, which can easily distinguished from the hyaluronidase band, as they appear also in gels without hyaluronan. Hyaluronidase activity was calculated by using bovine testicular hyaluronidase as a standard at pH 6.0 and 37°C and expressed in m units/μL. The sensitivity limit of this method is 1.8 m units of hyaluronidase activity. Densitometry analysis was performed using the software 'Image SXM' on a Macintosh computer. Patient's serum Hyal-1 activity was compared with that of healthy controls. As an illustration, Figure 1 shows the migration and activity of serum Hyal-1 from both a healthy donor (lane 1) and patient MC serum (lane 2) on the gel. Figure 2 shows the migration and activity of leukocyte hyaluronidase from a healthy donor (lane1), and from patient AW with deficient serum hyaluronidase (lane 2).

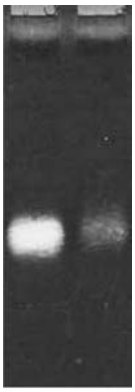

Figure 1. Serum Hyal-1 activity. Lane 1: serum from a healthy donor. Lane 2: serum from patient MC. Samples were analyzed on 20 μg/ml HA-5% polyacrylamide gel, 0.5 μl of serum per lane. Migration time 2.5 h.

Figure 2. Leukocyte hyaluronidase activity. Lane1: leukocyte extract from a healthy donor. The extract contained 1.6 mg protein. Lane 2: leukocyte extract from patient AW (patient with deficient serum hyaluronidase activity). The extract contained 0.65 mg protein. Samples were analyzed on 20 μg/ml HA-7% polyacrylamide gel. Migration time 2.5 h.

ß-*N*-acetylglucosaminidase and ß-glucuronidase activities

As HA is degraded by the combined action of hyaluronidase, ß-*N*-acetylglucosaminidases and ß-glucuronidase, we measured the activity of the latter two enzymes in serum [33,34]. Activities were measured by fluorometry with 4-methylumbelliferyl derivatives as substrates. Enzyme activity was expressed as nmol of liberated 4-methylumbelliferon/h/ml.

Hyaluronan

Concentration in serum was determined by an immuno-enzyme assay using hyaluronectin, a HA-binding protein [35].

Total sulfated glycosaminoglycans were determined in urine by using chondroitin sulfate as standard. The presence of chondroitin-4 and -6 sulfates, keratan sulfate, and heparan sulfate was determined by qualitative cellulose acetate electrophoresis [36].

Hyaluronidase, ß-*N*-acetylglucosaminidase and ß-glucuronidase activities, and hyaluronan concentration were measured on the same sample.

Results

Among 48 patients presenting with bone or connective tissue abnormalities like dysplasia and deformations and changes of bone structure, serum hyaluronidase activity was tested. Of these, 30 patients had disorders of unkown etiology with dysmorphic features that resembled the mucopolysaccharidosis phenotype, but without significant mucopolysaccharidoria, and 18 patients presented clinically or biochemically defined syndromes. In six patients low serum hyaluronidase activity was observed, three from the group with unkown etiology, and three from the group of well defined etiology (one patient with Sanfilippo A disease, one with achondroplasia and one with hypophosphotemic rickets). The activity of hyaluronidase, ß-*N*-acetylglucosaminidase and ß-glucuronidase and hyaluronan concentration were measured on the same serum sample. Leukocyte hyaluronidase activity was measured in 5 healthy donors and in the 3 patients where this was possible (Table 2).

Patients with disorders of unkown etiology and decreased levels of serum hyaluronidase

All three patients presenting with bone abnormalities, in particular shortened extremities, had normal liver, spleen, heart, teeth and cornea and were without neurological abnormalities or mental retardation. There were also no mucopolysaccharidoria. The HA concentration in serum was within control limits. Levels of calcium, phosphate and other routine clinical laboratory tests were also within normal limits.

Patient AW: female, followed from age nine to 15. She presented with dysostosis multiplex, epiphysis, scoliosis, and keratosis pilaris. Decreased stature (119 cm) with abnormally shortened lower extremities observed from early infancy. By age 15, her stature was normal (160 cm), but with persistent mild mental retardation (I.Q. 86). Kin observations were normal.

