THE BIOCHEMICAL STRUCTURE OF MAMMALIAN VITREOUS

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The vitreous gel is a highly hydrated transparent extracellular matrix with a water content estimated to be between 98% and 99.7%.¹ Vitreous therefore contains a very dilute dispersion of structural macromolecules that include hyaluronan (HA), proteoglycans, collagens and non-collagenous proteins. Additionally, the vitreous contains non-structural proteins including serum components which, except for iron binding proteins (lactoferrin and transferrin), are normally present in much lower concentrations than those found in serum.^{2,3} The vitreous contains low numbers of cells – hyalocytes – that are confined to the vitreous cortex and the vitreous base.

This review will concentrate on the structural macromolecules found within the vitreous gel. An understanding of these macromolecules and their interactions will provide new insights into how the vitreous gel is formed, what biochemical changes occur during degenerative liquefaction (syneresis), the molecular mechanisms by which genetic defects lead to vitreoretinal disease and, ultimately, to new biochemical therapies for vitreoretinal diseases.

In general extracellular matrices contain two macromolecular components that have complementary functions. There are fibrillar structures that are composed predominantly of protein, and glycosaminoglycans that are composed of carbohydrate. The fibrillar structures provide the tissue with mechanical strength and transmit tractional forces whereas the glycosaminoglycans inflate the matrix and space the fibrils apart.⁴ In vitreous the predominant fibrillar structures are collagen fibrils, but vitreous also contains fibrillin-containing microfibrils. Addition-

ally, most tissues, including the vitreous, contain a variety of other structural proteins and proteoglycans that are thought to contribute towards the formation and maintenance of this composite structure. Along with salts and water these structural macromolecules variously interact to determine the physical properties of the matrix.

GLYCOSAMINOGLYCANS

Glycosaminoglycans are long carbohydrate chains composed of repeating disaccharide (two sugar) units. All glycosaminoglycans, with the exception of HA, are attached to a protein core and the resultant molecule is called a proteoglycan. Following synthesis of a proteoglycan, its constituent glycosaminoglycan chains are variably modified by processes including sulphation, epimerisation and deacetylation prior to secretion into the extracellular environment.

The predominant glycosaminoglycan of mammalian vitreous is HA, but in other species, for example in chick vitreous, the predominant glycosaminoglycan is chondroitin sulphate.^{5,6} HA is composed of the repeating disaccharide unit $[\beta 1-4\text{glucuronic acid } \beta 1-3N\text{-acetylglucosamine}]_n$. This initial biosynthetic product is not modified before being secreted into the extracellular environment.

The overall concentration of HA in adult human vitreous has been estimated to be between 65 and 400 $\mu\text{g/ml}$ ⁷ and in adult bovine vitreous to be 430–555 $\mu\text{g/ml}$.⁸ However, the HA is not uniformly distributed throughout the vitreous, higher concentrations being present in the posterior vitreous and relatively lower concentrations in the anterior vitreous.^{9,10} Furthermore, there appears to be an age-dependent change in the concentration of HA, young animals having a lower concentration than adult animals.^{9,11} There are similar age-related changes in human vitreous, the concentration of HA increasing up to about the age of 20 years and remaining fairly constant until approximately 70 years when it again increases.¹² The HA of vitreous

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is polydisperse in molecular weight, but various estimates have been made of the average molecular weight in different species. The molecular weight of HA in human vitreous has been reported as 2–4 million.¹² The molecular weight of bovine vitreous HA has been investigated much more extensively and has been shown to be widely polydisperse, values between 1.7 million and 77 000 being obtained in one study.¹³ Furthermore, values for the average molecular weight have varied widely depending upon the methodologies used for isolation of HA and for molecular weight determination. By gel filtration chromatography, a weight-averaged molecular weight of 3 million in the newborn calf decreasing to 800 000–500 000 in old cattle was obtained.⁸ However, an average molecular weight value of as low as 63 000 was obtained for adult bovine vitreous by labelling the reducing end of HA with ¹⁴C followed by analysis by gel filtration and viscosity measurement.¹⁴ Clearly, widely varying values have been obtained, but estimates for the weight-averaged molecular weight of adult bovine vitreous HA have tended to fall in the range of 800 000 to 300 000.

