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Final Report of the Safety Assessment of Hyaluronic Acid, Potassium Hyaluronate, and Sodium Hyaluronate

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Hyaluronic acid, sodium hyaluronate, and potassium hyaluronate function in cosmetics as skin conditioning agents at concentrations up to 2%. Hyaluronic acid, primarily obtained from bacterial fermentation and rooster combs, does penetrate to the dermis. Hyaluronic acid was not toxic in a wide range of acute animal toxicity studies, over several species and with different exposure routes. Hyaluronic acid was not immunogenic, nor was it a sensitizer in animal studies. Hyaluronic acid was not a reproductive or developmental toxicant. Hyaluronic acid was not genotoxic. Hyaluronic acid likely does not play a causal role in cancer metastasis; rather, increased expression of hyaluronic acid genes may be a consequence of metastatic growth. Widespread clinical use of hyaluronic acid, primarily by injection, has been free of significant adverse reactions. Hyaluronic acid and its sodium and potassium salts are considered safe for use in cosmetics as described in the safety assessment.

Keywords: cosmetics; hyaluronic acid; safety

The safety of ingredients used in cosmetic formulations is reviewed by the Cosmetic Ingredient Review (CIR) program. Published studies relevant to assessing the safety of hyaluronic acid, sodium hyaluronate, and potassium hyaluronate as used in cosmetic products have been combined with unpublished data provided by interested parties. In a series of public meetings, with formal notice and comment opportunities for any interested party to provide additional data or comment, the CIR Expert Panel reviewed these data and reached a tentative and then final conclusion regarding safety of these ingredients as used in cosmetics.

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Chemistry

Definition and Structure

Hyaluronic acid (CAS No. 9004-61-9) is the natural glycosaminoglycan formed by bonding N-acetyl-D-glucosamine with glucuronic acid.[1] Disaccharide units are formed at the plasma membrane in vertebrates and some bacteria.[2-4] These units are linked together with 2 to 4 glycosidic bonds to a long, linear (unbranched) molecule, which grows into a random coil as it becomes longer. A completed hyaluronic acid molecule can reach 10 000 or more disaccharide pairs, a molecular mass of approximately 4 million Da. The molecule is considerably rigid, and as it grows longer, the overall shape is spherical. It also entangles with adjacent coils to create a continuous network. At concentrations higher than 0.1%, the chains of hyaluronic acid form a continuous network. Only about 0.1% of the volume of the molecule

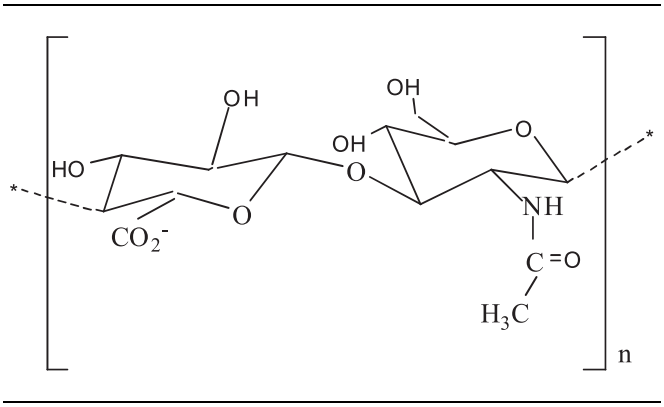

Figure 1. Structure of hyaluronic acid. *The repeating units, identical to the structure in brackets.

is hyaluronic acid, the rest being water which is mechanically immobilized within the coil. The rate of diffusion through the network is inversely related to the size of the polysaccharide molecules. The structure of the disaccharide is energetically very stable. The domain structure of hyaluronic acid allows small molecules such as water, electrolytes, and nutrients to freely diffuse through the solvent within the domain while large molecules such as proteins will be partially excluded from the domain because of their hydrodynamic sizes in solution.

