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Presence of hyaluronidase isoforms in nasal polyps

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Abstract. – BACKGROUND: Nasal polyps are benign lesions originating from the nasal mucosa or paranasal sinuses. The most important etiological factor seems to be increased hydration of epithelium and hyperplasia of the extracellular matrix, which may involve hyaluronan, a high molecular mass extracellular glycosaminoglycan. Degradation of hyaluronan proceeds through the action of specific hyaluronidases.

OBJECTIVE: The aim of the present study was to investigate the hydrodynamic size of hyaluronan and the presence of the various hyaluronidase isoforms in nasal polyps.

METHODS: Samples of polypoid mucosal tissue and normal nasal mucosa were obtained from twenty patients suffering from nasal polyposis. Zymographic analysis and western blotting were used to detect hyaluronidase activity.

RESULTS: The results indicated the presence of hyaluronan of small molecular mass in all samples examined. About one third of it has a mean molecular mass of 240 kDa, exactly that required for the expression of inflammatory response. Laboratory analysis suggested that degradation of hyaluronan occurred through the action of three hyaluronidase isoforms: Hyal-1, Hyal-2 and PH-20.

CONCLUSIONS: Since hyaluronan fragments of 200-250 kDa induce the expression of inflammatory cytokines, a specific role of hyaluronidases in the development or progression of nasal polyps may be concluded. Therefore, new treatment protocols may be proposed.

Key Words:
Allergy, Inflammation, Hyaluronan, Hyal-1, Hyal-2, PH-20

Introduction

Nasal polyps (NP) are described as benign lesions that originate from any portion of the nasal mucosa or paranasal sinuses. They consist a common ear-nose and throat (ENT) disease with high recurrence rate and they are found more commonly in adults than in children usually starting during the third or fourth decade of life, affecting 1%-4% of the population. They are also more common in males than females.

NP are typically round, smooth, semi-translucent masses usually located along the middle meatous and ethmoid sinuses, even if rarely emanating from the maxillary or sphenoid sinuses. They typically presented bilaterally as ethmoid polyps, arising from the ethmoid sinuses, but can also present unilaterally as antrochoanal polyps, arising from the maxillary sinuses. NP consist of edematous connective tissue that is covered by respiratory epithelium and results in the development of characteristic tumours that cause nasal airway obstruction, postnasal drainage, dull headaches, snoring, rhinorrhea and finally hyposmia or anosmia. The disease can be managed medically with nasal and/or oral administration of corticosteroids or surgically. Although some patients could be benefit from medical treatment, most of them are treated surgically. However, the possibility of recurrence is high. Recurrent operations in combination with the symptoms have a significant adverse impact on patient's quality of life.

The etiology and pathogenesis of NP are so far largely unknown and we have not yet fully understood the exact mechanisms underlying this disease. The most usual etiological factor is considered the presence of allergy, but recent studies have largely expanded the theories of disease pathogenesis, including chronic infection, genetic polymorphisms, alteration in aerodynamics with trapping of pollutants, epithelial cell defects or epithelial disruptions.

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One of the factors involved in the development of NP is the increased hydration of epithelium and hyperplasia of the extracellular matrix. This is usually the result of overproduction of hyaluronan (HA), a linear heteropolysaccharide with high molecular mass up to 10 MDa[1,2]. Its biosynthesis takes place through the action of three specific synthases named HAS1, HAS2, HAS3, which are membrane enzymes[3]. Biosynthesis of HA is also guided by its receptor, CD44, which is responsible for the control of the extracellular amounts of HA. The size of HA presents high diversity and many times chains of molecular mass lower than 100 kDa can be identified. These chains are considered to be responsible for the inflammation[4]. The degradation of HA occurred through a variety of mechanisms, of biologic (exoglycosidases and endoglycosidases) or chemical nature (mainly the various reactive oxygen species)[5]. Hyaluronidases are the enzymes specialized in acting upon the HA. They consist a family of 6 enzymes, named HYAL1, HYAL2, HYAL3, HYAL4, PH20 and PHYAL with different chromosomal localization of their genes. The first two and the fifth appear to have the most important role in the cellular functions[5].

Accumulation of HA in the extracellular matrix is due either to increased biosynthesis through increased expression of HA synthases or to decreased clearance of HA due to the decreased activity of hyaluronidases. However, and since the relatively low molecular mass HA chains are responsible for the inflammation, it is plausible to consider their accumulation as a result of the activity of hyaluronidases. The purpose, therefore, of the present study was the molecular size analysis of HA and the examination of the presence of hyaluronidases in NPs.