Biophysical data have shown that HA can adopt a random coil configuration in solution,¹⁵ and it has been suggested that HA molecules intertwine at low concentration and become entangled at higher concentrations.¹⁶ Evidence for weak HA–HA interactions has been obtained^{17,18} and such interactions contribute towards the biophysical properties of HA solutions. Recently, a model has been proposed whereby combinations of hydrophilic and hydrophobic interactions allow lateral aggregation of hyaluronan monomers into extended networks of sheets and tubular structures.¹⁹ Networks can be observed by rotary shadowing electron microscopy of hyaluronan in aqueous solution¹⁹ and of the HA of human and bovine vitreous.²⁰ However, in vitreous globular proteins were frequently seen at intersections in this HA network and it was suggested that these proteins may regulate network assembly or stabilise tertiary structure.²⁰ Our laboratory is currently attempting to isolate and identify these putative HA binding proteins.

COLLAGENS

The vitreous gel contains a low concentration of collagen estimated to be approximately 60 µg/ml in bovine eyes and approximately 300 µg/ml in human eyes.²¹ The collagen concentration is not uniform throughout the vitreous, the highest concentration being present at the vitreous base. The concentration then decreases towards the central and posterior part of the vitreous but increases in the peripheral cortical layer.²¹ The collagen concentration decreases during embryonic development in foetal bovine eyes but remains fairly constant after birth.⁹ In human

vitreous the collagen concentration decreases during the first few years of life but this parallels eye growth and so the total amount of collagen does not change significantly.¹² It has been suggested that since the total amount of collagen does not change during eye growth or adulthood there is no turnover of vitreous collagen and the same pool of collagen is present throughout life.¹² However, the identification of procollagen in adult bovine vitreous²² and evidence for an increased proportion of immature cross-links in adult bovine vitreous as compared with cartilage²³ suggest that there may be active turnover of vitreous collagen throughout life.

Major collagen fibrils have been identified in vitreous by electron microscopy and estimates of their diameter vary between 10 and 20 nm.^{12,24} These collagen fibrils are relatively thin compared with the major collagen fibrils in other tissues and do not branch.^{12,20}

Currently at least 18 different types of collagen molecule have been identified (collagen types I–XVIII).²⁵ All collagen molecules are composed of three polypeptide chains (α -chains). These α -chains contain collagenous regions which possess a characteristic amino acid sequence with glycine at every third residue while the other two residues frequently comprise the amino acids proline or hydroxyproline. The three chains (each having this amino acid sequence) wind around each other to form a collagenous triple helix. All collagen molecules also have non-collagenous regions. These non-collagenous regions are always present at the ends of the molecule but may additionally be interspersed between collagenous regions. In the case of the fibrillar collagens such as types II collagen the non-collagenous regions are short 'telopeptides' at either end of the triple helical region but, in molecules such as type VI collagen, the non-collagenous regions make up more than half of the molecular weight of the collagen molecule.

The major collagen fibrils of vitreous are heterotypic (i.e. they contain more than one collagen type) and they are formed from collagen types II, V/XI and IX (Fig. 1). The major heterotypic collagen fibrils of cartilage and nucleus pulposus are closely related to those of vitreous, containing collagen types II, XI and IX. Collagen types II and V/XI are fibrillar collagens that align in staggered arrays and then cross-link together to form long thin fibrils. By contrast, type IX collagen belongs to the FACIT (fibril-associated collagens with interrupted triple helices) group of collagens and has been shown to be regularly aligned (in a D-periodic distribution) along the surface of the major collagen fibrils.

