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Clinical uses of hyaluronan*

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Abstract. The availability of elastoviscous solutions of highly purified hyaluronan has created two new therapeutic methods in human and veterinary medicine: viscosurgery and viscosupplementation. Viscosurgical tools and implants are widely used in ophthalmology and have been suggested for use in otology. Viscosupplementation of joint fluid using elastoviscous hyaluronan solutions is widely used in the treatment of equine traumatic arthritis. It was also suggested for use in idiopathic osteoarthritis in humans, but this application has not received wide acceptance. Cross-linked forms of hyaluronan have been developed and given the generic name of hylans. Water-insoluble soft gels of hylans are ideally suitable as viscosurgical implants to prevent postoperative adhesions and to control scar formation. Hylan solutions are being used in arthroscopic viscosurgery. Hylan devices in various forms (gels, tubes, membranes) have been used in animal studies for matrix engineering, the purpose of which is to control and direct tissue regeneration and augmentation.

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In this paper we shall review past and present efforts to use hyaluronan as a therapeutic agent in human and veterinary medicine and explain the rationale behind its use. Our presentation will be limited to two areas of medical application—viscosurgery and viscosupplementation. We shall also discuss the reasons why cross-linked derivatives of hyaluronan were recently developed and will review their intended clinical uses.

Viscosurgery

Viscosurgery is the surgical procedure in which an elastoviscous surgical tool or implant is used to help carry out the objectives of the surgery. This new surgical procedure was developed during the 1970s (Balazs et al 1972, 1979, Balazs 1983). This development was directly related to the introduction of the

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*Paper presented by Professor T. C. Laurent, in Professor Balazs' absence.

first elastoviscous solution of a highly purified, high molecular mass sodium salt of hyaluronan in eye surgery.

Viscosurgical tools

Viscosurgical tools are applied during the surgical procedure and can act as space-makers to separate tissues and create the space for surgical manipulations and for the insertion of implants. They can serve as tissue protectors, that is, shock absorbers or elastoviscous coatings and lubricants for cell layers that are sensitive to mechanical damage caused by surgical instruments or implants. They act as soft surgical instruments with which to manipulate tissues, to move tissue debris or blood clots and to separate tissue adhesions. During surgery the elastoviscous solution or gel represents the medium in which the surgical procedure is carried out.

Viscosurgical implants

The benefits of viscosurgery go beyond the surgical procedure itself. Viscosurgical implants, when left in the site of surgery, will exclude blood cells and fibrinogen from the place where they are applied, thereby decreasing the possibility of adhesion formation. They form transparent molecular barriers between two tissue surfaces that must remain separated in order to function properly. Viscosurgical implants can also restrain postoperative bleeding and exudation, thereby aiding the normal healing process.

Mode of action and biocompatibility

Viscosurgical tools and implants function by their mechanical properties. The most important physical parameters that define their efficacy are viscosity, elasticity, plasticity or pseudoplasticity, and their permeability to molecules of various sizes. A viscosurgical tool or implant cannot have any diffusible component which is toxic or causes immunological reaction or inflammation. The inflammatory process that accompanies humoral or cellular immune reactions or foreign body reactions is detrimental and counter-productive to the objectives of viscosurgery. Therefore it is extremely important that the viscosurgical tool or implant is highly biocompatible with the tissues to which it is applied.

The use of hyaluronan in viscosurgery

The use of sodium hyaluronan for viscosurgery is a logical choice because of its biocompatibility and its unique rheological properties. The biocompatibility is understandable from the fact that this molecule in a very high polymeric form (relative molecular mass 4–6 million) is present at various concentrations in all connective tissue matrices of primates. Therefore its viscosurgical use in various connective tissue spaces represents only a temporary increase in the concentration of the native hyaluronan. Its biocompatibility is assured, provided that three criteria are met. First, during the purification process, all proteins that potentially can cause antibody formation must be removed. If the hyaluronan is prepared by bacterial fermentation, the removal of bacterial toxins and bacterial wall components is essential. Second, during the purification procedure, several potentially toxic chemicals are used which must be removed. Third, the hyaluronan molecules that are associated with the chemotactic agent must be removed. Thus, only the non-inflammatory fraction of Na-hyaluronan (NIF-NaHA) can be used for any medical application (see for review Balazs 1979, 1983, 1986, 1988). Hyaluronan has unique rheological properties; at a relatively low concentration ($\approx 0.02\%$) the molecules form a continuous molecular network which is extremely viscous at very low shear rates ($< 0.01\ s^{-1}$) and very elastic at relatively high frequencies (> 1 Hz). The extremely high shear rate dependency of the hyaluronan solution is called 'pseudoplasticity', because at low shear rates it behaves as a soft gel (plastic), yet it does not have a real yield point as soft gels have. With increasing shear rate, the viscosity drops dramatically because the molecules deform (change shape) under shear forces.