Materials and Methods

Chemicals

Phosphate buffer saline (PBS), phenylmethylsulfonyl fluoride, Alcian blue and Coomassie Brilliant blue were obtained from Serva (Darmstadt, Germany). Hyaluronic acid, hyaluronidase (type I) from bovine testes, hyaluronidase from streptomyces, benzamidine HCl, ε-amino-n-caproic acid, Triton X-100, N-ethylmaleimide, Na2EDTA, DEAE-cellulose and Sepharose CL-2B were obtained from Sigma Chemical Co (St Louis, MO, USA). Mouse polyclonal antibodies against hyaluroglucosaminidase 1 (Hyal-1), hyaluroglucosaminidase 2 (Hyal-2), hyaluroglucosaminidase 3 (Hyal-3) and sperm adhesion molecule 1 (PH-20/Spam-1) were obtained from Abnova Corporation (Taiwan). All other chemicals used throughout the study were of the highest available grade.

Tissue Source

Twenty patients with NP who underwent sinus surgery, were recruited from the Department of Otolaryngology-Head and Neck Surgery for this study. These patients had already treated medically with nasal steroids for a long time of period without substantial improvement. During the surgery, polypoid sinus mucosal tissue was collected and sent for biopsy. A part of the tissue was stored at –80°C for biochemical examination. Twenty samples of normal nasal mucosa from the same patients were also obtained from the inferior conchae and informed consent was obtained from all of them. The Ethical Committee of the University Hospital of Patras approved the study design.

Isolation and Characterization of HA

A small part of each polyp or normal nasal mucosa was finely diced and subjected to digestion with papain in 0.1 M sodium acetate pH 7.0 for 24 h at 37°C using 20 units of enzyme per g wet weight of tissue. Then, the solution was clarified after a brief centrifugation at 2,000 × g and the macromolecules were precipitated with 5 vols of ethanol. The precipitate was collected after centrifugation at 5,000 × g for 10 min, dissolved in 0.15 M sodium chloride and applied on a DEAE-cellulose column eluted with 3 bed vols of 0.15 M, 0.3 M and 1 M sodium chloride. The second eluate that contained all HA was collected, concentrated and applied on a precalibrated Sepharose CL-2B column eluted with 0.3 M sodium chloride.

Extraction and Zymographic Examination of Hyaluronidases

Each polyp or normal nasal mucosa was finely diced and the macromolecules contained were sequentially extracted for 3x24h periods at 4°C in the dark with PBS (10 mM disodium phosphate, 0.14 M NaCl, pH 7.4), 4 M guanidine hydrochloride (GdnHCl) – 0.05 M sodium acetate and 4 M GdnHCl – 0.05 M sodium acetate – 1% Triton X-100, using 10 vols of extraction buffer

per g of tissue. A protease inhibitor cocktail was included containing 5 mM benzamidine HCl, 0.4 mM phenylmethylsulfonyl fluoride, 10 mM N-ethylmaleimide, 0.1 M ε-amino-n-caproic acid and 0.01M Na_2EDTA. Each one of the extracts was stored at –20°C until use.

Hyaluronidase activity of the various extracts was examined using a HA zymography procedure, as described previously[6]. The samples were electrophoresed and at the end of the electrophoresis the gels were submerged in the suitable buffer (0.15 M NaCl– 0.1 M CH_3COONa pH 3.7) and incubated at 37°C for 16h. Then they were stained sequentially with Alcian blue to stain undegraded HA and with Coomassie blue to overstain the Alcian blue stained HA and the non enzymic protein bands. The enzyme activity appeared as white bands in a dark blue background. Finally, semi- quantification of the enzymatic units was achieved after scanning of the gel by a digital scanner (Epson-6100C/T, London, UK).

