Hyaluronic acid (hyaluronan): a review

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ABSTRACT: Hyaluronic acid (HA) is a high molecular weight biopolysacharide, discovered in 1934, by Karl Meyer and his assistant, John Palmer in the vitreous of bovine eyes. Hyaluronic acid is a naturally occurring biopolymer, which has important biological functions in bacteria and higher animals including humans. It is found in most connective tissues and is particularly concentrated in synovial fluid, the vitreous fluid of the eye, umbilical cords and chicken combs. It is naturally synthesized by a class of integral membrane proteins called hyaluronan synthases, and degraded by a family of enzymes called hyaluronidases. This review describes metabolisms, different physiological and pathological functions, basic pharmacological properties, and the clinical use of hyaluronic acid.

Keywords: hyaluronic acid; metabolism; toxicity

List of abbreviations

CD44 = cell surface glycoprotein; **CDC37** = intracellular HA-binding protein; **Da** = dalton; **DNA** = deoxynucleotid acid; **ECM** = extracellular matrix; **EM** = electron microscopy; **GHAP** = glial hyaluronate-binding protein; **GIT** = gastrointestinal tract; **HA** = hyaluronic acid; **HARE** = hyaluronic acid receptor for endocytosis; **HAS1, HAS2,** and **HAS3** = types of hyaluronan synthases 1, 2 and 3; **IHABP** = intracellular HA-binding protein; **IMP** = integral membrane protein; **IL-1** = interleukine 1; **LM** = light microscopy; **LYVE-1** = lymphatic vessel endocytic receptor; **MRHD** = maximum recommended human dose; **NS** = normal saline; **OA** = osteoarthrosis; **P-32** = protein-32; **RHAMM** = receptor for hyaluronic acid mediated mobility; **RHAMM/IHABP** = receptor for hyaluronic acid mediated mobility/intracellular HA-binding protein; **TDLo** = toxic dose low; **TIMP-1** = tissue inhibitor of matrix metalloproteiness 1;**TNF-α** = tumor necrosis factor alpha; **TSG-6** = tumor necrosis factor-α-stimulated gene-6; $t_{1/2}$ = half-life; **UDP** = uridine diphosphate

Contents

1. Introduction

Hyaluronic acid (HA) is a carbohydrate, more specifically a mucopolysaccharide, occurring naturally in all living organisms. It can be several thousands of sugars (carbohydrates) long. When not bound to other molecules, it binds to water giving it a stiff viscous quality similar to "Jello". The polysaccharide hyaluronan (HA) is a linear polyanion, with a poly repeating disaccharide structure [(1→3)-β-D-GlcNAc-(1→4)-β-D-GlcA-]. HA is found primarily in the extracellular matrix and pericellular matrix, but has also been shown to occur intracellularly. The biological functions of HA include maintenance of the elastoviscosity of liquid connective tissues such as joint synovial and eye vitreous fluid, control of tissue hydration and water transport, supramolecular assembly of proteoglycans in the extracellular matrix, and numerous receptor-mediated roles in cell detachment, mitosis, migration, tumor development and metastasis, and inflammation (Balazs et al., 1986; Toole et al., 2002; Turley et al., 2002; Hascall et al., 2004). Its function in the body is, amongst other things, to bind water and to lubricate movable parts of the body, such as joints and muscles. Its consistency and tissue-friendliness allows it to be used in skin-care products as an excellent moisturizer. Hyaluronic acid is one of the most hydrophilic (water-loving) molecules in nature and can be described as nature's moisturizer.

The unique viscoelastic nature of HA along with its biocompatibility and non-immunogenicity has led to its use in a number of clinical applications, including the supplementation of joint fluid in arthritis (Neo et al., 1997; Barbucci et al., 2002; Uthman et al., 2003; Medina et al., 2006), as a surgical aid in eye surgery, and to facilitate the healing and regeneration of surgical wounds. More recently, HA has been investigated as a drug delivery agent for various administration routes, including ophthalmic, nasal, pulmonary, parenteral and topical (Brown and Jones, 2005).

2. History

In 1934, Karl Meyer and his colleague John Palmer isolated a previously unknown chemical substance from the vitreous body of cows' eyes. They found that the substance contained two sugar molecules, one of which was uronic acid. For convenience, therfore, they proposed the name "hyaluronic acid". The popular name is derived from "hyalos", which is the Greek word for glass + uronic acid (Meyer and Palmer, 1934). At the time, they did not know that the substance which they had discovered would prove to be one of the most interesting and useful natural macromolecules. HA was first used commercially in 1942 when Endre Balazs applied for a patent to use it as a substitute for egg white in bakery products.