Type II Collagen

The predominant collagen in vitreous is type II collagen, accounting for approximately 70–80% of

Fig. 1. A schematic representation of the structure of the major heterotypic collagen fibrils of vitreous (not to scale). This proposed structure is largely speculative and based upon current knowledge of the structure of the constituent collagen molecules and how they assemble to form fibrils. (Drawing by Unit of Art in Medicine, University of Manchester.)

the total collagen.²⁶ It is composed of three identical α -chains [$\alpha 1(\text{II})_3$]. The type II collagen molecules are covalently cross-linked together in a 'quarter staggered' array to form the bulk of the major heterotypic collagen fibrils. When type II collagen molecules are secreted into the extracellular environment they are in a soluble precursor form: the procollagen. The procollagen has terminal extensions called the N-propeptide and the C-propeptide (Fig. 2). Once within the extracellular environment, however, the procollagen is 'processed' by specific enzymes called N-proteinase and C-proteinase that cleave off the N- and C-propeptides respectively. This process reduces the solubility of the collagen and allows it to participate in fibril formation.

It has been shown that the N-propeptide of type II collagen undergoes alternative splicing at the mRNA level where exon 2 is, or is not, expressed. In mature cartilage exon 2 of the type II collagen gene is not expressed resulting in a short N-propeptide. By contrast, in vitreous both splice variants are expressed, but the long form predominates resulting in an extended N-propeptide.²² The significance of these findings is not known. However, it has been demonstrated *in vitro* that incompletely processed type II collagen (with a retained N-propeptide) can be incorporated into fibrils.²⁷ Thus a proportion of the type II collagen molecules incorporated into collagen fibrils *in vivo* may possess an N-propeptide and this is assumed to protrude from the surface of the fibril. This retained N-propeptide may play a role in control of fibril diameter and/or in the interactions

of the collagen fibril with other extracellular matrix components. The different splice variants of the N-propeptide may have different functional properties in these respects.

Genetic analysis has shown that a proportion of patients with Stickler syndrome possess defects in the type II collagen gene (COL2A1) and this is frequently in the form of premature stop codon.²⁸⁻³⁰ Linkage to COL2A1 has also been established in one family with Kniest dysplasia.³⁰ A number of other chondrodysplasias in which ocular abnormalities are not described have been linked to

Fig. 2. Schematic diagram of the structure of type II procollagen. Thick lines represent triple helical regions of the molecule and thin lines non-collagenous regions. Short non-collagenous telopeptides (TP) remain after the molecule is fully processed. In some instances incomplete processing may take place, resulting in molecules with a retained N-propeptide being incorporated into the collagen fibril; it is thought that this retained N-propeptide would be present on the surface of the fibril.

COL2A1, including the lethal achondrogenesis and hypochondrogenesis, spondyloepiphyseal dysplasia and familial osteoarthritis/mild chondrodysplasia.³¹

Type V/XI Collagen

The major collagen fibrils of vitreous are thought to have a central core composed of collagen type V/XI (Fig. 1). Two of the three α -chains of this collagen were shown to be $\alpha 1(\text{IX})$ and $\alpha 2(\text{V})$ in bovine vitreous, but the composition of the third chain is unknown.³² That is to say one of these α -chains is a gene product conventionally associated with type XI collagen and the other with type V collagen. By contrast, the type XI collagen of cartilage is composed of the α -chains $\alpha 1(\text{XI})$, $\alpha 2(\text{XI})$ and $\alpha 3(\text{XI})$. Collagen types V and XI are very similar in structure and function and there are other examples of tissues containing mixtures of α -chains derived from these collagens. Therefore, it has recently been suggested that these two collagens should no longer be regarded as distinct entities, but that they should all be regarded as being part of the type V/XI collagen family.³³

The $\alpha 1(\text{XI})$ chain of type V/XI collagen exists in a complex series of splice variants within the N-propeptide.³³ This N-propeptide is not cleaved by an N-proteinase (the enzyme that removes the N-propeptide from, for example, type II collagen). The retained N-propeptide probably protrudes from the surface of the fibril (while the triple helical portion of the molecule is buried within the fibril) and may be involved in the control of fibril diameter and the interaction of the fibril with other extracellular matrix components.

