Hyaluronan and Other Carbohydrates in the Vitreus

I.B.

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Outline

Keywords
Vitreous • Gel vitreous • Liquid vitreous • Hyaluronan • Proteoglycan

Key Concepts

1. Adult vitreus may consist totally of gel (cattle, rabbit) or may be a combination of gel and liquid (humans, monkeys). The gel is composed of a network of a very thin, fibrous collagen network, and the liquid is viscoelastic hyaluronan.
2. Vitreus viscoelasticity is produced by a combination of the concentration and the molecular size of the hyaluronan molecules.
3. After birth no new collagen fibrils, but only hyaluronan (liquid vitreus), is produced to keep pace with and fill the globe as it develops. With aging, gel vitreus liquefies in nearly all animals and in humans.

I. Introduction

Why vitreus? Why any connective tissue? In the eye, vitreus is responsible for the stability and general good health of the adjacent structures retina, lens, ciliary body, and zonules. Unlike other connective tissues, vitreus fulfills its function of transparency with solidity in order to serve as a stabilizer and shock absorber for movement or mechanical impact that could harm these delicate tissues. In addition, its high permeability permits free diffusion of most molecules reaching its border [see chapter IV.A. Vitreous physiology]. The authors have spent several decades studying vitreus, despite the fact that many years ago someone once noted that there is nothing more to know about the vitreus; everything is known. This chapter explains how the

As elected by the authors of this chapter, *vitreus* will be used as a noun, designating the connective tissue surrounded by the lens, ciliary body, and retina. Thus, vitreus will replace vitreous humor, vitreous body, and vitreous (if used as a noun). The two rheological states of the vitreus will be designated as *gel vitreus* and *liquid vitreus. Vitreous* will be used, as an adjective, in the following manner: vitreous anomalies, vitreous strands, vitreous implants, etc. [1].

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hyaluronan (formerly hyaluronic acid, hyaluronate) content and function in human vitreus contribute to development and aging in this most interesting tissue. Most of the work on this subject has been done before the mid-1980s and was reviewed and summarized by us [1] and updated in 1992 and 1998 by Sebag [2, 3] and in 1994 by Balazs [4]. In the last three decades, there has been additional work on human and animal vitreus, noting the similarities and differences between them. Unfortunately, since 1998 until today fewer than ten nonclinical papers were published with the words "hyaluronan" and "vitreous" in the title. In this paper we will review only a select group of those papers that represent important research findings on this subject.

As early as 1852, the German pathologist Rudolf Virchow observed that there were two parts to vitreus: a viscous liquid that could pass through a filter and a white, fibrous residue that was retained on the filter [5]. It was not, however, until the work of Meyer and Palmer [6] in 1934 that this substance was isolated, characterized, and given the name hyaluronic acid (from hyaloid (glassy, vitreus)) and uronic acid (for a review on the history of vitreous research from 1852, see [7]). In 1986, the name *hyaluronan* was adopted to replace the inaccurate name *hyaluronic acid* [8].

II. Vitreus Structure

The adult human vitreus has three distinct parts: liquid vitreus, gel vitreus, and posterior vitreous cortex, 100–300 μm thick, which contains in its posterior and lateral portions the only cells in the vitreus (hyalocytes) and is attached to the basal lamina of the retina and ciliary body [9, 10]. Its anterior portion, known as the *annular gap* [11], represents a "diffusion barrier" for liquid vitreus so that it cannot directly flow, but can only diffuse, out of the vitreous space into the anterior chamber. This anterior portion of the cortical gel vitreus does not contain cells. In a few primates such as the owl monkey (*Aotus trivirgatus*) and bush baby (*Galago galago*) in whom the entire vitreous space is filled with viscous liquid vitreus, the cortical gel still exists as a thin shell completely surrounding the liquid [12].