These rheological properties of high polymeric ($M_r > 4$ million) hyaluronan dissolved in physiological buffer at low concentration ($\leqslant 1\%$) are unique and not matched by any other natural or synthetic polymer. These characteristics, combined with its obvious biocompatibility, make NIF-NaHA the ideal viscosurgical tool and implant. The efficacy of a viscosurgical implant depends on its mechanical properties as well as its residence time at the site of application. The residence time of NIF-NaHA (1% solution, M_r 1–4 million) was carefully studied in various tissues, such as the vitreus and anterior chamber of the eye, and in the joints (Denlinger et al 1980, Denlinger 1982, Schubert et al 1984). We found that the half-life time of the exogenous hyaluronan depends on molecular size and is the same as the regeneration time of endogenous hyaluronan. We also found that the half-life time of NIF-NaHA in the vitreus and the anterior chamber of owl monkey eyes was similar to that found in rabbit eyes using exogenous radiolabelled hyaluronan prepared from fibroblast cultures (Laurent 1982, Laurent & Fraser 1983). Thus the use of NIF-NaHA as a viscosurgical implant is limited by its residence time, which is similar to that of the endogenous hyaluronan in the same tissue compartment.

Hyaluronan in viscosurgery of vitreo-retinal diseases

The use of hyaluronan as a viscosurgical tool and implant was first suggested for the reattachment of the detached retina and for the replacement of the fibrous pathological vitreus (Balazs 1960, Balazs et al 1972). Viscosurgical objectives in so-called posterior segment (vitreus and retina) surgery are to push the detached retina back to its normal position and keep it there until the healing process between the pigment epithelium and the retina is complete. It is also used as a 'viscosurgical vice' to separate preretinal tissue membranes, to replace the vitreus with transparent inert medium and to regulate the intraocular pressure postoperatively (for reviews of clinical use see Meyer-Schwickerath 1982, Miller & Stegmann 1983, Eisner 1986, Rosen 1988).

Hyaluronan in viscosurgery of the anterior segment of the eye

Anterior segment surgery consists of procedures carried out in connection with corneal transplantation, cataract surgery, intraocular lens implantation and glaucoma surgery. The first application of hyaluronan was in corneal transplantation, when elastoviscous hyaluronan solution was used to protect the corneal endothelium from mechanical damage. The aim of using NIF-NaHA in corneal transplantation is to make the trephination of the recipient's cornea less traumatic, to break adhesions or synechiae, and to aid in the introduction of the donor cornea and protect its endothelial cell layer (Balazs et al 1972, Edmund 1976, Graue et al 1979, Polack et al 1981).

The widest use of NIF-NaHA is in connection with the removal of a cataractous lens and its replacement with a plastic intraocular lens. The elastoviscous solution serves as a space-maker by maintaining the anterior chamber during surgery; it acts as a cushion, protecting the endothelial cell layers (cornea, lens, iris) from mechanical damage by instruments and the plastic implant itself. It can be used as a soft instrument with which to dilate the pupil and to block capillary bleeding (Balazs et al 1979, Miller & Stegmann 1980, 1981).

Viscosurgical tools have been used in glaucoma surgery to help maintain the anterior chamber and to separate the tissues. NIF-NaHA as a viscosurgical implant helps to reduce scarring between the conjunctiva and the sclera and has proved to be an invaluable tool in the repair and reconstruction of traumatic injuries of the eye. For further details of the viscosurgical uses of hyaluronan in the anterior segment, the reader is referred to four books on this subject (Meyer-Schwickerath 1982, Miller & Stegmann 1983, Eisner 1986, Rosen 1988).

Hyaluronan as a viscosurgical implant in joints and tendons

In animal models, viscosurgical implants improved the healing process of damaged articular cartilage, decreased scar formation in subcutaneous wounds and reduced adhesion formation between traumatized tendons and tendon sheaths. Foreign body reactions around plastic implants could be reduced by the application of elastoviscous hyaluronan solutions around the implant

(Rydell & Balazs 1971, St. Onge et al 1980). Since non-elastoviscous solutions of hyaluronan did not produce the same effect, we suggested that the molecular network of hyaluronan forms an effective barrier for fibrinogen and blood cells. Because fibrinogen from the tissue exudate cannot penetrate the dense molecular network of hyaluronan, fibrin coagulum connecting the surfaces cannot form. Consequently, the scaffolding for fibrous tissue formation is absent, and the formation of adhesions or scar tissue is prevented. NIF-NaHA does not interfere with the clotting mechanism but, by its mechanical properties, prevents the formation of fibrin clots connecting wound surfaces. The viscosurgical implant does not interfere with wound healing, provided it is not used as a space separator between the healing surfaces.

Hyaluronan in viscosurgery of the ear

Animal experiments showed that elastoviscous solutions of NIF-NaHA, when used as viscosurgical implants during the healing of perforated tympanic membranes, influenced the healing process favourably. The healing was faster and the scar formation was reduced (Hellström & C. Laurent 1987). Absorbable gelatin sponges—commonly used in otosurgery—saturated with NIF-NaHA decreased the formation of fibrous tissue, which is known to develop around these sponges (C. Laurent et al 1986). These results confirmed much earlier findings that elastoviscous solutions of NIF-NaHA promote wound healing and decrease scar tissue formation and granulation around foreign bodies (Rydell & Balazs 1971). The viscosurgical use of NIF-NaHA implants to aid middle ear surgery and the healing of tympanic membranes has been advocated by Claude Laurent (1988). Some preliminary clinical studies have given further encouragement to the use of viscosurgical implants in otosurgery (Stenfors 1987a,b).