Linkage to the COL11A1 gene³⁴ (which produces the $\alpha 1(\text{XI})$ chain) and to the COL11A2 gene³⁵ (which produces the $\alpha 2(\text{XI})$ chain) has recently been established in two different pedigrees with Stickler syndrome. As might be predicted from the biochemical analyses the family with a defect in COL11A1 had vitreoretinal disease (as the $\alpha 1(\text{XI})$ chain is found in vitreous and cartilage) whereas the family with a defect in COL11A2 did not have abnormal vitreous (as the $\alpha 2(\text{XI})$ chain is found in cartilage but is not present in vitreous).

Type IX Collagen

The third type of collagen molecule found in the major collagen fibril of the vitreous is type IX collagen. This collagen is composed of three different α -chains: $\alpha 1(\text{IX})$, $\alpha 2(\text{IX})$ and $\alpha 3(\text{IX})$. Type IX collagen has a rather more complex structure than that of the collagens described above as it possesses three collagenous regions (COL1, COL2 and COL3) interspersed between four non-collagenous regions (NC1, NC2, NC3 and NC4) (Fig. 3). Unlike collagen types II and V/XI, this collagen is not cross-linked

within the major heterotypic fibril but is regularly distributed along, and is cross-linked onto, the surface of the fibril.^{36–38} COL1 and COL2 are thought to be aligned parallel with the surface of the collagen fibril and COL2 provides sites for cross-linking to type II collagen molecules.³⁸ However, rotary shadowing electron microscopy has demonstrated that NC3 acts as a hinged region allowing COL3 to extend in a strut-like manner away from the surface of the fibril with the NC4 domain attached to the end of this strut.³⁶ In cartilage a large globular NC4 domain can be seen at the end of this strut but in vitreous the large globular NC4 domain is not visible.^{36,37} The large globular NC4 domain of cartilage type IX collagen is almost entirely composed of an extended $\alpha 1(\text{IX})$ chain. By contrast, the $\alpha 1(\text{IX})$ chain of vitreous type IX collagen is short owing to the use of an alternative transcription start site and differential splicing; this results in a small NC4 domain.^{39–42} The potential significance of these findings is that the large globular NC4 domain of cartilage type IX collagen is highly positively charged (pI 10.4 in human cartilage type IX collagen) and may be capable of interaction with other matrix components, whereas the small NC4 domain of vitreous is not highly charged in physiological conditions.

A second important feature of type IX collagen is that it can exist in a proteoglycan form, i.e. a sulphated glycosaminoglycan chain may be covalently linked to the $\alpha 2(\text{IX})$ chain at the NC3 domain. In cartilage this glycosaminoglycan chain is only sometimes present. However, type IX collagen in vitreous always possesses a chondroitin sulphate glycosaminoglycan chain.^{26,42} Human vitreous gel was analysed by electron microscopy after staining with the cationic dye Cupromeronic blue and

Fig. 3. Schematic diagram of the structure of type IX collagen showing each of the three α -chains individually. Thick lines represent collagenous regions (COL1, COL2 and COL3) and thin lines non-collagenous regions (NC1, NC2, NC3 and NC4). The dotted line represents an extension of the $\alpha 1(\text{IX})$ chain in the NC4 domain that is present in cartilage but not present in vitreous (see text). Asterisks show the position of cross-links to type II collagen.