The first medical application of hyaluronan for humans was as a vitreous substitution/replacement during eye surgery in the late 1950s. The used hyaluronan was initially isolated from human umbilical cord, and shortly thereafter from rooster combs in a highly purified and high molecular weight form (Meyer and Palmer, 1934). The chemical structure of haluronan was essentially solved by Karl Mayer and his assocates in the 1950s. It was first isolated as an acid, but under physiological conditions it behaved like a salt (sodium hyaluronate).

The term "hyaluronan" was introduced in 1986 to conform with the international nomenclature of polysaccharides and is attributed to Endre Balazs (Balazs et al., 1986), who coined it to encompass the different forms the molecule can take, e.g, the acid form, hyaluronic acid, and the salts, such as sodium hyaluronate, which form at physiological pH (Laurent, 1989). HA was subsequently isolated from many other sources and the physicochemical structure properties, and biological role of this polysaccharide were studied in numerous laboratories (Kreil, 1995). This work has been summarized in a Ciba Foundation Symposium (Laurent, 1989) and a recent review (Laurent and Frazer, 1992).

3. Physicochemical and structural properties

Hyaluronan, an extracellular matrix component, is a high molecular weight glycosaminoglycan composed of disaccharide repeats of N-acetylglucosamine and glucuronic acid. This relatively simple structure is conserved throughout all mammals, suggesting that HA is a biomolecule of considerable importance (Chen and Abatangelo, 1999). In the body, HA occurs in the salt form, hyaluronate, and is found in high concentrations in several soft connective tissues, including skin, umbilical cord, synovial fluid, and vitreous humor. Significant amounts of HA are also found in lung, kidney, brain, and muscle tissues.

3.1. Chemical structure

The uronic acid and aminosugar in the disaccharide are D-glucuronic acid and D-N-acetylglucosamine, and are linked together through alternating beta-1,4 and beta-1,3 glycosidic bonds (see Figure 1). Both sugars are spatially related to glucose which in the beta configuration allows all of its bulky groups (the hydroxyls, the carboxylate moiety and the anomeric carbon on the adjacent sugar) to be in sterically favorable equatorial positions while all of the small hydrogen atoms occupy the less sterically favourable axial positions. Thus, the structure of the disaccharide is energetically very stable.

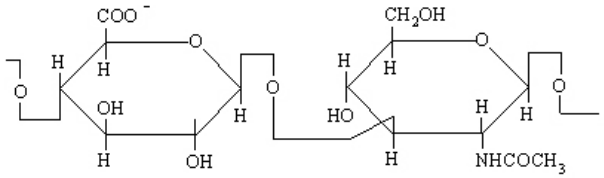

Figure 1. Chemical structure of HA

3.2. Solution structure

In a physiological solution, the backbone of a hyaluronan molecule is stiffened by a combination of the chemical structure of the disaccharide, internal hydrogen bonds, and interactions with the solvent. The axial hydrogen atoms form a non-polar, relatively hydrophobic face while the equatorial side chains form a more polar, hydrophilic face, thereby creating a twisting ribbon structure. Solutions of hyaluronan manifest very unusual rheological properties and are exceedingly lubricious and very hydrophilic. In solution, the hyaluronan polymer chain takes on the form of an expanded, random coil. These chains entangle with each other at very low concentrations, which may contribute to the unusual rheological properties. At higher concentrations, solutions have an extremely high but shear-dependent viscosity. A 1% solution is like jelly, but when it is put under pressure it moves easily and can be administered through a small-bore needle. It has therefore been called a "pseudo-plastic" material. The extraordinary rheological properties of hyaluronan solutions make them ideal as lubricants. There is evidence that hyaluronan separates most tissue surfaces that slide along each other. The extremely lubricious properties of hyaluronan, meanwhile, have been shown to reduce postoperative adhesion formation following abdominal and orthopedic surgery. As mentioned, the polymer in solution assumes a stiffened helical configuration, which can be attributed to hydrogen bonding between the hydroxyl groups along the chain. As a result, a coil structure is formed that traps approximately 1 000 times its weight in water (Cowman and Matsuoka, 2005).

3.3. Polymer structure

Hyaluronan synthase enzymes synthesize large, linear polymers of the repeating disaccharide structure of hyaluronan by alternating addition of glucuronic acid and N-acetylglucosamine to the growing chain using their activated nucleotide sugars (UDP – glucuronic acid and UDP-N-acetlyglucosamine) as substrates (Meyer and Palmer, 1934). The number of repeat disaccharides in a completed hyaluronan molecule can reach 10 000 or more, a molecular mass of ~4 million daltons (each disaccharide is ~400 daltons). The average length of a disaccharide is ~1 nm. Thus, a hyaluronan molecule of 10 000 repeats could extend 10 μm if stretched from end to end, a length approximately equal to the diameter of a human erythrocyte (Cowman and Matsuoka, 2005).