The human gel is made of collagen fibers, and in between the fibers is a large amount of hyaluronan. The gel vitreus fills the vitreous space in embryos and for a short period postnatally. After birth liquid vitreus is produced to keep pace with and fill the eyeball as it develops [13]. From development until adulthood, the vitreus doubles in volume. The concentration of hyaluronan in the normal human eye during this same period more than doubles increasing from approximately 0.07–1.8 mg/mL. The number of collagen fibrils responsible for the gel state of the vitreus (10–20 μm in size), forming a completely non-cross-linked, random network, remains constant. These fibers are very similar to type II collagen and crisscross the vitreous space, from basal lamina to basal lamina without detectable ends [2, 14]. Collagen fibers that are part of the gel vitreus are formed during prenatal development, and their number remains constant thereafter. There is no evidence that this collagen fibrillar network metabolizes in the normal adult vitreus. Following vitrectomy, the gel-forming collagen fibrils do not regenerate, provided that surgical trauma that could lead to wound-related connective tissue formation is minimal. The formation of liquid vitreus during normal growth and development of the eye is a result of the fact that the number of collagen fibrils does not increase, and hyaluronan (liquid vitreus) fills the space between the fibrils. The molecular size of hyaluronan is the same in young and old eyes, as well as in the liquid and gel vitreus [13, 15].

Also of note are differences in the topographical distribution of the insoluble (fibrillar) and soluble macromolecules in the human vitreus. The collagen fibrillar network is densest in the cortical vitreus gel next to the ciliary epithelium and near the ora serrata of the retina. In other areas of the cortical gel, it is less dense. Liquid vitreus, whether visualized during development or aging, is always located in the center and anterior part of the vitreus where the collagen fibrillar network is the least dense. Collapse of the sparse collagen network in this area (syneresis) can result in pools of liquid vitreus.

III. Hyaluronan

Hyaluronan is a unique, pure polysaccharide in the human and animal body – present in all intercellular matrices (also intercellular spaces) of the body. It is a polydisperse molecular system above the molecular size of 30,000; therefore, the molecular weight must always be expressed as an "average." Consequently the term "Dalton" cannot be used for characterizing hyaluronan because it is not monodisperse. The average molecular weight of hyaluronan varies in the human and animal body according to the intercellular space of the tissue where it is present. In vitreus the average molecular weight is very high; consequently the solution of the molecules represents a viscoelastic fluid system. The viscoelasticity of the hyaluronan solution depends not only on the average molecular weight but also on the concentration of the molecules. Hyaluronan is also unevenly distributed, being the highest in the posterior cortical gel in which the hyalocytes are

embedded. A concentration gradient toward the retrolental space exists and is maintained by a continuous production of hyaluronan by the hyalocytes. It must be pointed out, however, that the evidence for hyalocyte synthesis of hyaluronan is circumstantial and not direct.

The filtering effect of the basal laminae prevents the hyaluronan from diffusing into the intercellular spaces of the adjacent structures. Hyaluronan can slowly diffuse (it does not "flow") through the anterior cortical gel of vitreus into the posterior chamber [11]. From the posterior chamber, it is washed by the aqueous to the anterior chamber and leaves the eye through the trabecular meshwork to Schlemm's canal. The turnover time (half-life) of hyaluronan in the eyes of two monkey species was found to be several months, indicating a very slow diffusion (not flow) of these molecules out of the eye [1, 16]. This type of organization is typical of connective tissue matrices that separate tissues and protect them against friction and vibration caused by natural dislocation and stress (eye movements, heart beats, rubbing the eye). Examples of such matrices, in addition to vitreus, are synovial tissue and fasciae. These matrices are also more viscoelastic, as they protect against weak mechanical stress of higher frequency. Borzacchiello et al. [17] studied the rheological properties of vitreus in pigs, sheep, and rabbits in the frequency range of 0.05–10 Hz. The dynamic elastic modulus (G') was always higher than the viscous modulus (G") within this frequency range. It was possible to reproduce the rheological behavior of the animal vitreus qualitatively by increasing the molecular weight or the concentration of pure hyaluronan. This demonstrated that the molecular network of high average molecular weight hyaluronan can "mimic" the rheological properties of vitreus. Soluble protein concentration follows approximately the same distribution pattern as for hyaluronan, being highest in the posterior cortical gel vitreus and lowest in the central and anterior part [9, 18].