Western Blotting Identification of Hyaluronidases

The various extracts were subjected to sodium dodecyl sulphate-polycrylamide gels (SDS-PAGE) (T: 10%, C: 2.7%) according to Laemmli, using denaturing conditions in the presence of β-mercaptoethanol[6]. After electrophoresis, the gels were immersed in 0.15 M iodoacetamide in 0.05 M Tris-HCl pH 8.3 for 15 min at room temperature to block free β-mercaptoethanol. Thereafter, the protein bands were electrotransferred to nitrocellulose (Immobilon NC) (Millipore, London, UK) membranes at constant current of 80 mA at 4°C for 20 h in 0.05 M Tris-HCl pH 8.3. The membranes were washed with PBS containing 0.1% Tween 20 (PBS-T) and blocked with 5% dry skimmed milk in PBS-T. They were then incubated with the respective polyclonal antibody (see above) against either Hyal-1, Hyal-3 or PH-20 in an appropriate dilution in PBS-T for 1 h at room temperature. After repeated washings with PBS-T, the membranes were incubated with second antibody (goat anti-mouse IgG) (see above) peroxidase-conjugated (1:5000) in PBS-T, for 1 h at room temperature and washed exhaustively with PBS-T. The immunoreacting bands were visualized by enhanced chemiluminescence method (ECL, Millipore, London, UK), according to the manufacturer's instructions and by exposure to Agfa Curix X-ray film.

Statistical Analysis

Statistically significant differences between normal nasal mucosa and nasal polyps were estimated using the One Way Anova test. Statistical significance was determined as value of $p < 0.05$.

Results

Size – Distribution of Hyaluronan

The DEAE-cellulose eluates were examined for the presence of HA by enzymatic treatment with streptomyces hyaluronidase that specifically degrades HA and none of the other glycosaminoglycans. All HA was found to be present in 0.3 M sodium chloride and its molecular mass distribution was examined by gel chromatography on a precalibrated Sepharose CL-2B column eluted with 0.3 M sodium chloride (Figure 1). As it can be observed from all samples examined, nasal mucosa contained high molecular mass HA,

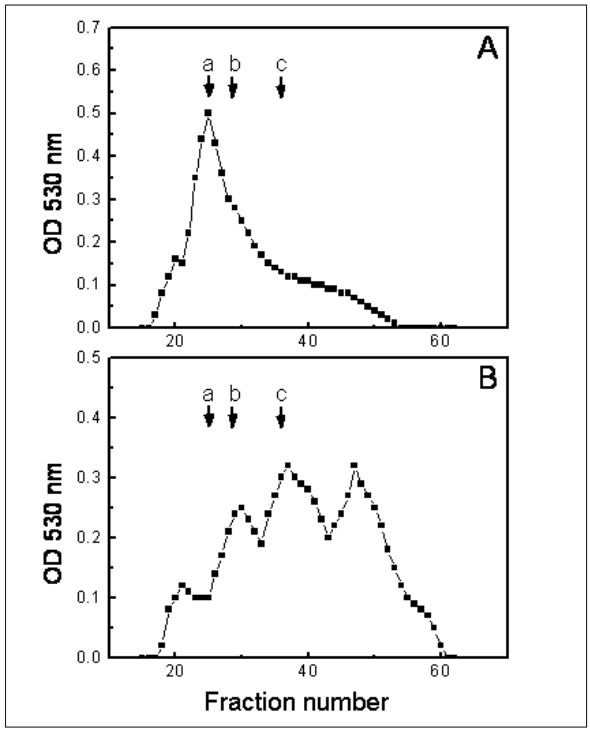

Figure 1. Size distribution of hyaluronan on Sepharose CL-2B. The 0.3 M NaCl eluate of DEAE-cellulose ion exchange chromatography was applied on a Sepharose CL-2B (0.8 × 140 cm) eluted with 0.3 M NaCl. Fractions of 1.2 ml were collected and analysed for their uronic acid content. ***A,*** Normal nasal mucosa. ***B,*** Nasal polyps. Arrows indicate the elusion position of molecular mass standards. a: 1,000 kDa, b: 600 kDa, c: 250 kDa. For details, see text.

whereas almost all of HA of NPs was included in the column, eluted as three separate populations, corresponding to molecular mass of 580 kDa, 240 kDa and 80 kDa. This observation indicated a high HA degrading activity in the NPs, a hypothesis that was examined further in details.

Extractability of HA – Degrading Activity

HA zymography was performed using 5 ml, 100 ml and 100 ml of the three sequential extracts, respectively, as found after preliminary experiments. In the case of normal mucosa samples, the results indicated the absence of any HA-degrading activity in all extracts examined (Figure 2A). In the case of NP, the results indicated the presence of a HA-lysis band, corresponding to a molecular mass of about 72 kDa (Figure 2A), which was present mainly in the PBS extracts. This observation was in accordance with the previous findings, regarding the low molecular mass of HA in NP.