Viscosupplementation

Viscosupplementation is also a new medical concept. When the normal viscoelasticity of a solid tissue compartment or the elastoviscosity of a liquid tissue compartment is decreased under pathological conditions, the normal functioning and the regeneration processes in such tissues are impaired. 'Viscosupplementation' designates the therapeutic process by which the normal rheological state of such compartments is restored or augmented by introducing viscosupplementary devices. These devices stay in the tissue compartment for various periods of time, depending on the nature of the viscosupplement and the physiology of the tissue compartment.

Mode of action of viscosupplementation in the joint

The elastoviscosity of the synovial fluid of the joint is entirely due to its hyaluronan content. The elastoviscous hyaluronan permeates the lamina splendens of the articular cartilage surface and the intercellular matrix of the synovial tissue and capsule. The collagen matrix of the intercellular space is filled with viscoelastic hyaluronan solution. The movement of the joint generates a flow of synovial fluid which maintains a continuous exchange of fluid between these tissues. Therefore, the elastoviscous properties of the synovial fluid reflect the rheological properties of the intercellular matrices (lamina splendens, synovial tissue, capsule, intraarticular ligaments). In human and equine arthritis and arthrosis (chronic or acute), the elasticity and viscosity of the synovial fluid are much lower than in the normal joint. The cause of this decrease in rheological properties is twofold. First, the molecular size of the individual hyaluronan molecules is smaller and, second, as a result of exudation, the concentration of hyaluronan is lower. Because of these inferior rheological properties, the protective, shock-absorbing and barrier properties of the hyaluronan in the intercellular matrix are diminished. The central hypothesis of the role of hyaluronan in joint pathology is that this leads to severe alteration of the matrix and disturbance of cell function. Under the mechanical stress caused by the movement of the joint, fibrillation and dislocation of the collagen network of the cartilage surface and the synovial tissue occur. The folding and stretching of the synovial membrane during joint movement can damage the hyaluronan-producing cells. The mechanically induced pain receptors (nociceptors) in the capsule thus are unprotected because of loss of the shock-absorbing effect of the elastoviscous matrix surrounding them.

Viscosupplementation restores the normal rheological environment of the network of collagen fibres and cells and instantly provides protection, shock absorption and a barrier effect. In other words, the supplementation and augmentation of the rheological properties supplies an elastoviscous shield under which the regeneration may occur. The efficacy of viscosupplementation depends on the rheological properties of the elastoviscous device and its residence time in the joint (for review see Balazs 1974, Balazs & Gibbs 1970, Balazs et al 1981, Denlinger 1982, Balazs & Denlinger 1984, 1985a).

Hyaluronan in joint viscosupplementation

The high molecular mass, non-inflammatory fraction of Na-hyaluronan (NIF-NaHA) was first used as a viscosupplement in the joints of dogs after the cartilage was artificially injured. NIF-NaHA was then introduced into the joint, creating an elastoviscous shield over the damaged cartilage. Under this shield the cartilage wound healed, the inflammatory reaction of the synovial tissue decreased and the capsular pain was reduced (Rydell & Balazs 1971). Similar observations were made on the natural traumatic arthritis of race horses. The painful arthritic condition of the joint could be reduced, and the normal function of the joint was restored, after viscosupplementation of the synovial fluid with NIF-NaHA (Rydell & Balazs 1971, Rydell et al 1970; for review see Balazs & Denlinger 1985b). NIF-NaHA was the first commercially available and widely sold viscosupplement for equine joints. Less elastoviscous and less pure hyaluronan solutions are now also available for viscosupplementation in equine arthritis.

In the early 1970s these studies were extended to human osteoarthritic knee joints. Several clinical investigations showed that NIF-NaHA decreased the pain and restored the painless function of the knee joint in severe, acute idiopathic osteoarthritis (osteoarthrosis). This effect, however, did not last longer than 6–8 weeks, and idiopathic osteoarthritic joints with associated chondromalacia did not respond to viscosupplementation (Balazs 1974, Peyron & Balazs 1974, Weiss et al 1981, Balazs & Denlinger 1985a). Elastoviscous hyaluronan solution as a viscosupplement for the treatment of human idiopathic arthritis is available in some countries; however, its use has not gained general acceptance. This is because the solutions presently available for viscosupplementation do not remain long enough in the joint and their rheological properties are not sufficient to provide a long-lasting protective viscoelastic shield for the joint tissues.

The use of cross-linked hyaluronan in viscosurgery, viscosupplementation and matrix engineering

The native, pure hyaluronan fraction (NIF-NaHA) even in its largest polymeric form ($M_r \approx 6$ million) and in a relatively high concentration ($\approx 2\%$) did not provide the rheological properties needed for the efficacious use of viscosurgical tools and implants in certain medical conditions. For example, in vitreoretinal diseases a more solid viscosurgical implant was needed which remained in the eye for a longer period than the native hyaluronan. In glaucoma surgery the 2% NIF-NaHA did not provide the solidity needed to maintain the anterior chamber and prevent the scarring down of the filtration bleb. Most importantly, NIF-NaHA solutions did not provide sufficiently solid and long-lasting separation of connective tissue surfaces to efficiently prevent formation of adhesions and scar formation and they did not stay long enough in the tissues of the joint to provide the most effective viscosupplementation for this tissue.