The hyaluronan molecule in vitreus has approximately the same average molecular weight in humans and animals, except in the case of hyaluronan in vitreus of macaques (ages 6–21 years), which was found to be significantly lower than that found in adult human liquid vitreus (2.9×10^6 versus 4.6×10^6) [19]. The hyaluronan molecular network consists of a spheroidal system with a molecular weight range of two to ten million and a diameter about ten times larger (200–300 nm) than that of the individual collagen fibrils. The domain of the hyaluronan molecular network consists of polysaccharide chains of 1–2 nm long random coils in which the space between the coils is filled with water. This special structure, with random distribution of thin molecular networks with relatively low concentration, is responsible for vitreus transparency. Reports of hyaluronan with lower-than-normal molecular size are thought to be due to artifacts resulting from damage to the molecule during the purification process or, as is the case in bovine vitreus, exposure to atmospheric oxygen during dissection. This rapid oxygen-sensitive degradation system does not manifest itself in vitreus of other species [10, 20].

There are considerable variations in the concentration of hyaluronan among species, with the highest concentrations found in the vitreus of the owl monkey, cattle, human, and rhesus monkey and the lowest in guinea pig, rabbit, cat, dog, and chicken [1]. In searching for an appropriate animal model for human vitreus, the rhesus monkey (Macaca mulatta) eye was found to have the most similarities to the human eye with regard to physical state, collagen, hyaluronan content, and aging [19]. The hyaluronan concentration is about ten times higher in the vitreus than in the aqueous in both macaque and human eyes. In both species, the hyaluronan concentration is similar and changes with age in the same manner [19, 21].

There are only a few connective tissue compartments that contain mainly one major structural polysaccharide – hyaluronan – and these are the vitreus, the umbilical cord, the rooster comb, and the synovial tissue around the joint space. In all of these tissues, one of the functions of hyaluronan is to stabilize a collagen fibrillar network. This interaction between the collagen fibrils and hyaluronan molecules is predominantly frictional, meaning that these two elements of the vitreus can be separated by mechanical forces such as filtration and centrifugation without denaturing or destroying them. Either of these two mechanical processes, however, will destroy the gel itself, as the collagen fibrils accumulate at the bottom of the centrifuge tube or on the filter. In animals that have a predominantly gel vitreus (rabbit, dog, cat, rodent, chicken), the concentration of hyaluronan is low and the collagen fibril concentration is higher than in species which have more hyaluronan in their vitreus. Thus, one can surmise that the role of hyaluronan as a stabilizer of collagen fibrils becomes more and more important as the human eye grows during development and the distance between the collagen fibrils increases.

Hyaluronan plays an important role in the physical properties of the vitreus. It was suggested by several authors that this provides an important "linkage" between the collagen-gel network of the vitreus and hyaluronan. One must realize that an interaction between the collagen network and the hyaluronan filling the space in between does not create a permanent contact (covalent binding) between the collagen and hyaluronan [22].

IV. Proteoglycans of the Vitreus

While the major molecular components of the human (as well as most animals) vitreus are the collagen and hyaluronan, there are also proteoglycans present in smaller quantities. The name proteoglycan indicates that the molecules are made up of a molecular complex of proteins and carbohydrates of various types. These are present in the liquid vitreus, but are not attached to the collagen fibers or to hyaluronan. One such proteoglycan is called *versican* [23–25]. This large molecule has a mass of 37 kDa (kDa) and contains a large amount of sulfated disaccharides. The presence of versican-like proteoglycans in different species represents a common structural component of the mammalian vitreus. A second important proteoglycan is *type IX collagen*. Like versican, it also contains both uronic acid and chondroitin sulfate [23, 26, 27].