In the presence of K^+, NH_4^+, Rb^+, and Cs^+, an antiparallel double-helical structure forms with hydrophobic and hydrophilic pockets between adjacent double helices.[5] Under physiological conditions (pH 7), this water soluble molecule exists in its salt form as sodium hyaluronate.[6] The structure of hyaluronic acid is shown in Figure 1.[4]

According to the *International Cosmetic Ingredient Dictionary and Handbook*, hyaluronic acid is also known as hyaluronan.[7] Trade names for hyaluronic acid and trade name mixtures containing hyaluronic acid are listed in Table 1.

Sodium hyaluronate (CAS no. 9067-32-7), also known as hyaluronic acid sodium and hyaluronate sodium, is the sodium salt of hyaluronic acid.[7] Other technical names are: hyaluronic acid, sodium salt; sodium hyaluronate (1); sodium hyaluronate (2); and sodium hyaluronate (3); and sodium hyaluronate solution. Its trade names and trade name mixtures are listed in Table 1. Potassium hyaluronate (CAS no. 31799-91-4) is the potassium salt of hyaluronic acid, also known as hyaluronic acid, potassium salt.[7]

Physical and Chemical Properties

Various authors have reported the water retention properties of hyaluronic acid. Hyaluronic acid has a greater capacity to hold water than any other natural or synthetic polymer.[8] One gram of hyaluronic acid can hold up to 6 L of water.[9]

Polymer Network

The structural factors underlying hyaluronic acid's unique properties are its high molecular weight and large molecular volume.[10] The large molecular volume forces the overlap of individual hyaluronic acid molecular domains, resulting in extensive chain entanglement and chain–chain interaction.[10-13]

Concentration (expressed in g/cc) and intrinsic viscosity (limiting viscosity number, expressed in cc/g), which are measures of molecular volume, are related to the ability of hyaluronic acid to form viscoelastic polymeric networks. The product of concentration and intrinsic viscosity has been called the coil overlap parameter, which expresses the degree of network formation.[14] Concentration and molecular volume (size) are totally interdependent in determining the physical properties of a hyaluronic acid solution.[13] They stated that the smaller the size of the individual hyaluronic acid molecules, the higher the concentration necessary for a viscoelastic network to form.

The carboxyl groups fully dissociate at physiological pH[15]; the structure is sensitive to ionic strength and pH. The molecule expands at lower ionic strength due to repulsions between the charges. This increases viscosity.[16,17]

The chains, when entangling, also interact with each other and form stretches of double helices so that the network becomes mechanically more firm.[18,19] Each glucuronate unit carries an anionic charge at physiological pH associated with its carboxylate group. There are often hundreds of negative charges fixed to each chain. These charges are balanced by mobile cations such as Na^+, K^+, Ca^{++}, and Mg^{++}. The charges are important in determining solubility in water because hyaluronic acid, converted into an uncharged polymer by fully esterifying with methyl groups, is insoluble. The molecule has the properties of a highly hydrophilic (polyhydroxylic) material simultaneously with hydrophobic domains characteristic of lipids.

Table 1. Trade Names for Hyaluronic Acid and Sodium Hyaluronate and Trade Name Mixtures Containing These Ingredients[7]