Quantitative analysis of the zymography gels (Figure 2B) showed that about 95% of the HA-degrading activity was extracted with PBS, indicating a possible extracellular presence of the enzyme or its loose connection with the cellular membrane.

Western Blotting Identification of Hyaluronidases

The presence of the various isoforms of hyaluronidases was examined with western blotting. As it is shown in Figure 3, all of the samples were positive for the presence of Hyal-1, Hyal-2 and PH-20. Hyal-1 migrated as a double band, the smaller having a molecular mass of 72 kDa

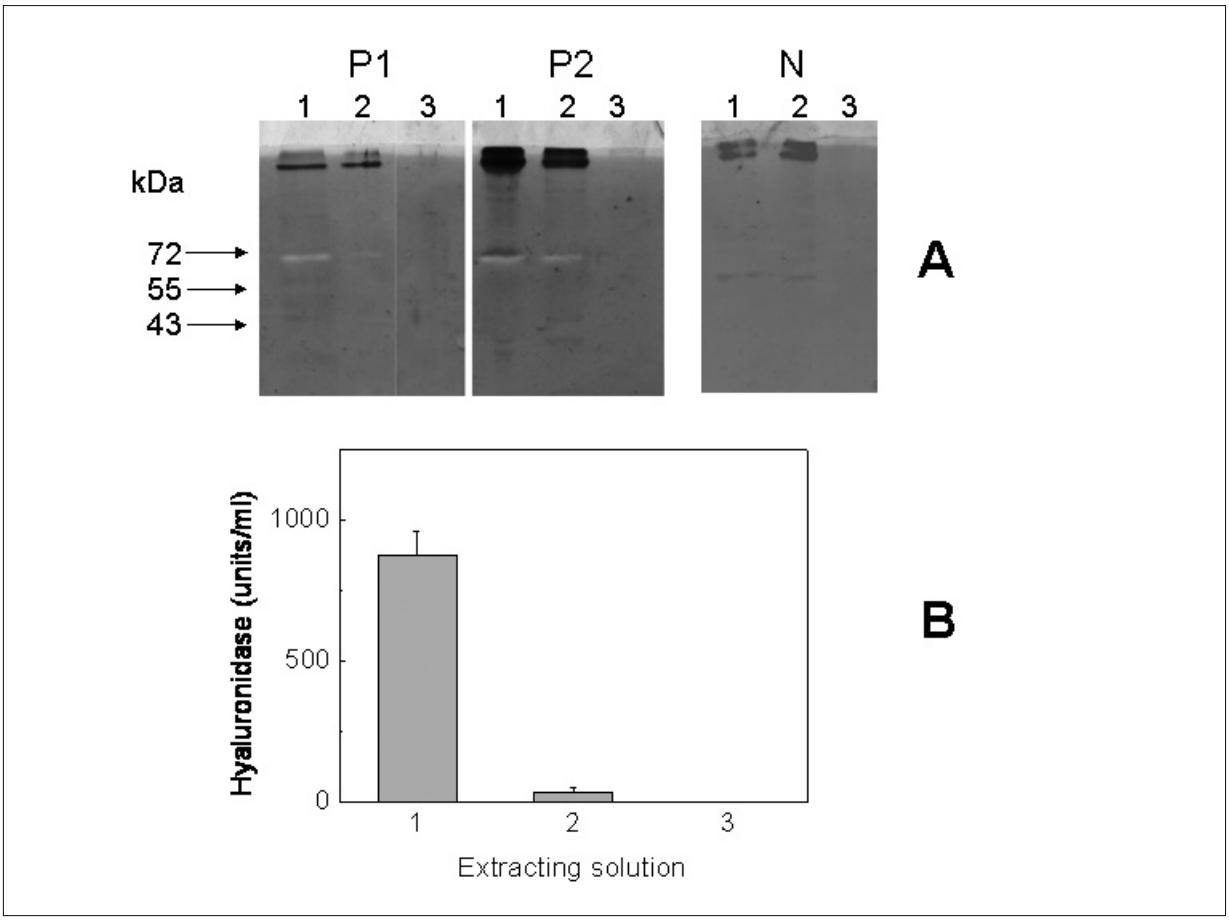

Figure 2. Zymographic analysis of HA degrading activity in NPs. ***A,*** The three sequential extracts (1, 2 and 3) of each nasal polyp (P) and nasal mucosa (N) were subjected to HA zymography, as described in the text. The results of two different NPs and one normal sample are presented. Arrows indicate the migration of protein molecular mass markers. ***B,*** Quantitative analysis of the HA degrading activity in the NPs.