By using two distinctly different cross-linking processes, we produced hyaluronan derivatives in which the two otherwise unaltered hyaluronan chains are permanently bridged by the cross-linking agent. The resulting molecular entity, which has significantly altered rheological properties, we called hylan. This generic name designates hyaluronan molecular chains cross-linked with various methods without changing the characteristic carboxylic and *N*-acetyl groups of the polysaccharide chain. In one process the hylan molecules are cross-linked with formaldehyde through a specific protein bridge (Balazs et al 1987). The total protein content of hylan is less than 1% of the polysaccharide content. The resulting molecular entities can have an average M_r up to 25 million. Solutions of hylan have distinctly different rheological properties from solutions containing comparable concentrations of hyaluronan of the highest molecular size. Hylan solutions have very high viscosity at low shear ($<0.01\ s^{-1}$) and very high elasticity at high frequencies (>1 Hz). They are also more pseudoplastic than hyaluronan solutions.

Another process produces unaltered hyaluronan chains bridged by sulphonyl diethyl cross-links. The resulting soft gel contains 0.3 to 0.7% solid, hydrated with 99.3–99.7% physiological salt solution. The rheological properties of slurrys made from this gel are distinctly different from those of hylan solutions (Balazs & Leshchiner 1985, 1986, 1987). Hylan solutions and gels are degraded by all the enzymes that degrade hyaluronan, and their catabolic pathway is identical with that of the native hyaluronan molecule. Preclinical animal studies have shown that hylans are non-antigenic, non-toxic and non-inflammatory and do not elicit foreign body reactions.

The antiadhesion effect of hylan gels was tested after severe surgical trauma in the long toe extensor tendon of the rabbit (Weiss et al 1986, 1987). Gels with distinct rheological properties were extremely effective in diminishing adhesion formation between the tendon and tendon sheath without interfering with the healing of the tendon wound itself.

Exploratory clinical studies indicate that hylans are efficacious viscosurgical tools in arthroscopy of the joint. It was demonstrated that they protect the articular cartilage from scuffs caused by instruments during the surgical procedure. The lubricating properties of hylans help the movement and operation of motorized instruments. Viscosurgery with hylan facilitates the control of tissue movements, visualization during surgery and the collection of blood and tissue debris. The benefits of arthroscopic viscosurgery were demonstrated in the knee joint, but they are especially pertinent in small joints such as the temporomandibular joint. Preliminary clinical studies in equine and human arthritis indicate that hylans are well suited for viscosupplementation in the arthritic joint.

The development of water-insoluble hylan has opened up a new field of medical application. The cross-linking process yields not only extremely elastoviscous solutions and soft gels, but also membranes, tubes, sleeves, envelopes and coatings for other non-natural biomaterials (dacron, polyurethane). Solid copolymers of hyaluronan with other carbohydrates (cellulose, gums, etc.) and proteins (collagens) were developed. These various forms of hylans maintain the original high biocompatibility of the native hyaluronan and improve the biocompatibility of other polymers. Connective and neural tissues, epithelium and blood cells react differently to various hylan devices. Some cells find support on the surface of the hylan devices; others do not attach to them. Connective tissue capsule formation around hylan depends on the size, permeability and surface characteristics of the device. Hylan devices therefore can be used to engineer the intercellular matrix for tissue regeneration and augmentation.

Matrix engineering with hylan devices is in its early exploratory stage. Studies in tissue culture and in various animal species and preliminary clinical investigations indicate that chemically modified and cross-linked hyaluronan provides a novel tool for a broad spectrum of therapeutic applications in medicine.

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DISCUSSION

Claude Laurent: Dr Balazs referred to our studies on the use of hyaluronan in middle ear surgery. Before hyaluronan could be used in humans it was necessary to do studies in animal models. The rat is a suitable model for middle ear research because of the many similarities to the human middle ear. The tympanic membrane consists of a larger acoustic part, the pars tensa, rich in collagen fibres, and a smaller upper part, the pars flaccida. The pars tensa has an outer layer of keratinizing stratified squamous epithelium, a middle fibrous connective tissue layer (containing predominantly collagen fibres arrayed in a typical pattern of inner circular and outer radial fibres) and an inner thin mucosal layer facing the middle ear cavity. Our research group has been studying the occurrence of endogenous hyaluronan in normal and diseased middle ears. We have also studied the fate and effects of hyaluronan applied in the middle ear.

Significant concentrations of HA were found at all tissue sites in the normal rat middle ear, and in the pars flaccida the concentrations were some 20–30-fold higher than in other tissue sites. This is interesting, as the pars flaccida is structurally different from the rest of the tympanic membrane, for example in fibre composition, occurrence of mast cells and innervation. The two parts of the tympanic membrane also differ in their involvement in middle ear pathology.