3.4. Synthesis

The cellular synthesis of HA is a unique and highly controlled process. Most glycosaminoglycans are

made in the cell's Golgi networks. HA is naturally synthesized by a class of integral membrane proteins called hyaluronan synthases, of which vertebrates have three types: HAS1, HAS2, and HAS3 (Lee and Spicer, 2000). Secondary structure predictions and homology modeling indicate an integral membrane protein (IMP). An integral membrane protein is a protein molecule (or assembly of proteins) that in most cases spans the biological membrane with which it is associated (especially the plasma membrane) or which, is sufficiently embedded in the membrane to remain with it during the initial steps of biochemical purification (in contrast to peripheral membrane proteins). Hyaluronan synthase enzymes synthesize large, linear polymers of the repeating disaccharide structure of hyaluronan by alternate addition of glucuronic acid and N-acetylglucosamine to the growing chain using their activated nucleotide sugars (UDP = glucuronic acid and UDP-N-acetlyglucosamine) as substrates.

3.5. Degradation

In mammals, the enzymatic degradation of HA results from the action of three types of enzymes: hyaluronidase (hyase), b-D-glucuronidase, and β-N-acetyl-hexosaminidase. Throughout the body, these enzymes are found in various forms, intracellularly and in serum. In general, hyase cleaves high molecular weight HA into smaller oligosaccharides while β-D-glucuronidase and β-N-acetylhexosaminidase further degrade the oligosaccharide fragments by removing nonreducing terminal sugars (Leach and Schmidt, 2004).

The degradation products of hyaluronan, oligosaccharides and very low molecular weight hyaluronan, exhibit pro-angiogenic properties (Mio and Stern, 2002). By catalyzing the hydrolysis of hyaluronic acid, a major constituent of the interstitial barrier, hyaluronidase lowers the viscosity of hyaluronic acid, thereby increasing tissue permeability. It is, therefore, used in medicine in conjunction with other drugs in order to speed their dispersion and delivery. The most common application is in ophthalmic surgery, in which it is used in combination with local anesthetics. Some bacteria, such as *Staphylococcus aureus*, *Streptococcus pyogenes et pneumoniae* and *Clostridium perfringens*, produce hyaluronidase as a means of increasing mobility through the body's tissues and as an antigenic disguise that prevents their recognition by phagocytes of the immune system (Ponnuraj and Jedrzejas, 2000; Lin and Stern, 2001; Lokeshwar et al., 2002; Hajjaji et al., 2005; Kim et al., 2005; Girish and Kemparaju, 2006).

4. Mechanism of action

Although the predominant mechanism of HA is unknown, *in vivo*, *in vitro*, and clinical studies demonstrate various physiological effects of exogenous HA.

Hyaluronic acid possesses a number of protective physiochemical functions that may provide some additional chondroprotective effects *in vivo* and may explain its longer term effects on articular cartilage. Hyaluronic acid can reduce nerve impulses and nerve sensitivity associated with pain. In experimental osteoarthritis, this glycosaminoglycan has protective effects on cartilage (Akmal et al., 2005); exogenous hyaluronic acid is known to be incorporated into cartilage (Antonas et al., 1973).

Exogenous HA enhances chondrocyte HA and proteoglycan synthesis, reduces the production and activity of proinflammatory mediators and matrix metalloproteinases, and alters the behavior of immune cells. These functions are manifested in the scavenging of reactive oxygen-derived free radicals, the inhibition of immune complex adherence to polymorphonuclear cells, the inhibition of leukocyte and macrophage migration and aggregation (Balazs and Denlinger, 1984) and the regulation of fibroblast proliferation. Many of the physiological effects of exogenous HA may be functions of its molecular weight (Noble, 2002; Uthman et al, 2003; Hascall et al., 2004; Medina et al., 2006).

Hyaluronan is highly hygroscopic and this property is believed to be important for modulating tissue hydration and osmotic balance (Dechert et al., 2006). In addition to its function as a passive structural molecule, hyaluronan also acts as a signaling molecule by interacting with cell surface receptors and regulating cell proliferation, migration, and differentiation. Hyaluronan is essential for embryogenesis and is likely also important in tumorigenesis (Kosaki et al., 1999; Camenisch et al., 2000).

Hyaluronan functions are diverse. Because of its hygroscopic properties, hyaluronan significantly influences hydration and the physical properties of the extracellular matrix. Hyaluronan is also capable of interacting with a number of receptors resulting

in the activation of signaling cascades that influence cell migration, proliferation, and gene expression (Turley et al., 2002; Taylor et al., 2004).