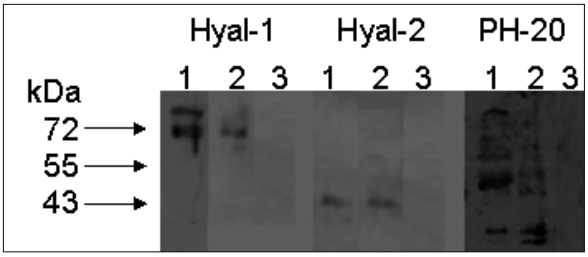

Figure 3. Western blotting examination of hyaluronidase isoforms. The three sequential extracts of NPs were subjected to western blotting using specific antibodies for each one of Hyal-1 (*A*), Hyal-2 (*B*) and PH-20 (*C*). Arrows indicate the migration of protein molecular mass markers. For details, see text.

corresponding to the lysis band observed in Figure 2. Hyal-2 migrated as a single band with a molecular mass of about 45 kDa. PH-20, on the other hand, gave a more complicated pattern, the main bands having molecular mass of 55 and 35 kDa. All enzymes were present in the PBS and 4 M GdnHCl extracts, in accordance with the zymography results.

Discussion

The investigation of extracellular matrix (ECM) components for their participation in nasal polyps formation and hydration is a subject of various studies[7,9]. The results obtained suggested that a very significant ECM macromolecule, HA, is not involved in these processes, since its amounts do not alter compared to normal mucosa. However, it is well known that ECM formation in NP is a result of inflammation, which usually proceeds through the participation of small-sized HA chains. The present work was, therefore, undertaken to investigate the size distribution of HA in NP and the possible implication of hyaluronidases in the inflammation.

The first observation of the study was the presence of HA of low molecular mass in NP. Indeed, more than 90% of the isolated HA was well included in the chromatography column and separated to three different fractions of 580, 240 and 80 kDa, whereas the major proportion of HA of nasal mucosa has a molecular mass of 1000 kDa. It is well known that HA chains with a molecular mass of about 200-250 kDa, has the ability to induce inflammatory cytokines[10-17], such as macrophage inflammatory protein-1a, macrophage inflammatory protein-1b, cytokine responsive gene-2, onocyte chemoattractant protein-1, interleukin-8, interleukin-1b, tumor necrosis factor (TNF)-α, and interleukin-12. Since the 240 kDa HA population was the major observed in the NP and represented about one third of the total HA, the assumption of an induction of degradation of HA to further induce the inflammatory response seemed to be correct. This assumption was additionally supported by the observation of even smaller HA chains, which also represented a significant proportion of HA.

HA chains of small size are usually produced from the action of the various hyaluronidases. Indeed, significant HA degrading activity was detected in the NP, the vast majority of which (about 95%) was observed extracellularly, suggesting that it directly acted to the ECM macromolecules. The HA degrading activity was absent from normal nasal mucosa. These findings strongly support the above observations, regarding the presence of low molecular mass HA chains.

The HA degrading activity was characterized using western blotting. Three different hyaluronidase isoforms were observed, namely, Hyal-1, Hyal-2 and PH-20. The first two enzymes are of lysosomal origin and have an acidic optimum pH, whereas PH-20 is active in a wide range of pH values, from 4.0 to 7.0. All enzymes can degrade HA to tetra- and disaccharides, although Hyal-2 usually leaves products of about 20 kDa molecular mass. However, and since the pH of the ECM of the NPs is in the neutral range, the activity of the enzymes seems to be down-regulated and, thus, the presence of degradation products larger than oligosaccharides is explained. It is not known the cells producing the different hyaluronidase isoforms. Preliminary studies suggested that the enzymes were not of fibroblast origin. This subject is now under investigation.

The characteristic of NP is the infiltration of eosinophiles, which generally participate in allergic inflammatory reactions. Expression of inflammatory cytokines, such as TGF-β, is induced by HA fragments in the 200-250 kDa range in eosinophils[18], in addition to monocytes and lymphocytes. This is indicative of a specific role of hyaluronidases in the development or progression of NPs and opens new avenues for the investigations of new formulations for their treatment.

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