When hyaluronan (tritium-labelled Healon) was applied in the rat middle ear with the Eustachian tube open, it was eliminated within 12 hours through the Eustachian tube. Radioactivity peaked after three hours in the nasopharynx and was almost zero after 12 hours. When the Eustachian tube was blocked with a small rubber plug, hyaluronan was neither absorbed in the middle ear nor degraded. So it is eliminated only through the Eustachian tube when applied in the rat middle ear.

The rat tympanic membrane has proved to be an excellent model for studying wound healing. It is extended in the air space and is easy to examine with an otomicroscope through the external auditory canal. You can make standardized wounds in this structure, where you always cut through the same tissue layers. It is also easy to apply substances to the wounded tympanic membrane. We have been studying, in addition to hyaluronan, the effects of fibronectin, carboxypolymethylene and hydrocortisone.

A fascinating aspect of this wound model is that the healing tissue has to bridge a gap over an air space: there is no underlying stroma over which the tissue can grow out. We have applied different preparations of hyaluronan to standardized wounds made in the upper posterior quadrant of the rat tympanic membrane, applying HA once daily until healing was observed. About half of the hyaluronan-treated tympanic membranes healed before the first wounded tympanic membrane in the control group closed. So the application of hyaluronan enhanced the healing rate. This effect depended on the concentration of HA (a higher concentration being superior to a lower one), not on its molecular mass or its viscosity.

We also graded the quality of scar area in the tympanic membrane. It was clear that scar opacity was better in the HA-treated groups than in the controls. In histological sections, the hyaluronan-treated tympanic membranes were thinner than the controls and the fibrous connective tissue layer was well restored, with abundant densely packed collagen fibres oriented parallel to the surface. We also saw a few fibroblasts with their long axis oriented parallel to the tympanic membrane surface. The improvement in scar quality was invariably obtained in the presence of hyaluronan, irrespective of its rheological properties.

Supporting substances are needed in middle ear surgery. Gelatin sponge (an artificial material made from a non-specific beef protein) has been used for almost 30 years for this purpose. However, many ear surgeons have found unexpected fibrous connective tissue changes after using this material. In our rat model, exogenous hyaluronan (Healon) mixed with gelatin sponge and put in the middle ear of normal rats induced much less fibrous connective tissue than the sponge alone. From our experimental data we can now suggest that HA be used in otosurgical procedures as an alternative to myringoplasty (a tissue transplantation to a perforated tympanic membrane). In Sweden we do about

3000 such operations a year, probably corresponding to about 21 000 in Britain annually. Hyaluronan, in a suitable form, should also be useful in supporting ossicular chain reconstructions, where we need a scaffold, and as a support for tympanic membrane grafts. It could counteract the formation of granulation tissue and adhesions in the middle ear and it may also promote re-epithelialization in wounded mucosa and skin. It may also enhance the biocompatibility of artificial implants that are sometimes inserted into the middle ear cavity.

Weigel: You get significant effects, including improvement in scar quality, independent of the rheological properties of the preparations. This must mean either that HA is interacting with something else, or that perhaps its angiogenic ability is involved. Would you like to speculate on what is going on here? To what extent have you looked at various HA sizes?

Claude Laurent: We have been examining events in the early stage after wounding the tympanic membrane and putting HA into the perforation gap. Histologically, in 1–3 days, we see a sheet of keratin and hyaluronan in the gap. Through this sheet, keratinocytes move straight through, without any supporting stroma under them. It appears that the hyaluronan–keratin sheet acts as a scaffold in the gap, and maybe it also promotes migration of cells right through it. An improved scar quality is obtained in the healed tympanic membrane, irrespective of the molecular mass and concentration of the HA, provided that the preparation has remained long enough in the perforation gap. (A low molecular mass, low concentration preparation disappears rapidly and then this effect is not seen.)

Weigel: How small is the average size of HA at which you still see the healing effect?

Claude Laurent: The smallest preparation used was a 1% solution of 700 000 Da hyaluronan. That is still a big molecule, but with a low viscosity.

Richard Margolis: In practice, hyaluronan is considerably less satisfactory as a vitreous replacement than just saline. Why should exogenous HA clog up the trabeculae and block aqueous outflow more than endogenous HA?

Balazs(comment added in proof): Since the early 1960s I have tried to find the proper elastoviscous substance for replacing the diseased vitreus. I have never heard or read that hyaluronan in its purest (non-inflammatory) form is less satisfactory than saline for vitreous replacement after vitrectomy and/or retinal surgery. It is true that hyaluronan preparations now available to the eye surgeons are not sufficiently elastoviscous to be of benefit in pushing back and holding the detached retina in place, and others are not sufficiently purified and thus cause inflammatory reactions in the eye. For this reason even the purest and most elastoviscous preparation of hyaluronan has not fulfilled the expectations of the eye surgeons, and only a few use hyaluronan as a vitreous substitute.

Torvard Laurent: The exogenous hyaluronan is so much more concentrated.

The normal vitreous body contains 0.2 mg HA per ml, whereas the HA used clinically (Healon) is 10 mg per ml—a 50 times higher concentration. Furthermore, the normal vitreous body is stabilized by a collagen network that keeps the hyaluronan as a gel. Healon does not have this gel character, so it just flows out from the vitreous cavity.