Trade Names of Hyaluronic Acid		
AEC Hyaluronic Acid	Biomatrix	Hyaluronic Acid SOL
Trade Name Mixtures of Hyaluronic Acid		
Amisil-HA	BioCare BHA-10	BioCare HA-24 Bio
BioCare Polymer BHA-10	BioCare Polymer HA-24	BioCare SA
Biosil Basics HMV - Hair Moisture Complex	Biosil Basics HMW - Hair Moisture Complex	Cromoist HYA
Cromoist WHYA	Glycoderm P	Lipocare HA/EC
Molecularsource LPC	PHYTO/CER.HA	
Trade Names of Sodium Hyaluronate		
Actimoist	AEC Sodium Hyaluronate	Avian Sodium Hyaluronate Powder
Avian Sodium Hyaluronate Solution	Bio-HE	Dekluron
HTL MYP Hyaluronic Acid	Hyaluronate Na F93	Hyaluronate Na F100
Hyaluronate Na 1.0% Gel	Hyaluronate Na P85	Hyaluronate Na P90
Hyaluronate Na P93	Hyaluronate Na P100	Hyaluronic Acid FCH-150
Hyaluronic Acid FCH-200	Hyaluronic Acid (Na)	Hyaluronic Acid, Sodium Salt
Hyaluronsan HA-L510	Hyaluronsan HA-M5070	Hyaluronsan HA-Q
Hyaluronsan HA-QSS	Hyaluronsan Solution HA-Q1P	Hyasol
Hyasol-BT	LMW Hyaluronic Acid Na Salt	Nikkol Sodium Hyaluronate
OriStart SH	RITA HA C-1-C	RITA HA C-1-P
Saccaluronate CW	Sodium Hyalronate HA-Q	Restylane
Hylaform		
Trade Name Mixtures of Sodium Hyaluronate		
Actiglide	Advanced Moisture Complex	Aragoline
Atecoron	Bellsilk HA	Biocrystal
Brookosome H	Chronosphere FHC/HA Blend	Chronosphere Hyaluronic
Collagen-Hyaluronic	Collagen-Hyaluronic Acid-Jelly	Desaron
EASHAVE	Essential Vital Elements - S	Gelhyperm (Avocado Oil)
Gelhyperm (Jojoba Oil)	Gelhyperm (Macadamia Nut Oil)	Gelhyperm (Seabukthorn Oil)
Gelhyperm (Wheat Germ Oil)	HA-Sol 2%	Hyaluronic Acid 1%
Hydralphatine 3P	Hydroxan	Hydroxan BG
Hydroxan CH	Iricalmin	Polyson HQ
Quiditat NwH	Ritacomplex DF 15	Ritacomplex DF 26
Rovisome H A	Saccaluronate CC	Saccaluronate LC
Spherica HA	Thioglycans	Toshiki BINS-3

Physical Form

Hyaluronic acid is available to the cosmetic formulator as a highly purified, freeze-dried powder or as an aqueous solution, and as its potassium or sodium salt.[20] In this form, hyaluronic acid slowly but fully dissolves in water to give viscous, clear to slightly opalescent and colorless solutions, which must be preserved in cosmetic usage. The viscosity can vary with the method of its preparation, decreasing sharply in the presence of electrolytes. Sodium hyaluronate is supplied as a white fiber-like or creamy white powder with a very faint odor. According to this author, it usually contains not less than 98% of the salt, although there are products with lower levels. It does not usually lose more than 10% of its weight on drying and normally gives not more than 20% of residue on incineration. A 0.2% water solution of sodium hyaluronate can have a pH range of 5.5 to 7.5. During the purification of sodium hyaluronate, which involves the removal of lipids, proteins, and nucleic acids, its molecular weight quickly drops. Sodium hyaluronate dissolves slowly but completely in water to give a clear to faintly opalescent colorless and highly viscous solution. The salt is soluble in sodium chloride solution but almost insoluble in organic solvents. Up to 90% of the salt can be insoluble in ethanol. This author also stated that aqueous solutions of sodium hyaluronate must be preserved, using 0.4% to 0.75% phenoxyethanol, for example, as the material is a rich source of nutrients for microbes.

Method of Manufacture/Sources

Hyaluronic acid and sodium hyaluronate historically are derived from rooster combs but can be prepared from human umbilical cords.[21] Hyaluronic acid is present in the perivascular connective tissue of the umbilical cord and is known as Wharton's jelly.[13] Hyaluronic acid also has been derived from bovine tracheas and bovine vitreous.[22] Hyaluronic acid and sodium hyaluronate of high molecular weight and purity are difficult to prepare because long chains of these molecules are easily broken by shear forces and are easily degraded by free radicals produced by ultraviolet radiation and oxidative agents.[1,13,23]

Billek and Billek described a process to produce a completely "protein-free" solution of hyaluronic acid.[8] A patent issued in Germany and several other European countries is for the process of purifying the vitreous bodies of pig eyes by lowering the pH to 4.2. The proteins form an insoluble complex with the hyaluronic acid that can be separated by centrifuging. The remaining clear, highly viscous solution is then adjusted to a physiological pH of 7.0, put into ampules, and sterilized.