Table 2. Serum and leukocytes hyaluronidases activities and hyaluronan concentration, ß-*N*-acetylglucosaminidase and ß-glucuronidase activities in the sera of patients with low Hyal-1 activity and in I-cell disease patients

		Serum hyaluronidase activity[a] *(m units/μl)*		*Leukocyte hyaluronidase*[a]	*Serum hyaluronan concentration (ng/ml)*	*Serum β-N-acetyl glucosaminidase activity (nmoles 4-methyl-umbelliferone/ml.h)*	*Serum β-glucuronidase activity (nmoles 4-methyl umbelliferone/ml.h)*
Healthy donors		6.0 $\pm$ 1.0[f]		Normal	22.4 $\pm$ 16.7[g] [5]	650 $\pm$ 37[g]	27.7 $\pm$ 6.5[g]
		N = 100[b]		N=5[b]	N=100[b]	N = 6[b]	N = 6[b]
Patients with		6.0 $\pm$ 1.0[f]		Normal	18.7 $\pm$ 9.3[g]	Very variable	Very variable
unknown causes		P = 27[c]		P = 1[c]	P = 6[c]	P = 27[c]	P = 27[c]
of the symptoms	AW	3.0 (6d)[d]	4.2 (3d)[e]	Abnormal	9–13 (2d)[d]	514–400 (2d)[d]	66–59 (2d)[d]
	MC	2.2 (7d)[d]	6.0 (3d)[e]		13–19 (2d)[d]	429–380 (2d)[d]	29.5–27 (2d)[d]
	KW	2.8 (2d)[d]	3.3 (3d)[e]	Normal	13–13 (2d)[d]	384–300 (2d)[d]	32–24 (2d)[d]
Sanfilippo disease	JK	3.3 (5d)[d]			91–113 (2d)[d]	1412 (1d)[d]	159 (1d)[d]
Achondroplasia	BD	2.0 (3d)[d]	4.0 (3d)[e]		20–21(2d)[d]	400–370 (2d)[d]	14–18 (2d)[d]
Hypophosphotemic rickets	EO	2.2 (5d)[d]	5.4 (3d)[e]		20–21 (2d)[d]	360 (1d)[d]	32 (1d)[d]
I-cell disease	MW	6.0 (4d)[d]				12060 (1d)[d]	1820 (1d)[d]
I-cell disease	KG	5.5 (3d)[d]			87–117 (2d)[d]	6400 (1d)[d]	

[a]semi quantitative zymographic procedure, [b]number of healthy donors, [c]patients with unknown causes of symptoms with normal serum Hyal-1 activity, [d]in parentheses, number of determinations on the same sample of serum Hyal-1, ß-*N*-acetylglucosaminidase and ß-glucuronidase activities and hyaluronan concentration, [e]number of Hyal-1 serum activity determination after one year, [f]mean $\pm$ SE, [g]mean $\pm$ SD.

At nine years of age a slight increase in urinary mucopolysaccharides was observed, Hyal-1 activity was only 50% of normal (Figure 1), but it rose to 70% after one year, and became normal at age 15. At the age of nine, the activity of leukocyte hyaluronidase was higher and the electrophoretic migration slower than in controls (Figure 2), HA concentration and ß-*N*-acetylglucosaminidase activity were normal but as ß-glucuronidase activity in serum was twice as high as in controls.

Patient MC: male, followed from age six to 12. The patient's stature increased only slightly during that period, from 120 to 135 cm. He presented with dysostosis multiplex, dysplasia epiphysialis, scoliosis, sternal protusion, and muscle atrophy. Kin were normal. Hyal-1 activity was only 36% of controls at age 6, but reached a normal level within one year. At age 6, HA concentration, and ß-glucuronidase activity in serum were normal, but ß-*N*-acetylglucosaminidase activity was 60% of controls.

Patient KW: male, followed from age 10 to 15, during which time his stature increased from 106 cm to 136 cm. He presented with joint deformation, swelling of the joints and surrounding areas, dystrophia, sternal protusion, spondoepiphysialis, short extremities, and coarse facial features. Mental development and observations of kin were normal**.** Hyal-1 activity at 10 years of age was 47% of controls, one year later, 55%, five years later 73% and 5.4 years later 78%. Leukocyte hyaluronidase migration and activity were normal. Serum HA concentration and ß-glucuronidase activity were normal, but ß-*N*-acetylglucosaminidase activity was 50% compared to controls.