Balazs (comment added in proof): We showed that the half-life of hyaluronan in the anterior chamber of a primate (owl monkey) eye, depending on the viscosity of the solution, is one or two days and in the vitreus of two primate species (owl monkey and macaque) is 20–60 days. This difference is due to the different fluid flow conditions in the two tissue compartments. In the anterior chamber the residence time is controlled by the flow of the aqueus, which rapidly dilutes the injected hyaluronan and washes it out through the trabecular network to the blood. In the vitreus, where there is no fluid flow, HA diffuses through the collagen network barrier of the anterior segment of the vitreus into the posterior chamber, and from there flows with the aqueus to the anterior chamber. In other words, the residence time of hyaluronan, both endogenous and exogenous, is controlled by diffusion in the vitreus and by the flow of aqueus in the posterior and anterior chambers of the eye.

We have studied development and ageing in more than a thousand human and monkey eyes. We found no evidence that the so-called liquefaction of the vitreous gel is related to the degradation of the collagen gel or to changes in the concentration or molecular mass of the hyaluronan present in the vitreus. In primates (including human) the increase in volume of the liquid vitreus and the simultaneous decrease in volume of the gel vitreus is due entirely to the shrinkage (syneresis) of the collagen gel network. The reason for this shrinkage is not well understood.

Kuettner: Have anyone tried adding collagen fibres to the HA?

Torvard Laurent: This was Endre Balazs's first idea. He made a collagen solution at low temperature and injected it into the eye where, at 37 °C, it formed a gel. It was not transparent, however, so it could not be used.

Kuettner: Those experiments were done with type I collagen, but the eye is now known to contain type II collagen. Could you mix HA and type II collagen and inject a similar concentration as exists in the eye?

Torvard Laurent: That would probably work now.

Balazs (comment added in proof): My first idea in the 1960s was to mix heat-gelable (at 37 °C) tropocollagen with an elastoviscous solution of hyaluronan and use that as a 'reconstituted vitreus'. Using human umbilical cords as a source for both collagen and hyaluronan we found that (in monkey eyes) the gel formed in the eye was well tolerated. We could not find a physiological solution (pH, ionic strength and osmolarity) in which the *in vivo* collagen fibre formation was such that the gel formed was sufficiently transparent. In all cases the collagen fibres formed varied greatly in thickness. These fibres, which were much thicker than the native collagen fibres in the vitreus, scattered the light to such a degree that the image formed by the lens on the retina was distorted. After this failure we started to use pure elastoviscous solutions of hyaluronan.

Orkin: Dr Laurent, are you able to wound just one portion of the tympanic membrane—the pars tensa, as opposed to the pars flaccida? Is the wound a full or partial thickness wound? And are there differences in the results in relation to these variables?

Claude Laurent: We always use a full thickness wound of the pars tensa. The pars flaccida is more elastic and difficult to wound, but it is still possible to make standardized wounds there. They have not been much studied yet.

Orkin: You administered the hyaluronan through the external ear canal. Is there any evidence that hyaluronan would be of clinical use in less acute diseases of the tympanic membrane, such as otitis?

Claude Laurent: Our group has begun experimental studies of the effects of exogenous hyaluronan in otitis media. So far we cannot say whether it could be used to influence that condition.

Orkin: Is there any evidence that hyaluronate passes across the tympanic membrane?

Claude Laurent: It doesn't cross an intact tympanic membrane. Our Finnish colleague, Dr Stenfors, is treating patients with chronic tympanic membrane perforations with hyaluronan. About 60% of the patients with small or medium-sized dry perforations healed without surgery as a result (Stenfors 1987). We are now completing a clinical study of 64 patients with chronic tympanic membrane perforations who are given either treatment with HA once daily for seven days or more conservative treatment with a rice-paper patch over the perforation. We have not got the results yet.

Warren Knudson: Dr Balazs used to talk about making a gel out of pure HA by lowering the pH to 2.5. What is going on there? Does it go back to a liquid state if you return to neutral pH, or does it remain a gel?

Balazs (comment added in proof): The hylan gel I referred to is a truly cross-linked gel which has nothing to do with the interesting physical state of hyaluronan at pH 2.5. In 1966 we described the dramatically enhanced elastoviscosity at this low pH as a 'jelly' in order to avoid confusion of this rheological state with true gel formation. This jelly is not stable at any other pH than 2.5. The hylan gel we are talking about here has nothing to do with the hyaluronan jelly. Hylan gels are stable under physiological conditions and are also heat stable.

Fraser: I am interested, from a biological point of view, in the way the tympanic membrane heals itself so naturally with HA. I wonder what the functional result is. Can you do electroaudiograms on rats to see what the frequency response is, Dr Laurent? In other words, is that beautiful healing process retuning the membrane at the same time?