Two of the hyaluronic acid gels manufactured specifically for dermal augmentation are from different sources.[24] Hylaform is produced by extraction from rooster combs, has a high molecular weight but a lower concentration (6 mg/mL), and its viscoelastic properties have a more elastic tendency. Restylane is produced by bacterial fermentation, has a lower molecular weight but a higher concentration (20 mg/mL), and its viscoelastic properties have a more viscous tendency.

In cosmetics, the only sources of hyaluronic acid used are from bacterial fermentation and rooster combs, with molecular weights between 5 and 1800 kD.[25] These 2 processes are described below.

From Bacteria

Hyaluronic acid of low molecular weight has been found in the capsules of bacteria such as Group A and C hemolytic *Streptococci* and *Pneumococcus* type II stain D39R.[26-32] Bacterial hyaluronic acid is industrially produced from *Streptococcus zooepidemicus* and *Streptococcus equi* where the microorganism produces hyaluronic acid and lactic acid from carbon and nitrogen sources.[9,22,31] Other bacteria that produce hyaluronic acid include *Streptococcus dysgalactiae*, *Staphylococcus aureus*, and *Streptococcus pyogenes*.[31,33-37]

To produce hyaluronic acid, bacteria was collected from a surface swab of guinea pig conjunctiva. *S zooedidemicus* was isolated and transferred to a Sakaguchi flask containing culture medium.[31] At the maximum cell titer, the culturing process was terminated, and benzyl alcohol was added for sterilization. After activated carbon and alumina were added to the growth media and agitated, the liquid was then filtered until transparent. Hyaluronic acid was precipitated by the addition of sodium and methanol. After thoroughly removing the methanol, the mixture was dried to a white powder.

From Cockscombs

Balazs was issued a patent in 1981 for sodium hyaluronate extracted from cockscombs to be used in cosmetics.[8,38] This product contained protein (50% to 400% relative to the extracted hyaluronic acid). By heating the extract to 100°C, some of the polysaccharide is broken down, leaving part of the product as high molecular weight (HMW) sodium hyaluronate and the rest as low molecular weight (LMW) sodium hyaluronate.

Crosslinking

Hyaluronic acid chains are crosslinked to stabilize the polysaccharide in such a way as to not affect the 2 specific groups of the molecule, the carboxylic and N-acetyl groups.[39] This crosslinking process produces a less dense structure than that of the native hyaluronic acid, resulting in a partial specific volume of 0.63 cc/g compared with hyaluronic acid's 0.57 cc/g (both in 0.15 N NaCl). Because only a limited number of the polysaccharide chains of hyaluronic acid are permanently associated through a methylene–bridge-protein–methylene bond, the hydrated molecules form an elastoviscous solution, which is called hylan fluid. The other hyaluronic acid crosslinking process utilizes vinyl sulfone, which reacts with the hydroxyl groups of the polysaccharide chain to form an infinite network through sulfonyl-bisethyl crosslinks.

Hyaluronic acid used for soft tissue augmentation is stabilized with carbon bridges every 2 to 500 U of disaccharide.[40] It is an epoxy, also used in many household glues, which hydrolyzes irreversibly into harmless carbon chains in hours.

Hylan B gel, sold as Hylaform (Biomatrix, Inc., Ridgefield, NJ) for tissue augmentation is stabilized

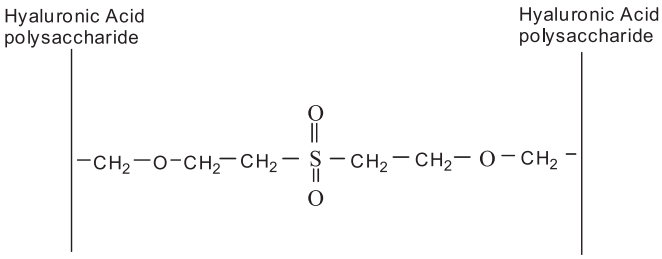

Figure 2. Chemical structure of the crosslink in insoluble Hylan B gel.[41]

hyaluronic acid.[41] The biomaterial retains the biocompatibility and biological properties of hyaluronic acid, but its residence time in dermal tissue is increased by introduction of sulfonyl-bis-ethyl crosslinks between the polysaccharide chains as shown in Figure 2.