The most important question from the point of view of stability of the vitreus is whether or not these proteoglycan interact with collagen fibers or with hyaluronan [28]. If an interaction exists, then the hyaluronan could not be removed from the gel part of the vitreus by washing with water. The proteoglycans could also be removed from the gel. In general, recent opinion is that if a direct interaction between hyaluronan and collagen fibers exists at all, it is very weak. Recent (1990–2008) studies were carried out using animal vitreus (cattle, pig, rabbit, sheep, and goat). The first biochemical evidence that hyaluronan-binding macromolecules are present in the vitreous was published in 1998 [24]. Noulas et al. [22] published investigations on pig vitreus gel. The aim was to study the "macromolecular composition of the vitreus," with particular interest in hyaluronan-binding proteoglycans. Their study confirmed the presence of hyaluronan and that the pig vitreus contained lower levels than human vitreus. This was the first time the existence of a population of hyaluronan-binding proteoglycans in the pig vitreus gel was shown. These molecules show structural and immunological characteristics similar to versican. Their findings are in agreement with the data obtained in human vitreus, as well as from bovine vitreus gel [24]. The molar ratio of versican and link proteins is approximately 1:1, suggesting that they form aggregates with hyaluronan in cattle vitreus. The functional significance of versican and link proteins remains uncertain.

V. Aging of the Vitreus

The human vitreus continues to develop after birth, nearly doubling its volume (Figure I.B-1). This volume increase occurs in parallel with the production of hyaluronan that fills the enlarging vitreous body and provide support and stabilization for the collagen fibrillar network (Figure I.B-2). This stabilizing effect is especially important because the production of collagen fibers does not continue after birth and the space between the fibrils increases as the gel becomes less and less dense.

No liquid vitreus is found in human eyes below the age of 4 years [13]. As the eye develops, up to the age of 20 years, the liquid volume slowly increases and fills about 20 % of the total vitreous volume. This may increase in some cases to 50 % in old adult eyes. In animals that have a predominantly gel vitreus (cattle, dogs, sheep, cats, horses, rabbits, and most monkeys), liquid vitreus does not form [30], but in humans and rhesus monkeys, aging results in important and parallel changes in the vitreus. From the age of 45–50 years in humans, the volume of the liquid vitreus increases as the volume of the gel vitreus steadily decreases (Figure I.B-1).

From early adulthood to old age, the hyaluronan concentration in the vitreus does not change in either the gel or liquid vitreus (Figure I.B-2 [31–35]). After 70 years, an increase in hyaluronan concentration was found in most individuals studied, with a greater increase in the liquid than the gel vitreus with no change in molecular size of the hyaluronan [1, 13, 30]. Eisner [36] has described this process as beginning with the occurrence of narrow channels and then pockets and pools of liquid vitreus that begin to coalesce after 40–45 years of age, resulting in a central liquid pocket that in later years occupies slightly more than half the volume of the vitreus. A parallel result is the increase in collagen concentration in the gel vitreus during the aging process due to the collapse or contraction of the collagen fibrillar network during the formation of these channels and pools of liquid vitreus (see [1] for review).

Another aging change that occurs in the vitreus and has only been documented in the human eye is called posterior vitreous detachment [37, 38] [see chapter II.C. Vitreous Aging and PVD]. Since this change occurs in a large number of the aging population and usually does not lead to any serious pathological change or impairment of vision, it is assumed to be part of the normal aging process. The fundamental structural changes that influence the vitreus during aging are a thickening of the basal lamina of the retina accompanied by an increase in the volume of the liquid vitreus with simultaneous collapse of the gel and aggregation/coalescence of the collagen fibrils. The rhesus monkey (*Macaca mulatta*) vitreus is the only one described thus far that shows developmental and aging changed similar to those that occur in humans. Posterior vitreous detachment, however, was not observed in rhesus monkeys as old as 21 years to 30 years. Posterior vitreous detachment can also