4.1. Interactions with hyaladherins

HA plays several important organizational roles in the extracellular matrix (ECM) by binding with cells and other components through specific and nonspecific interactions. Hyaluronan-binding proteins are constituents of the extracellular matrix, and stabilize its integrity. Hyaluronan receptors are involved in cellular signal transduction; one receptor family includes the binding proteins aggrecan, link protein, versican and neurocan and the receptors CD44, TSG6 (Kahmann et al., 2000), GHAP (Liu et al., 2001), and LYVE-1 (Banerji et al., 1999). The RHAMM receptor is an unrelated hyaluronan-binding protein, and the hyaluronan binding sites contain a motif of a minimal site of interaction with hyaluronan. This is represented by B(X7) B, where B is any basic amino acid except histidine, and X is at least one basic amino acid and any other moiety except acidic residues. CD44 and RHAMM have attracted much attention, because they are believed to be involved in metastasis (Toole, 1997; Ahrens et al., 2001; Noble, 2002; Toole et al., 2002).

CD44 is a structurally variable and multifunctional cell surface glycoprotein expressed on most cell types (Karjalainen et al., 2000). To date, it is the best characterized transmembrane hyaluronan receptor and because of its wide distribution is considered to be the major hyaluronan receptor on most cell types (Tzircotis et al., 2005). Many functions of CD44 are mediated through interaction with its ligand hyaluronan, a ubiquitous extracellular polysaccharide (Toole, 1997). Hyaluronan is abundant in soft connective tissues, but also in epithelial and neural tissues.

Low and intermediate molecular weight HA (2×10^4–4.5×10^5 Da) stimulates gene expression in macrophages, endothelial cells, eosinophils and certain epithelial cells (McKee et al., 1996; Oertli et al., 1998). Hyaluronan degradation products are purported to contribute to scar formation. Fetal wounds heal without scar formation and wound fluid HA is of high molecular weight. When hyaluronidase is added to generate HA fragments, there is increased scar formation. These data support the theory that high molecular weight HA promotes cell quiescence and supports tissue integrity, whereas generation of HA breakdown products is a signal that injury has occurred and initiates an inflammatory response (Chen and Abatangelo, 1999).

The role of CD44 in HA-binding and signaling has recently been investigated in hematopoietic cells from CD44-deficient mice (Schmits et al., 1997; Protin et al., 1999). CD44-deficient mice develop normally and exhibit minor abnormalities in hematopoiesis and lymphocyte recirculation (Schmits et al., 1997; Protin et al., 1999). The induction of inflammatory gene expression in response to hyaluronan was observed in the absence of CD44 in bone marrow cultures and dendritic cells. These data suggest that there are CD44-independent

Table 1. Normal values of kinetic parameters of HA in animals

Species	Compartment*	$t_{½}$ (min)	Extraction ratio (%)	Plasma clearance (mg/day)	Total daily turnover (mg/day)	K_m (μg/l)	V_{max} (μg/min)	Method**
Pig	hepatic		50	332	71			3
	splanchnic		23	150	24.3			3
	renal		14	41	8.9			3
	urine			11	2.9			3
Sheep	plasma	2–7	50	215	37	120	88	2
Rabbit	plasma	2–5			20–50		100	1
Rat	plasma	1.4						1

*compartment over which the parameter was determined; $t_{1/2}$ = half-life of hyaluronan; K_m = the Michaelis-Menten constant; V_{max} = the maximum metabolic rate

**method used for determination of kinetic parameters: 1 = bolus dose of labeled HA; 2 = infusion of unlabelled HA and kinetic modeling; 3 = direct measurement of HA concentration over eliminating organ

mechanisms for the induction of gene expression by HA (Noble, 2002).

RHAMM (Receptor for HA-Mediated Mobility), has been found on cell surfaces, as well as in the cytosol and nucleus (Leach and Schmidt, 2004). It has been implicated in regulating cellular responses to growth factors and plays a role in cell migration, particularly for fibroblasts and smooth cells (Toole, 1997).

Table 2. Concentration and turnover of HA in different tissues (values within parentheses represent total amount recovered in the cavity, or injected)

Tissue and species	Concentration of HA in		$t_{1/2}$ (days)
	tissue (μg/ml)	injectate (mg/ml)	
Vitreous body			
man	100–400		
rhesus monkey	100–180	10	10–20
owl monkey	300–900	10	20–30
rabbit	14–52	0.02	70
Anterion chamber			
man	1.1		
owl monkey	11.4	10	0.2–0.6
cynomolgus		10	0.8
rabbit	1.1	10	0.3–0.5
rabbit	1.1	0.02	0.04–0.06
Joints			
horse	300–500	10	1
rabbit	(134)	0.3	0.5
rabbit	3 800	20	0.5
Pleura			
rabbit	(0.76)	(0.03–0.05)	0.4–1.0
Pericard			
rabbit	5	10	3–4
rabbit	5	0.06	3–4
Peritoneum			
rabbit	(2–93)	10	1.2
rabbit		0.07	0.1
Skeletal muscle			
rabbit	26–28*	10	1.25
Amniotic fluid			
sheep 12 week	5.1	tracer	3–8
sheep 15–17 week	1.9	tracer	0.5–0.8
Skin			
rabbit			1.9– .7
rat	840*		2.6–4.5
rabbit		0.07	0.5
rabbit		10	2