Restylane (Q-Med Scandinavia Inc, Uppsala, Sweden) is produced in cultures of *Equine streptococci* by fermentation in the presence of sugar.[24] The fermentation material is then alcohol precipitated, filtered, and dried. The hyaluronic acid chains are then chemically stabilized through permanent epoxidic crosslinks that the manufacturer reports to alter only 1% of the hyaluronan molecular network. The material is heat sterilized in its final container.

Manna et al compared hyaluronic acid derivatives from rooster combs (Hylaform, subjected to crosslinking) and streptococcus (Restylane, stabilized as declared by the manufacturer), which were developed for soft tissue augmentation.[42] The researchers found that Hylaform functions as a strong hydrogel, has a minor quantity of crosslinked hyaluronic acid (about one fourth that of Restylane), and contains a low amount of protein (about one fourth that of Restylane). Restylane functions as a weak hydrogel and contains protein resulting from bacterial fermentation or added to enable crosslinking reaction. Hyaluronic acid and sodium hyaluronate may be crosslinked.[43]

Analytical Methods

Originally it was necessary to purify hyaluronic acid from tissue in order to measure the amount of hexosamine or hexuronic acid, which results in an accuracy within 100 μg.[2] Hyaluronate lyase from *Streptomyces hyalurolyticus* was also employed to degrade hyaluronic acid in the presence of other polysaccharides, and the resulting unsaturated disaccharides can be analyzed within 1 μg.

Hyaluronic acid can be detected and measured to within 1 ng in biological samples by specific radioassay making use of proteins with affinity for hyaluronic acid that are present in cartilage.[44] Proteins with affinity for hyaluronic acid also can be used for detection of the polymer using techniques similar to immunohistochemical methods.[45]

An absolute, weight average determination of molecular weight is obtained by using light scattering.[46] Milas et al suggested using this method to determine the range of molecular weights in a sample in combination with steric exclusion chromatography and refractometric detectors.[22]

Impurities

When hyaluronic acid is derived from animal sources, proteins may be present, which can affect the nonimmunogenic and immunogenic properties of the hyaluronic acid preparation.[6] To achieve high purity, a certain degree of depolymerization occurs, resulting in a lower grade product. For example, bovine hyaluronic acid (prepared by Etapharam, Vienna) contained approximately 10% of its total macromolecular content in protein.[47]

Proteins that bind to hyaluronic acid include hyaluronidases, antibodies, proteoglycans, and link proteins, cell-surface receptors for hyaluronic acid, and hyaluronic acid–binding proteins isolated from tissues and fluids.[2]

Balazs was granted a patent for "ultrapure" sodium hyaluronic acid.[8] The essential step that was different from other processes was to treat the hyaluronic acid solution, which had already had the proteins largely removed, with chloroform for up to 5 days. The purpose is to eliminate "a relatively undefined fraction ('inflammatory [H]yaluronic [A]cid') that is supposed to cause inflammation after injection into the eye."

The purity of hyaluronic acid may vary from one commercial batch to another.[48] The batch of human umbilical cord hyaluronic acid used in this study had <2% protein and <3% chondroitin sulfate. Using chondroitinase and protease digestion followed by thermic denaturation and dialysis against dissociating buffer to get rid of smaller fragments obtains an electrophoretically homogeneous hyaluronic acid preparation.

Clinically evident inflammatory reactions to viscous solutions of sodium hyaluronate are uncommon but may be related to impurities or deficiencies in formulation.[49] Reports of severe intraocular inflammation after cataract extraction with Viscoat (sodium hyaluronate; Alcon Laboratories, Fort Worth, TX) led to a recall of that product in 1987. The authors stated that the inflammation was probably due to the presence of endotoxins or other protein impurities in the visoelastic material, although the lots involved did meet standards for purity. The authors stated that this episode highlights the difficulty that manufacturers may have in detecting and eliminating impurities in long-chain HMW polymers from biological sources.