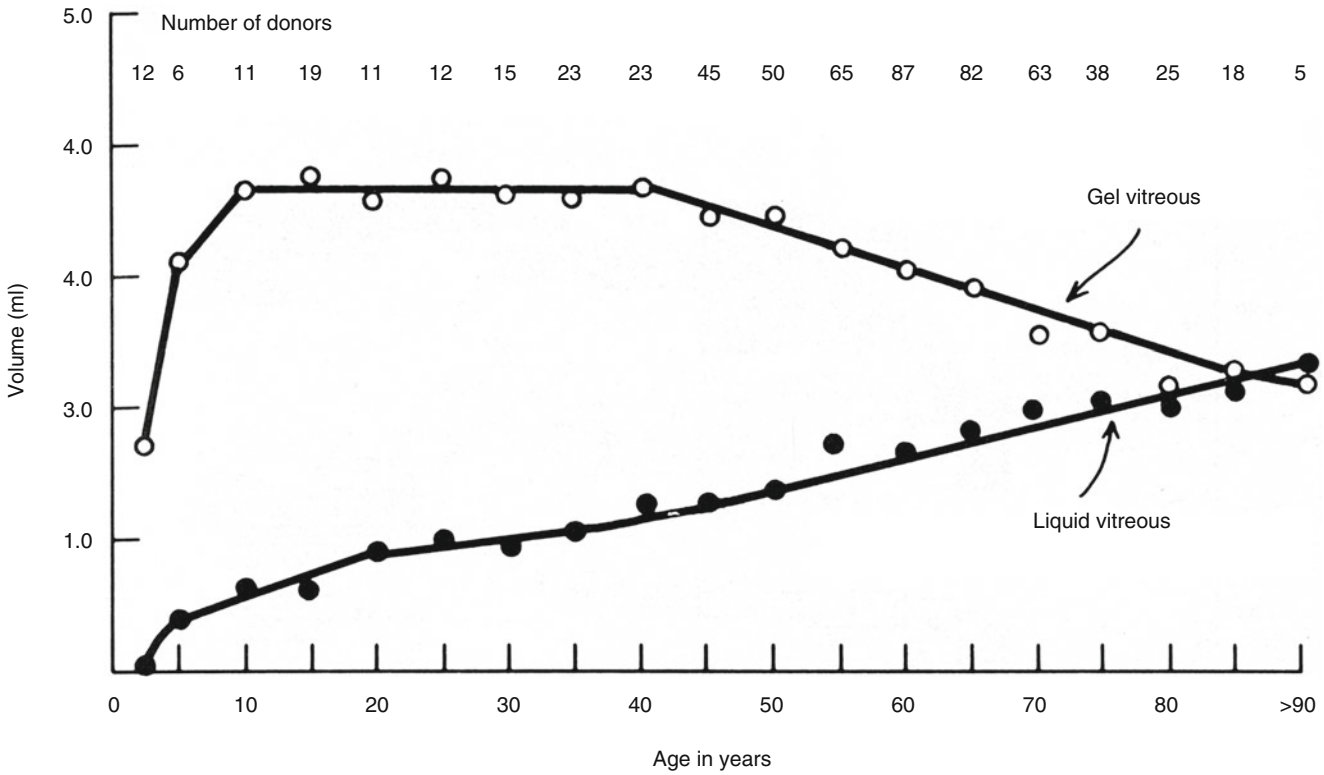

Figure I.B-1 Rheology of human vitreous [29]. Fresh unfixed human eyes were studied postmortem and the volumes of gel vitreous (*open circles*) as well as liquid vitreous (*closed circles*) were measured. The *y*-axis displays the volume in ml and the *x*-axis the age groups of the donors. Each point represents the mean value obtained from various numbers of donor eyes (shown at *top of graph*). It can be appreciated that after growth of the eye ceases (age 10–20), the gel volume is stable until age 40 when it begins to steadily decline throughout the remainder of life. The volume of liquid vitreous increases throughout life

occur at any age as a result of chronic intraocular inflammation, severe trauma to the eye, and in aphakic or highly myopic young individuals [19]. Molecular weight determinations of hyaluronan from human diabetic and nondiabetic vitreus showed no significant difference [39], although there are known changes in vitreous collagen [see chapter I.E. Diabetic vitreopathy]. Itakura et al. [40], using eyes from patients with diabetic retinopathy or macular holes, found that there was a significant decrease in the "level" (concentration) of hyaluronan in the vitreus with aging under these pathological conditions. In our opinion their reported data does not prove their assumption.