*μg/g

5. Pharmacokinetics

The normal systemic kinetics of HA is well established in several species including man. The removal of HA from the circulation is very efficient, with a half-life of 2–6 min and a total normal turnover of 10–100 mg/day in the adult human (Table 1 and 2). The main uptake from the blood takes place in the liver endothelial cells. However, evidence for a role of the kidney in the elimination of HA is accumulating. Recently published data suggest that the elimination kinetics of HA from the systemic circulation may be influenced by a number of factors, such as saturation of the elimination caused by an increased lymphatic input of HA to the circulation, alteration of the blood flow over the eliminating organ and competition with other macromolecular substances such as chondroitin sulphate or proteoglycans. Many of these factors may be operative during different disease states, and may therefore partly explain the observed differences between normal and pathological HA kinetics. The normal and pathological turnover of hyaluronan from the circulation has been determined in many different species, including man by many different authors using different techniques (Table 3).

5.1. Absorption rate and concentration in plasma

After *i.v.* injection of a bolus dose of [^{14}C]-HA in rabbits, it was shown that 98% of the administered dose had disappeared from the systemic circulation within 6 h after the administration (Lebel, 1991).

Similar results were also obtained in man, where 55% and 85% of the acetyl content after *i.v.* injection of [^{3}H]HA, was completely oxidized after 3 h and 24 h, respectively (Laurent and Fraser, 1992).

It is known that the major part of the elimination of HA from the blood circulation takes place in

Table 3. Kinetics parameters of hyaluronan in man and animals during different disease states (Lebel, 1991)

Species	Disease	Compartment*	$t_{½}$ (min)	Extraction ratio (%)	Plasma clearance (mg/day)	Total daily turnover (mg/day)	Method***
Man	primary bililary cirrh.	plasma	6–72		50–510**	69–115	1
	rheumatoid arthritis	plasma	2–3		970–2 060*	33–167	1
	kidney disease	splanchnic		33			3
		renal		22			
	alcoholic cirrhosis	splanchnic		14		61.9	3
		renal		5			
	non-cirrhotic alcoholic	splanchnic		36		8.9	3
	liver disease	renal		5			3
	rheumatoid arthritis	urine				0.5	3
	primary bililary cirrhosis	urine				0.9	3
	Werner's sy	urine				3.3	3
Pig	fecal peritonitis	hepatic		36	84	65	3
Sheep	endotoxin infusion	plasma	7–19				1
	TNF-alpha infusion	plasma	3–10				1
Rat	experimental arthritis	plasma	1–2				1

*compartment over which the parameter was determined; $t_{½}$ = half-life of hyaluronan

**blood clearance

***method used for determination of kinetic parameters: 1 = bolus dose of labeled HA; 3 = direct measurement of HA concentration over eliminating organ

the liver (Fraser et al., 1981) via receptor-mediated endocytosis in the sinusoidal liver endothelial cells (Bentsen et al., 1989; Smedsrod, 1991).

5.2. Distribution

HA is widely distributed in body tissues and intracellular fluids, including the aqueous and vitreous humour, and synovial fluid; it is a component of the ground substance or tissue cement surrounding cells (Laurent and Reed, 1991; Toole, 1997). It is not known whether hyaluronate sodium is distributed into breast milk.

5.3. Excretion (elimination)

5.3.1. Renal excretion

By direct measurement of HA in urine it can be calculated that approximately 1% of the normal daily turnover of HA from the systemic circulation in man is filtered via the kidneys. Similar results were obtained in studies on man (Lebel, 1991) and in a study on rabbits (Fraser et al., 1981).

Recently, the extraction ratio and clearance over the kidney in pig were reported to be 14% and 41 ml per min, using the method of measuring directly over the organ. In this study, it was also determined that the renal clearance was approximately three times the urinary clearance (Bentsen et al., 1989).

5.3.2. Hepatic elimination

Direct measurement of the difference of the endogenous concentration over a specific organ and knowledge of the blood flow enables calculation of the extraction ratio or clearance directly over a specific organ.

By use of this method Bentsen et al. (1989) determined the hepatosplanchnic extraction ratio and clearance of hyaluronan in man to be 33% and 250 ml/min, respectively.

The hepatic extraction ratio and clearance have also been determined in pigs by measurement directly over the organ and were found to be 23% and 150 ml/min, respectively (Bentsen et al., 1989). In a similar study on pigs, using the same method of direct determination, the extraction ratio and clearance over the liver were determined to be 49% and 332 ml/min, respectively. The reason for the discrepancies between these two studies is not known (Lebel, 1991).

5.3.3. Pulmonary excretion

Within 100 h, 63% and 20% of the administered dose was excreted and recovered in the respiratory gas (as $^{14}CO_2$) (Lebel, 1991).