Three of the 7 hyaluronic acid preparations tested (source not given) contained significant levels of DNA in the range of 0.03 mg to 0.15 mg of DNA per milligram of hyaluronic acid with molecular weights ranging from 500 to >20 000 base pairs.[50]

Hyaluronic acid is easier to produce, extract, and purify as a polymer free of proteins from bacterial sources.[31] In contrast to animal sources, hyaluronic acid from a bacterial source exhibits superior reproducibility, high yields, and high degree of purity.[6] Hyaluronic acid for tissue augmentation from both rooster comb and bacterial sources is very pure and contains only low levels of impurities.[24]

Micheels concluded that hyaluronic acid was impure based on cited studies that showed that the hyaluronic acid extracted from sources other than rooster combs can be immunologically reactive.[40]

Based on the observation that avian influenza H5N1 virus is sensitive to heat, the 70°C temperature reached in the extraction of hyaluronic acid from cockscombs will also kill the virus.[51]

Use

Cosmetic Use

As given in the *International Cosmetic Ingredient Dictionary and Handbook*, hyaluronic acid functions as a skin-conditioning agent and/or as a viscosity increasing agent in cosmetic formulations.[7] Voluntary industry reports to the US Food and Drug Administration (FDA) include use in 223 products.[52] Hyaluronic acid is used in cosmetics in concentrations up to 1%.[53] The available data on frequency of use and use concentration as a function of product category are reported in Table 2.

Hyaluronic acid is used in "anti-aging" skin preparations.[6] LMW hyaluronic acid has been shown to increase the moisture level of damaged skin and to accelerate damage repair.[54] HMW hyaluronic acid solutions, when applied to the surface of the skin, form a hydrated viscoelastic film.[13] The film is permeable to air and cutaneous respiration is not obstructed while it "fixes" moisture to the skin surface.

Sodium hyaluronate is a skin conditioning agent–miscellaneous and is reportedly used in 601 products.[7,52] The maximum concentration of use for sodium hyaluronate is 2% in a body lotion.[53] At an application rate of 1 mg/cm^2 of a product, 0.02 mg hyaluronic acid/cm^2 of skin is contributed. The frequency of use as a function of cosmetic product category is reported in Table 2.

Potassium hyaluronate is a skin conditioning agent–miscellaneous and is reportedly used in 11 products.[7,52] No use concentrations were reported by industry.[53] The frequency of use as a function of cosmetic product category is reported in Table 2.

There are no restrictions of the use of hyaluronic acid, sodium hyaluronate, or potassium hyaluronate in cosmetics in Japan.[55] Hyaluronic acid, sodium hyaluronate, and potassium hyaluronate are not included among the substances listed as prohibited from use in cosmetic products marketed in the European Union.[56]

Cosmetics Aerosols

Hyaluronic acid, sodium hyaluronate, and potassium hyaluronate are used in products for which the application may be by aerosol spray.[52,53] Jensen and O'Brien reviewed the potential adverse effects of inhaled aerosols, which depend on the specific chemical species, the concentration, the duration of the exposure, and the site of deposition within the respiratory system.[57] Particle size is the most important factor affecting the location of deposition. The determination of the health consequences of exposure to an aerosol requires an analysis of the inhalation and deposition of the aerosol within the human respiratory system. The toxic action of an aerosol may be related to the number of particles, their surface area, or the mass deposited. Many occupational diseases are associated with the deposition of particles within a certain region of the respiratory tract. The aerosol properties associated with the location of deposition in the respiratory system are particle

Table 2. Cosmetic Product Uses and Concentrations for Hyaluronic Acid, Sodium Hyaluronate, and Potassium Hyaluronate