The age-related partial liquefaction of human vitreous was studied by electron microscopy, and a breakdown of collagen fibers into smaller fragments was found [41]. The mechanism of the process – especially from the point of view of its cellular or extracellular origin – could not be determined. However, they "tentatively concluded that an extracellular process is involved." However, in biomicroscopic studies of macaque eyes, it was found that the coalescence of the liquid pockets results from dislocation and collapse of the tractus vitrealis rather than a breakdown of the collagen into smaller fragments [19].

VI. Miscellaneous Considerations

A. Species Variations

In fish eyes, Balazs, for the first time in 1956 [42], found that gel vitreus is attached to the entire retina and that in front of it is a viscoelastic liquid vitreus that completely surrounds the rigid, spherical lens. Balazs gave the name ichthyosan A to this very viscoelastic liquid that surrounds the lens and ichthyosan V to the gel in front of the retina. These two molecular complexes are non-covalent aggregates composed of hyaluronan, a sulfate-free chondroitin proteoglycan, and a keratin-like molecule [43, 44]. Ichthyosan V is in between the collagen fibrils in the vitreus and stabilizes the structure of the vitreous gel. The anterior chamber is

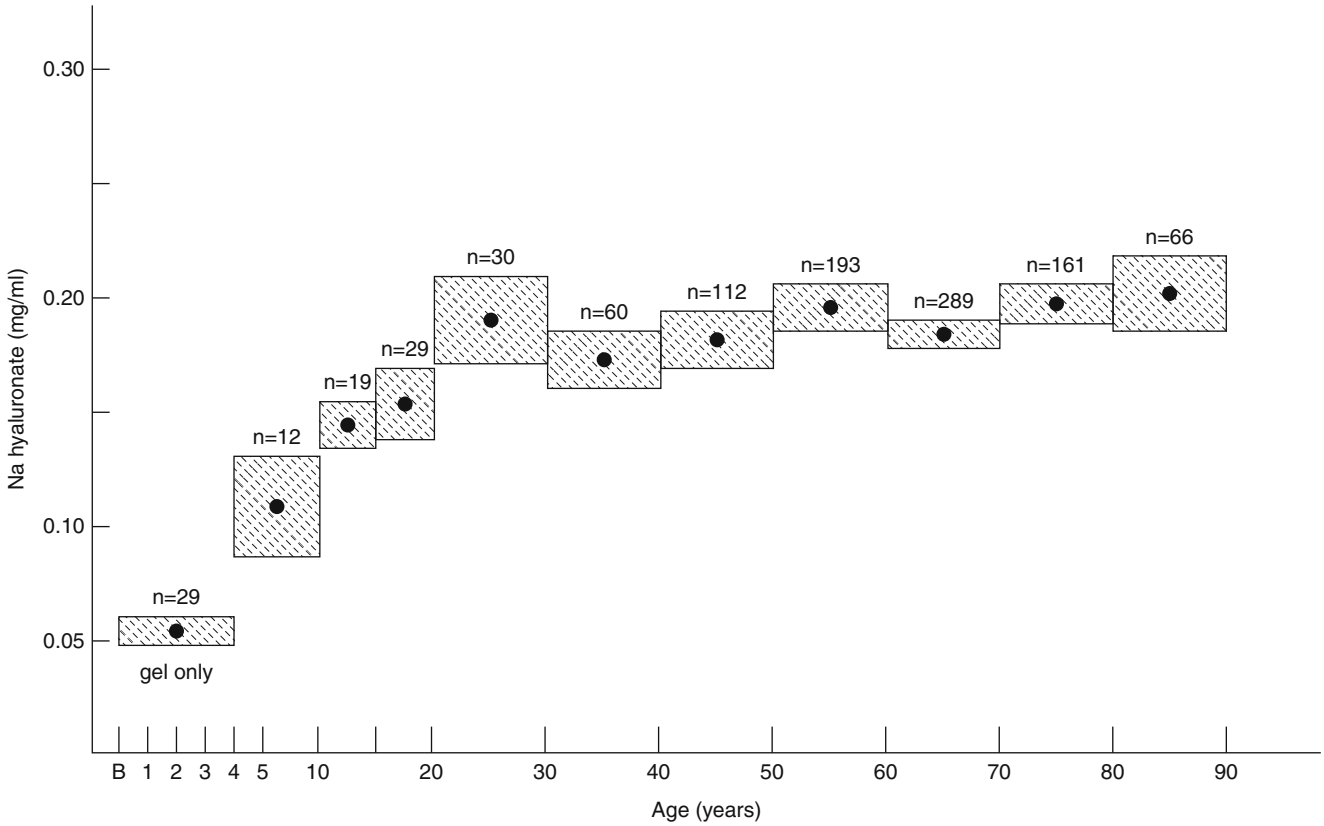

Figure I.B-2 Concentration of hyaluronan in human gel vitreous during life [30]. The sides of the boxes indicate the standard error of the means; the horizontal shows the age range of individuals included in the group. The number of samples in each group is indicated by "n". There is a steady increase in hyaluronan concentration until a plateau is reached at about age 25

filled with the same liquid as the vitreus. Because the lens cannot be deformed in fish eyes, it accommodates (focuses) by moving the lens back, forward, and sideways in this very elastoviscous liquid. The average molecular weight of ichthyosan varies from 5.2 to 13.0 million in various species of fish [43, 44].

B. Hyaluronidase and the Vitreus

Hyaluronidase has been found in the human and animal vitreus, but at physiological pH in the range of 6.5–8.0, it has no activity [45]. Consequently, hyaluronan is not affected by degrading enzymes in the vitreus in vivo [31–35]. Highly purified ovine hyaluronidase (Vitrase® [46]) has been prepared for the treatment of vitreous hemorrhage in human eyes. No serious safety issues were reported after a single intravitreal injection of this enzyme. Iritis, manifesting as an acute, self-limited inflammation, was the most common adverse event [47, 48]. This agent failed FDA testing and is thus not indicated for intraocular use.