5.3.4. GIT excretion

The total amount of excretion into bile within 24 h was reported to be very low, 0.7% of the administered dose. Similarly, the total amount of excretion into feces, within 100 h of administration, was also very small, about 0.5% of the administered dose (Lebel, 1991).

6. Toxicity

6.1. Cytotoxicity

Jansen et al. (2004) investigated the possible cytotoxic effects, biocompatibility and degradation of a hyaluronan-based conduit for peripheral nerve repair. The results show that a hyaluronan-based conduit is not cytotoxic and shows good biocompatibility.

Hyaluronan is highly non-antigenic and non-immunogenic, owing to its high structural homology across species, and poor interaction with blood components (Amarnath et al., 2006).

6.2. Neurotoxicity

Because HA has an anti-inflammatory effect and prevents and/or reduces tissue adhesion, it was believed that HA epidurally-administered during epidural adhesiolysis procedures could alleviate the condition of patients with chronic lower back pain. For this reason, the following clinical trial evaluation of epidurally-administered HA neurotoxicity was performed by light microscopy (LM) and electron microscopy (EM) in rabbits. Twenty rabbits were randomly divided into two groups, a normal saline (NS) group (n = 10) and a HA group (n = 10). Saline (0.2 ml/kg of 0.9% solution) and the same volume of HA were injected into the epidural space. No rabbits showed any sensory-motor or

behavioural changes during the three-week period, except for one rabbit in the NS group that showed decreased appetite and activity, and weight loss. By LM, abnormal findings were observed in two rabbits in the NS group; these were thought to be the result of trauma and infection associated with epidural catheterization. EM findings showed no significant neurotoxic findings in either group. In conclusion, epidurally-administered HA did not cause neurotoxicity in rabbits (Lim et al., 2003).

6.3. Carcinogenicity

HA is responsible for various functions within the extracellular matrix such as cell growth, differentiation, and migration (Jaracz et al., 2005; Paiva et al., 2005). A wide range of activities can be explained by a large number of Ha-binding receptors such as cell surface glycoprotein CD44, the receptor for hyaluronic acid-mediated motility (RHAMM), and several other receptors possessing Ha-binding motifs, for example: transmembrane protein layilin, hyaluronic acid receptor for endocytosis (HARE), lymphatic vessel endocytic receptor (LYVE-1), and also intracellular HA-binding proteins including CDC37, RHAMM/IHABP, P-32, and IHABP4 (Underhill, 1992; Forsberg et al., 1994; Pohl et al., 2000; Pure and Cuff, 2001; Toole, 2001; Weigel et al., 2002; Hascall et al., 2004; Hajjaji et al., 2005; Nawrat et al., 2005; Hill et al., 2006; Iacob and Knudson, 2006). It has been shown that the HA level is elevated in various cancer cells (Lin and Stern, 2001) and it is believed to form a less dense matrix, thus enhancing the cell's motililty as well as invasive ability into other tissues (Hill et al., 2006).

It is well known that various tumors (epithelial, ovarial, colon, stomach and acute leukemia) overexpress HA-binding receptors CD44 and RHAMM. Consequently, these tumor cells are characterised by enhanced binding and internalization of HA.

CD44-Ha interactions play various important physiological roles, including mediation or promotion of macrophage aggregation, cell migration, chondrocyte pericellular matrix assembly, and leukocyte activation.

Paradoxically, both HA and the enzymes that eliminate HA, hyaluronidases, can correlate with cancer progression. It has been shown that the over expression of hyaluronic acid synthases increases the HA level, which leads to the acceleration of tumor growth and metastasis. On the other hand, exogenous oligometric HA inhibits tumor progression, most likely by competing with endogenous polymeric HA.

6.4. Mutagenicity

Sister Chromatid Exchange Assay. Under the conditions of the assay, the sodium hyaluronate Orthovisc® solution (High Molecular Weight Hyaluronan) was not considered mutagenic to Chinese Hamster Ovary cells (Product information Orthovisc®, 2004).

Chromosomal Aberration Assay. Under the conditions of the assay, the Orthovisc® solution was not considered mutagenic to Chinese Hamster Ovary cells (Product information Orthovisc®, 2004).

Ames Salmonella/Mammalian Microsome Mutagenicity Assay. Under the conditions of the assay, the Orthovisc® solution was not considered mutagenic to *Salmonella typhimurium* tester strains (Product information Orthovisc®, 2004).

6.5. Reproductive toxicity

No evidence of impairment of fertility was seen in rats and rabbits given hyaluronate sodium in doses of up to 1.43 mg per kg of body weight, approximately 11 times the maximum recommended human dose (MRHD), per treatment cycle.

Reproductive toxicity studies, including multigenerational studies, have been performed in rats and rabbits at doses of up to 11 times the anticipated human dose (1.43 mg/kg per treatment cycle) and have revealed no evidence of impaired fertility or harm to the experimental animal foetus caused by intra-articular injections of hyaluronate sodium.