sulphated glycosaminoglycans were shown to be regularly distributed along, and extending away from, the surface of the major collagen fibrils.⁴³ Similar results were obtained in our laboratory for bovine vitreous with the cationic dye Alcian blue (unpublished observations). These sulphated glycosaminoglycans are partially or exclusively derived from type IX collagen proteoglycan. They sometimes appeared to bridge adjacent collagen fibrils and it was suggested that this appearance was due to weak interactions between chondroitin sulphate chains derived from adjacent collagen fibrils.⁴³ This interesting hypothesis provides one explanation of how the collagen fibrils of vitreous are spaced apart. However, such studies should be interpreted with caution as the sulphated glycosaminoglycans are only visible by electron microscopy because they are collapsed onto the cationic dye, whereas *in vivo* they are highly hydrated, occupy a comparatively large volume and would participate in different intermolecular and intramolecular interactions.

Type VI Collagen

We have recently demonstrated the presence of another type of collagen fibril in vitreous that is composed exclusively of type VI collagen.⁴⁴ These type VI collagen microfibrils have been identified by their characteristic appearance on rotary shadowing electron microscopy of human and bovine vitreous and by Western blotting of an extract from bovine vitreous for type VI collagen. Type VI collagen microfibrils are probably present in smaller amounts than the major heterotypic collagen fibrils but may nonetheless be important structural components within the vitreous as they can bind type II collagen and may be able to interact with hyaluronan.

NON-COLLAGENOUS GLYCOPROTEINS/PROTEOGLYCAN

The vitreous contains a number of other structural glycoproteins and proteoglycans but very few of these have been identified or characterised. Vitreous has been shown, by rotary shadowing electron microscopy, to contain fibrillin-containing microfibrils.^{37,45} The zonules appear to be exclusively composed of bundles of these microfibrils. Fibrillin-containing microfibrils are currently of particular interest as defects in the fibrillin gene (fibrillin being the major structural molecule of these microfibrils) have been directly implicated in Marfan syndrome. The structural role of these microfibrils in vitreous currently remains unclear.

A glycoprotein that is identical to, or very similar to, cartilage oligomeric matrix protein (COMP) has been identified in vitreous by Western blotting.⁴⁶ COMP is an acidic glycoprotein with a characteristic five-armed structure that has also been identified in

cartilage and tendon.⁴⁷ Little is known about the function of this molecule or its role in the structure of vitreous.

MACROMOLECULAR ASSEMBLY AND AGE-RELATED LIQUEFACTION OF VITREOUS

Human vitreous at birth is in the form of a gel, but with age this gel undergoes progressive liquefaction.¹² This process of liquefaction is evident after the age of 4 years and by the time the eye reaches full size (14–18 years of age) approximately 20% of the vitreous cavity is liquid. By the age of between 80 and 90 years more than 50% of the vitreous is liquefied.

Collagen fibrils are clearly an essential component of the vitreous gel, as removal of collagen fibrils by high-speed centrifugation or collagenase digestion results in the liquefaction of vitreous. During vitreous liquefaction the collagen fibrils remain within the gel component, i.e. the collagen fibrils aggregate together in the residual gel leaving areas of liquefied vitreous (lacunae) devoid of collagen fibrils.^{12,48} For most of adult life the concentration of HA in human vitreous is the same in both the liquid and gel compartments.¹² However, the concentration of HA increases in the liquid compartment after the age of 70 years while remaining unchanged in the gel compartment. The molecular weight of HA in the liquid and gel compartments was reported as being the same.¹²

CONCLUDING REMARKS

The vitreous is a highly specialised extracellular matrix that is of fundamental importance in the pathogenesis of a number of common eye disorders including rhegmatogenous retinal detachment, macular hole formation and the vasoproliferative retinopathies. However, remarkably little is known about the structural macromolecules of the vitreous gel (other than hyaluronan and the collagens), how these macromolecules interact to form the vitreous gel and how these interactions are altered by genetic abnormalities or during the degenerative liquefaction associated with ageing. Efforts to understand these molecular mechanisms will not only reap rewards in the understanding of these disease processes but also will allow the development of biochemical agents that can be used therapeutically to create a 'chemical vitrectomy'.

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