C. Hyaluronan and its Derivatives as Viscoelastics in Medicine

After more than 15 years of research and development to produce hyaluronan that could be used therapeutically in humans and animals, Balazs [49] published the first comprehensive report on this subject. Two conclusions could be drawn from this work: first hyaluronan could be purified in such a way that the fraction of the molecule that was responsible for the production of acute inflammation in animals and humans was removed and second the medical utility of hyaluronan is based on its mechanical properties of viscosity and elasticity, and thus its ability to be a physical barrier, rather than having a chemical effect. The first such non-inflammatory fraction of sodium hyaluronate (known by the acronym NIF-NaHA) was tested during the late 1960s for two medical applications: use in humans and animals (mainly horses) to treat traumatic and idiopathic osteoarthritis and for ophthalmic surgery in the anterior chamber and in the vitreus [49–52].

The concept of using hyaluronan for viscosurgery [53–55] in the eye was based on its rheological properties. Solutions

of hyaluronan and its derivatives have a very high viscosity at low shear (e.g., during the insertion of a plastic lens) where they can be used to make and maintain space, and they exhibit low viscosity and high elasticity at high shear, making it possible to deliver the solutions through narrow channels such as a small gauge needle with ease. In such cases the elastoviscous solution serves as a shock-absorbing body and serves to protect delicate tissues and cell layers, such as the corneal endothelium, the iris, and the retina [56]. In 1982, the first clinical symposium was organized [57] to review the clinical benefits of viscosurgery with Healon®, the name given by Balazs to the first viscosurgical tool made available for ophthalmic viscosurgery.

Abbreviations

FDA	Food and Drug Administration
kDa	Kilodaltons
mg	Milligrams
NIF-NaHA	Noninflammatory fraction of sodium hyaluronate
mL	Milliliters
nm	Nanometers
PVD	Posterior vitreous detachment
µm	microns

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