7. Efficacy and applications

7.1. Chondroprotective effects

The physical properties of HA are important but there is evidence to suggest that HA may provide both physiochemical and pharmacological advantages. Chondrocytes express the glykoprotein CD44 on their cell surface. This has the capacity to function as a HA receptor and so may be involved in biochemical interactions with chondrocytes. The effect of a HA injection may be mediated via CD44 inteactions (Akmal et al., 2005).

7.2. Chondroprotective effects *in vitro*

The chondroprotective effects of hyaluronic acid, e.g., that it stimulates the production of tissue inhibitors of matrix metalloproteineses (TIMP-1) by chondrocytes, inhibits neutrophil-mediated cartilage degradation and attenuates IL-1 induced matrix degeneration and chondrocyte cytotoxicity have been observed *in vitro* (Gerwin et al., 2006). Articular chondrocytes cultured in the presence of HA have a significantly greater rate of DNA proliferation and extracellular matrix production, compared with chondrocytes cultured without HA (Akmal et al., 2005).

7.3. Chondroprotective effects *in vivo*

HA has been experimentally studied as a potential agent of therapeutic intervention in osteoarthrosis (OA). Hyaluronid acid has been applied to the therapy of experimental OA. Investigations have shown that intra-articular injection of HA reduces arthritic lesions in experimental animal models of articular cartilage injury (Balazs and Denlinger, 1989; Neo et al., 1997; Kim et al., 2001; Moreland, 2003; Leach and Schmidt, 2004; Ding et al., 2005; Roth et al., 2005; Echigo et al., 2006).

7.4. Orthopaedic applications

HA plays a vital role in the development of cartilage, the maintance of the sinovial fluid and the regeneration of tendons (Toole, 1997, 2001). High concentrations of HA have been found in the ECM of all adult joint tissues, including the sinovial fluid and the outer layer cartilage (Leach and Schmidt, 2004). In part because of its viscoelastic nature and ability to form highly hydrated matrices, HA acts in the joint as a lubricant and shock absorber.

The pathologic changes of synovial fluid hyaluronic acid, with its decreased molecular weight and concentration, led to the concept of viscosupplementation.

7.4.1. Viscosupplementation

Viscosupplementation is a novel, safe, and possibly effective form of local treatment for osteoarthritis (Uthman et al., 2003). Viscosupplementation with HA products helps to improve the physiological environment in an osteoarthritic joint by supplementing the shock absorption and lubrication properties of osteoarthritic synovial fluid. The rationale for using viscosupplementation is to restore the protective viscoelasticity of synovial hyaluronan, decrease pain, and improve mobility. The immediate benefits of viscosupplementation are the relief of pain. Longer-term benefits are believed to include the return of joint mobility by the restoration of transsynovial flow and, ultimately, the metabolic and rheologic homeostasis of the joint (Wang et al., 2004).

Viscosupplementation came into clinical use in Japan and Italy in 1987, in Canada in 1992, in Europe in 1995, and in the United States in 1997. Two hyaluronic acid products are currently available in the United States: naturally occurring hyaluronan (Hyalgan) and synthetic hylan G-F 20 (Synvisc). Hylans are cross-linked hyaluronic acids, which gives them a higher molecular weight and increased elastoviscous properties. The higher molecular weight of hylan may make it more efficacious than hyaluronic acid because of its enhanced elastoviscous properties and its longer persistence in the joint space (Wen, 2000).

7.5. Antiadhesion applications

As HA is highly hydrophilic, it is a polymer that is well suited to applications requiring minimal cellular adhesion. Postoperative adhesions, which form between adjacent tissue layers following surgery, impede wound healing and often require additional surgical procedures to be repaired successfully. Barriers made from cross-linked HA have been effectively used to prevent such adhesions from forming. Furthermore, the adhesion of bacteria to biomaterials can induce infections and constitute a great risk to the patient; with this in mind, esterified HA has also been used to prevent bacterial adhesion to dental implants, intraocular lenses, and catheters (Leach and Schmidt, 2004).

7.6. Ophthalmology

HA, a natural component of the vitreous humor of the eye, has found many successful applications in ophthalmologic surgery. HA is particularly useful as a spacefilling matrix in the eye; thus, intraocular injection of HA during surgery is used to maintain the shape of the anterior chamber. Furthermore, HA solutions also serve as a viscosity-enhancing component of eye drops and as an adjuvant to eye tissue repair.

7.7. Dermatology and wound-healing applications

HA is naturally present in high concentrations in the skin and soft connective tissues. Therefore, HA is an appropriate choice for a matrix to support dermal regeneration and augmentation. For example, Prestwich and co-workers found that cross-linked HA hydrogel films accelerate the healing of full-thickness wounds, presumably by providing a highly hydrated and nonimmunogenic environment that is conducive to tissue repair. Hyaff scaffolds cultured *in vitro* with keratinocytes and fibroblasts have been used to create materials similar to skin, including two distinct epidermal and dermal-like tissue layers. Moreover, as a result of its ability to form hydrated, expanded matrices, HA has also been successfully used in cosmetic applications such as soft tissue augmentation (Leach and Schmidt, 2004; Dechert et al., 2006).