Claude Laurent: Before we can use a new substance in middle ear surgery we must know that it is not ototoxic and doesn't harm inner ear function. We applied high concentration HA repeatedly to the middle ears of rats and studied their hearing by brainstem audiometry. There was a transient effect on the auditory brainstem thresholds but it was reversible and no ototoxicity was found. In that study, we applied hyaluronan through a perforation of the tympanic membrane that healed in two or three days and the hearing after healing was unaffected. How it works in humans we shall see in a clinical study that we are doing now.

Reference

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General discussion

Torvard Laurent: Klaus Kuettner has suggested that we discuss free radical effects on hyaluronan, in view of new data that Mike Bayliss has.

Bayliss: We find that the age changes seen in human cartilage show a natural decrease in the size of hyaluronic acid, but only down to a molecular mass of 500 000, so there is a very limited clipping (Holmes et al 1988). It would be interesting to consider what mechanisms would result in such a limited reduction in molecular mass.

Kuettner: The point is that the hyaluronan synthesized is large (1.5 to 2×10^6 Da).

Torvard Laurent: How do you know that it is synthesized as 1.5 to two million dalton hyaluronan?

Bayliss: We don't know the exact size, only that it is excluded from S-1000. It is always very large. The newly synthesized HA could be different sizes at different ages; we don't know this. But it is very limited clipping, down to 500 000 Da, so there must be a mechanism for this, and we wondered whether free radicals could generate it. We know that they can generate that size of HA, but is there a mechanism in cartilage for generating those free radicals?

Torvard Laurent: Perhaps you will find some agent, in very small amount, that makes a few clips (an enzyme?). You need only three clips to reduce the M_r from two million to 500 000. To come down to 150 000, you would need at least twelve.

Bayliss: Yes. Free radicals are just one possibility. The other, as you say, is a hyaluronidase that works at neutral pH, because it must work in the matrix. You wouldn't need much of it; one would not want a large amount of an enzyme of that type around in the ECM.

Prehm: Can you really say that a smaller HA chain is not synthesized?

Bayliss: Yes; we showed that the newly synthesized HA in the tissue is always very large, compared with endogenous HA.

Mason: Something that puzzles me is why, when you intentionally degrade high molecular mass hyaluronan in the laboratory, with time-honoured methods like mixing it with impure ascorbate, you get cleavage only down to a certain size. You are usually left with a fragment that is quite large—between 20 000 and 50 000. Why doesn't it go down to a hexa- or tetrasaccharide?

Torvard Laurent: To go down from 25 000 to a hexasaccharide you may have to increase the dose of the degrading agent tenfold. In experiments with Robert Cleland and Lennart Rodén (Cleland et al 1969), we showed that if you degrade with ascorbic acid you get a polydisperse product in which some of the fractions were of low molecular mass.

Scott: Lars Sundblad also did that and obtained fragments that were characterized as disaccharides (Skanse & Sundblad 1943).

Mason: Extrapolating from that, could it be that if you are getting cleavage by oxygen free radicals, the extent of it is limited because there are so few oxygen radicals in tissues?

Torvard Laurent: Exactly. Also, there are enormous amounts of proteoglycans in cartilage, which scavenge most oxygen radicals generated.

Scott: On another point, hyaluronic acid is usually found in tissues containing collagen, yet not much is known about the precise interaction of HA with collagen. Bjorn Öbrink (1973), using light scattering in solution, found that HA increased the rate at which collagen molecules aggregate. Tentatively, this was explained as an excluded volume effect, which increased the activity of the collagen molecule in the HA solution. I'm not completely convinced. Most of the time, in the tissue, we are dealing not with single collagen molecules in solution, but with collagen fibrils. David Swann 15 years ago (personal communication) suggested that hyaluronate in the vitreous was to a certain extent held together by (and had a higher viscosity because of) interactions with small amounts of collagen. If that is so, it is an important phenomenon. Arguing by analogy with proteoglycans (Scott 1988), the interaction of HA with collagen fibrils would be different from that with collagen as a single molecule in solution.

Torvard Laurent: Ulf Larsson at the Karolinska Institute (Larsson et al 1987) has studied the formation of fibrin from fibrinogen, by dynamic light scattering, which gives the diffusion coefficient (*D*). As the fibrin monomers are polymerized, the average *D* value decreases: the larger the aggregates are, the more slowly they move in the solution. You would expect the *D* value to fall continuously. In fact, it oscillated. A possible explanation of the phenomenon could be ordered convection in the system (Preston et al 1980). When the fibrin monomers start to aggregate, microscopical density inhomogeneities could develop, resulting in convective flows which could order the system into structures. As a matter of fact, as seen in the electron microscope, the fibrin gel has a definite structure (Blombäck et al 1984). What the oscillations in *D* value would indicate is not diffusion, but movements due to flow of the structures. If this hypothesis is correct, the process of fibrin formation ought to be strongly influenced by viscosity, for example by the presence of hyaluronan. The effect of hyaluronan on collagen polymerization could be similar.

As we approach the end of the symposium, we ought to consider the need for new technology within the field of hyaluronan studies. A great breakthrough has come in the last few years as a result of technological innovations, such as more sensitive and specific techniques for measuring and visualizing hyaluronan.

Delpech: We need new techniques for calibrating fragments of HA properly, because we don't know the size of the molecules we are dealing with. It is easy to do this for small oligosaccharides, using gel chromatography, or for high molecular mass molecules, because they are in the void volumes of gel columns. Between 100 000 and one million molecular mass we have no easy technique.