Product Category	Ingredient Uses[52]	Use Concentrations[53](%)
Hyaluronic Acid		
Bath products		
Other	-	0.001
Eye makeup		
Eye shadow	15	0.02
Eye lotions	5	-
Eye makeup remover	2	0.001
Other	11	0.07
Fragrance products		
Powders	1	-
Noncoloring hair care products		
Conditioners	2	-
Permanent waves	1	-
Tonics, dressings, etc	1	-
Hair coloring products		
Rinses	1	-
Makeup		
Blushers	7	0.02
Face powders	5	0.00005
Foundations	24	0.002
Lipsticks	-	0.01
Makeup bases	22	-
Other	-	0.001
Nail care products		
Cuticle softeners	1	0.001
Other	1	0.01
Personal hygiene products		
Other	1	-
Shaving products		
Aftershave lotions	1	-
Shaving creams	3	0.3
Skin care products		
Skin cleansing creams, lotions, liquids, and pads	6	0.001
Face and neck creams, lotions, powders, and sprays	8	0.1
Body and hand creams, lotions, powders, and sprays	12	0.001-1
Foot powders and sprays	1	-
Moisturizers	37	0.001-0.1
Night creams, lotions, powders, and sprays	17	0.02
Paste masks/mud packs	13	0.001
Other	23	0.001
Suntan products		
Suntan gels, creams, liquids, and sprays	1	-
Indoor tanning preparations	1	0.001
Total uses/ranges for hyaluronic acid	223	0.00005-1
Sodium Hyaluronate		
Baby products		
Shampoos	-	0.5
Lotions, oils, powders, and creams	-	0.5
Other	-	0.5
Bath products		
Oils, tablets, and salts	-	0.5
Soaps and detergents	1	0.001-0.5
Bubble baths	-	0.001-0.5
Capsules	-	0.5

(continued)

Table 2. (continued)

Product Category	Ingredient Uses[52]	Use Concentrations[53](%)
Other	-	0.001-0.5
Eye makeup		
Eyebrow pencils	3	0.5
Eyeliners	4	0.001-0.5
Eye shadow	11	0.0001-0.5
Eye lotions	6	0.001-0.7
Mascara	9	0.0001-0.5
Other	16	0.0001-0.5
Fragrance products		
Perfumes	1	0.5
Powders	1	0.5
Sachets	-	0.5
Other	-	0.0002
Noncoloring hair care products		
Conditioners	5	0.001-0.5
Sprays/aerosol fixatives	-	0.5
Straighteners	1	0.5
Permanent waves	-	0.5
Rinses	-	0.001-0.5
Shampoos	6	0.001-0.5
Tonics, dressings, etc	-	0.02-0.5
Wave sets	-	0.5
Other	-	0.5
Hair coloring products		
Dyes and colors	-	0.5
Tints	-	0.5
Rinses	-	0.5
Color sprays	-	0.5
Lighteners with color	-	0.5
Bleaches	-	0.5
Other	2	0.5
Makeup		
Blushers	20	0.001-0.5
Face powders	15	0.0005-0.5
Foundations	27	0.001-0.5
Leg and body paints	1	0.5
Lipsticks	96	0.0002-0.5
Makeup bases	15	0.002-0.5
Rouges	10	0.0001-0.5
Makeup fixatives	3	0.05-0.5
Other	17	0.0001-0.5
Nail care products		
Basecoats and undercoats	-	0.5
Cuticle softeners	-	0.01-0.5
Creams and lotions	-	0.5
Nail extenders	-	0.5
Nail polishes and enamels	-	0.5
Nail polish and enamel removers	-	0.5
Other	-	0.5
Personal hygiene products		
Underarm deodorants	-	0.5
Feminine deodorants	-	0.001
Other	-	0.5
Shaving products		
Aftershave lotions	6	0.001-0.5

(continued)

Table 2. (continued)

Product Category	Ingredient Uses[52]	Use Concentrations[53](%)
Beard softeners	-	0.5
Men's talcum	-	0.5
Preshave lotions	-	0.5
Shaving creams	-	0.001-0.5
Shaving soaps	-	0.5
Other	2	0.5
Skin care products		
Skin cleansing creams, lotions, liquids, and pads	20	0.000001-0.5
Depilatories	-	0.5
Face and neck creams, lotions, powders, and sprays	48	0.005-1
Body and hand creams, lotions, powders, and sprays	25	0.0001-2
Foot powders and sprays	-	1
Moisturizers	151	0.000001-1
Night creams, lotions, powders, and sprays	11	0.0001-1
Paste masks/mud packs	12	0.005-0.5
Skin fresheners	10	0.05-0.5
Other	38	0.001-1
Suntan products		
Suntan gels, creams, liquids, and sprays	4	0.000001-1
Indoor tanning preparations	1	0.001-0.5
Other	3	0.001-0.5
Total uses/ranges for sodium hyaluronate	601	0.000001-2
Potassium Hyaluronate		
Skin care products		
Face and neck creams, lotions, powders, and sprays	6	-
Moisturizers	4	-
Paste masks/mud packs	1	-
Total uses/ranges for potassium hyaluronate	11	None reported