7.8. Cardiovascular applications

In a manner related to its antiadhesive properties, HA has also proven to be effective for increasing the blood compatibilities of cardiovascular implants such as vascular grafts and stents. For example, biomaterial surfaces treated with cross-linked HA have been associated with reduced platelet adhesion and thrombus formation (Leach and Schmidt, 2004). Furthermore, sulfated HA derivatives can act as heparin mimics (Barbucci et al., 1995); in fact, HA derivatives with higher degrees of sulfation are associated with increased abilities to prevent blood coagulation (as measured by longer times required for whole blood clotting) (Barbucci et al., 1995).

8. Tabular overview

Table 4. Data on reproductive effects of hyaluronan in animals

Effect	Route	Organism	Dose of TDLo (mg/kg)	Duration	Source/No./pp/year/
T16; T31; T73	subcutaneous	rat	189	multigenerations	OYYAA2 29, 139, 1985
T46; T86	subcutaneous	rat	220	7-17D preg	OYYAA2 29, 111, 1985
T85	subcutaneous	rat	77	7-17D preg	OYYAA2 29, 111, 1985
T81	subcutaneous	rat	660	7-17D preg	OYYAA2 29, 111, 1985
T12	intraperitoneal	rabbit	91	6-18D preg	OYYAA2 29, 131, 1985
T03	parenteral	rabbit	52	91D male	OYYAA2 28, 1 041, 1984

T03 – prostate, seminal vesicle, Cowperr's glands, accessory glands; T12 = ovaries, fallopian tubes; T16 = parturition; T31 = extra embryonic structures; T46 = musculosceletal system; T73 = sex ratio; T81 = growth statistics; T85 = behavioral; T86 = physical; OYYAA2 = Oyo Yakuri Pharmacometrics

TDLo (Toxic Dose Low): the lowest dose of a substance introduced by any route, other than inhalation, over any given period of time and reported to produce any toxic effect in humans or to produce tumorigenic or reproductive effects in animals or humans

Table 5. Other multiple dose data of hyaluronan in animals

Effect	Route	Organism	Dose of TDLo (mg/kg)	Duration	Source/No./pp/year/
U01; U05; U06	oral	rat	2 275	13W-I	YACHDS 27, 5 809 ,1993
M16; P05; P72	intraperitoneal	rat	1 680	4W-I	YACHDS 13, 2 763, 1985

M16 = other changes in urine composition; P05 = normocytic anemia; P72 = changes in leukocyte (WBC) count; U01 = weight loss or decreased weight gain; U05 = changes in Na^+; U06 = body temperature decrease; YACHDS = Yakuri to Chiryo, Pharmacology and Therapeutics

TDLo (Toxic Dose Low): the lowest dose of a substance introduced by any route, other than inhalation, over any given period of time and reported to produce any toxic effect in humans or to produce tumorigenic or reproductive effects in animals or humans

Table 6. Data on the reproductive effects of hyaluronan in humans

Effect	Route	Organism	Dose of TDLo (ml/kg)	Source/No./pp/year/
J18; J30; Y55	intrapleural	human	0.036	CEXPB9 30, 203, 2003

J18 = pleural thickening; J30 = other changes; Y55 = effect on inflammation or mediation of inflammation; CEXPB9 = Clinical and Experimental Pharmacology and Physiology

TDLo (Toxic Dose Low): the lowest dose of a substance introduced by any route, other than inhalation, over any given period of time and reported to produce any toxic effect in humans or to produce tumorigenic or reproductive effects in animals or humans

9. Conclusion

Hyaluronic acid has been used for more than 20 years in many products throughout the world. Because of its biocompatibility, biodegradability, and readily modified chemical structure, HA has been extensively investigated in drug-delivery applications. A variety of commercially available preparations of HA derivatives and cross-linked HA materials have been developed for drug delivery; these materials are created in forms such as films, microspheres, liposomes, fibers, and hydrogels. Through multidisciplinary discoveries about the structure, properties, biological activity, and chemical modification of this unique polymer, HA has found success in an extraordinarily broad range of biomedical applications. Future clinical therapies of HA-derived materials critically rely on a more detailed understanding of the effects of HA molecular weight and concentration and how this biomolecule specifically interacts with cells and ECM components in the body. The increased use of these materials will require finely tuned and controllable interactions between HA and its environment. Work in these areas is underway; for example, adhesive peptide sequences have been covalently bound to HA materials. Also, environmentally responsive materials have been synthesized from HA. These materials can be created to swell or degrade in response to inflammation, electrical stimulation, and heat.

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