Torvard Laurent: Pharmacia has HA fractions with which their gel columns are calibrated. Ove Wik at Pharmacia degrades Healon by autoclaving it for varying periods and he has characterized the products by light scattering.

Richard Margolis: How stable are they on storage? Stored hyaluronan solutions undergo a certain amount of degradation, I believe.

Torvard Laurent: Healon in the syringes in which it is distributed keeps its high viscosity and molecular mass, for a long time. One problem that we have encountered is that contamination with copper or iron leads to degradation of HA.

Weigel: We have become interested in HA sizes while trying to examine the intracellular processing of HA. With cetylpyridinium chloride (CPC) precipitation under fairly typical conditions (3% HA at room temperature and physiological ionic strength) we found the threshold size for precipitation to be fairly high. Oligosaccharides of 54 to 70 sugars are in the region in which there is a change from complete precipitation to non-precipitation; oligosaccharides of fewer than 54 sugars do not precipitate under those conditions. To do those studies we purified oligosaccharide standards, in order to generate 'ladders' of fragments on polyacrylamide gels. I therefore have fractions available for those who may be interested, in the range up to about 50 saccharide units. They appear to be stable (in terms of the patterns obtained on polyacrylamide gels) for several years. However, I could not find any information on the size cut-off under a particular set of conditions for precipitation.

Scott: I developed an equation (Scott 1961) that relates the molecular mass with the salt concentration at which the polyanion precipitate is obtained. This equation fitted hyaluronate, chondroitin sulphate, keratan sulphate, polyacrylate and polymethacrylate (Scott 1973). From one calibrated point you can therefore predict the molecular mass precipitated at another salt concentration.

Weigel: This experimental situation is at one CPC concentration and one ionic strength, and examining the size dependence of HA precipitation.

Scott: The equation predicts quite a strong dependence of salt concentration on molecular mass, below certain size limits (Scott 1973). At a moderately high molecular mass the dependency is almost nil. Molecular mass fractionations can be successful if the chemical structure of the polyanion is uniform. If it is not, as occasionally happens with the keratan and chondroitin sulphates, with varying degrees of sulphation, for instance, you can't use the equation directly. On hyaluronate you can (see Laurent & Scott 1964).

Then comes a difficult phenomenon. At 'high' concentrations of CPC (>0.01 M) *small* molecular mass fragments may redissolve in CPC micelles after they have been precipitated. You can titrate HA by adding CPC, molecule for molecule to the solution, until complete precipitation. If you continue adding CPC, the precipitate dissolves—it is a detergent effect. One must avoid too large an excess of CPC when trying to precipitate oligosaccharides quantitatively at room temperature. Alternatively, you can lower the temperature of the solution to 4 °C, when the CPC crystallizes out, and among the crystals you find precipitated polyanion—even the low molecular mass material.

Warren Knudson: Are there other standards besides HA oligosaccharides that would be easier to use for sizing HA, such as dextrans?

Torvard Laurent: The calibration curves for dextran and hyaluronate or chondroitin sulphate are very different. You cannot use dextran (Laurent et al 1969).

Toole: In the area of the HA-binding proteins, molecular biological approaches will clarify many problems, as they are doing in other fields. Immunological cross-reactivity and peptide mapping will give us some information, but the DNA sequence gives the most information, particularly in determining whether there are 'families' of HA-binding proteins and in studying the regulation of expression.

Engel: I agree that molecular biology is needed in this field, but a more integrated approach is also needed. Cellular interactions with the extracellular matrix are so complicated that we miss a lot by just looking at one molecule. This applies for example to localization studies, where results from different laboratories are often difficult to compare. Therefore the localizations of a variety of important molecules should be studied simultaneously. It also applies to complex cellular assays. One may demonstrate that one molecule does this or that, but there might be another molecule that has an even more dramatic effect but is not being looked at.

Torvard Laurent: We certainly need a balance between studies of single substances and surveys of the integrated matrix structures. We have concentrated on one compound in this symposium, but there are other symposia dealing with the whole panorama.

As a final point, on the clinical side, are there any diseases with defective synthesis or defective degradation of hyaluronan? In Werner's syndrome, there is a high concentration of hyaluronan in serum and urine (for references see Laurent et al 1987); there may be other diseases.

Scott: There is a hydrocephalic mouse where, in sternal cartilage, Breen et al (1973) found more than 100% increase in HA, in the heterozygote. The homozygous hydrocephalic mouse had only 50% of the normal amount of HA. That is, there was hyaluronate increase in a fairly 'normal' animal.

Mason: With regard to degradative pathways, from Bryan Toole's work in early embryos, the changes in hyaluronan in tissues are so dramatic in their effects on development that one suspects that if there were a defect, the embryo would not survive; so we may never find such a disease.

Torvard Laurent: I also expect that, but I am still fishing!

Toole: Andrew Copp and Merton Bernfield (1988) have been working on a mutant mouse where there is a decreased accumulation of hyaluronic acid in specific areas of the embryo where deformities arise.

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