Patients with clinically or biochemically defined syndromes, and decreased levels of Hyal-1

Patient JK: (Sanfilippo-A): male, was studied at age 18**.** He presented with lumbar scoliosis, sternal protusion, joints stiffness, mental and motor deterioration, together with muscle atrophy, and hepatomegaly. His Hyal-1 level was 55% of normal. Serum HA concentration was five times higher than controls. ß-*N*-acetylglucosaminidase and ß-glucuronidase activities were increased 2 and 6 fold, respectively.

Patient BD: (achondroplasia): female, followed from age nine to 10, with an increase in stature from 81 to 90 cm. She presented with shortened extremities and lumbar lordosis. Liver, spleen, and heart were normal. Observations of kin were normal**.** Her Hyal-1 activity at age nine was 33% compared with that of healthy controls and 70% at 10. Her serum hyaluronan concentration was normal, and ß-*N*-acetyglucosaminidase and ß-glucuronidase activities were decreased to about 60% of control.

Patient EO: (hypophosphotemic rickets): female, followed from age eight to 10. She presented with dysplasia or pseudorachitism, enlargement of epiphyseal joints due to cartilagenous hypertrophy, and hypophosphatasia. Her conditions improved after vitamin D treatment. The stature increased from 106 to 145 cm. At age eight her Hyal-1 activity was 37% of normal and one year later 90%. Her hyaluronan concentration

was normal, ß-*N*-acetyglucosaminidase activity was 50% of normal and ß-glucuronidase activity was normal.

Patients MW and KG: (I-cell disease): both patients were both two years old females. Their sera had a normal hyaluronidase activity, as already reported [4,37]. Serum HA concentration for KG, was about four times higher than controls. Serum ß-*N*-acetylglucosaminidase activity in MW was increased 18 fold and in KG 10 fold compared to normal, ß-glucuronidase activity in patient MW was increased 65 fold.

Discussion

We reported here six cases of serum hyaluronidase deficiency in patients with various dysmorphic syndromes, three with MPS-like morphological disorders of unknown cause, and three patients with different well-characterized diseases with morphological abnormalities. One patient with Sanfilippo-A disease had a low level of serum hyaluronidase activity accompanied by an increased concentration of hyaluronan, as well as increased ß-*N*-acetylglucosaminidase and ß-glucuronidase activities in serum. One patient out of 7 with achondroplasia had a persistent Hyal-1 partial deficiency, and another had hypophosphotemic rickets. The two patients with I-cell disease had normal levels of serum hyaluronidase [4,37], while most plasma lysosomal enzymes activities were very much increased [38]. In contrast, the HA concentration in serum, measured in only one patient with I-cell disease, was much increased, as were ß-*N*-acetylglucosaminidase and ß-glucuronidase activities in serum.

We did not find any patient with levels of serum Hyal-1 activity higher than normal. Nor did we find any patient totally lacking in serum hyaluronidase activity. This is not surprising, since the established role of hyaluronidase in embryonic morphogenesis suggests that a genetic deficiency might result in an embryonic lethal condition [16–18]. A Hyal-1 partial deficiency might result from a mutation of the HYAL1 gene, resulting in a less effective enzyme and a permanent deficit. The transient deficiency we observed in patients AW and MC in childhood, which improved during puberty, suggests the involvement of sex hormone.

These results raise the problem of what contribution of circulating Hyal-1 makes to normal development. A deficiency, either permanent or limited to infancy, could play a role in the pathology of patients: low stature, skeletal, and joint defects. This would not be surprising, considering that hyaluronidases degrade the major glycosaminoglycans components of connective tissue and cartilage. In the three patients with deficient Hyal-1 activity of unknown cause, only patient AW had a slight mental retardation. The patients MC and KW with unknown causes of symptoms, the patient with achondroplasia and the patient with hypophosphotemic rickets had normal mental development. It is worth while stressing that, in spite of the fact that HA plays a mayor structural role in the extracellular matrix of the brain [35,39,40], the HYAL1 gene is not expressed in this tissue [2].

In conclusion, aberrant circulating Hyal-1 might have different causes: a mutation of the HYAL1 gene resulting in less effective Hyal-1 enzyme, down regulation by other genes that control development, or a condition secondary to other genetic diseases [41]. Each of the six patients with decreased levels of serum hyaluronidase exhibited a wide spectrum of clinical abnormalities, in particular shortened extremities, that may have been the consequence of modifications of serum hyaluronidase.

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