size and density. The parameter most closely associated with this regional deposition is the aerodynamic diameter, d_a, defined as the diameter of a sphere of unit density possessing the same terminal setting velocity as the particle in question. These authors reported that the mean aerodynamic diameter of respirable particles is 4.25 ± 1.5 μm. This value may be compared with diameters of anhydrous hair spray particles of 60 to 80 μm (typically, <1% are below 10 μm) and pump hair sprays with particle diameters of $\geq$80 μm.[58]

Noncosmetic Use

General Medical

Hyaluronic acid has been used to simulate neutrophil function in patients with extreme susceptibility to bacterial infections.[59,60] Hyaluronic acid is also used in ophthalmic surgery, treating joint inflammation in racehorses, drug delivery, orthopedics, cardiovascular aids, and wound healing.[6,61]

Hyaluronic acid has been tested as a treatment for lipodystrophy in AIDS patients.[62] Injections of hyaluronans is considered effective in relieving the pain and effects of osteoarthritis (OA).[63,64] Hyaluronic acid application also has potential in facilitating the regrowth of severed nerves.[65]

Eye. Hyaluronic acid at 0.1% provides therapeutic benefit in dry eyes, which is because hyaluronic acid has a long residence time in the conjunctival sac and prolonged contact with the cornea.[66,67] It also has been suggested for use in glaucoma and other eye surgery techniques.[68-71]

Surgery. Hyaluronic acid has also been used in cochlear implant surgery to lubricate the electrode on insertion as well as tympanic membrane repair and otosurgery.[72-74] Hyaluronic acid also has been used in urological surgery, particularly for vesicoureteral reflux Krauss.[75]

Table 3. Summary of Drug Delivery Applications of Hyaluronic Acid[277]

Route	Role of Hyaluronic Acid	Therapeutic Agents
Ophthalmic	Increased ocular residence of drug, which can lead to increased bioavailability	Pilocarpine, tropicamide, timolol, gentamycin, |tobramycin, arecaidine polyester, (S) aceclidine
Nasal	Bioadhesion resulting in increased bioavailability	Xylometazoline, vasopressin, gentamycin
Pulmonary	Absorption enhancer and dissolution rate modification	Insulin
Parenteral	Drug carrier and facilitator of liposomal entrapment	Taxol, superoxide dismutase, human recombinant insulin-like growth factor, doxorubicin, paclitaxel (Rosato et al[278])
Implant	Dissolution rate modification	Insulin
Gene	Dissolution rate modification and protection	Plasmid DNA/monoclonal antibodies

Sodium hyaluronate may be the solution best suited to provide a sufficiently thick mucosal layer by endoscopic injection for endoscopic mucosal resection resulting in fewer complications because the raised effect is maintained longer.[76] However, hyaluronic acid may stimulate cell growth of any residual tumor cells.[77] Hyaluronic acid has potential use in anesthesia because its presence prolongs the effects of tetrodotoxin blocks on rabbit nerves.[78]

Respiratory Function. Inhalation of hyaluronic acid (at respirable particle sizes) by persons with pulmonary emphysema may improve the elastic properties of the lungs thus improving respiratory function.[79]

Drug Delivery. Hyaluronic acid is used as a drug delivery agent.[80] Hyaluronic acid may be used as a skin substitute delivering antibiotics to burns or skin dressing and to accelerate healing.[81,82] The uses of hyaluronic acid in drug delivery are shown in Table 3.

FDA Approvals. Deflux (Q-Med Scandinavia Inc, Uppsala, Sweden) is used to treat children who have vesicoureteral reflux, an abnormal condition in which urine flows backwards from the bladder to the kidneys.[83] This condition causes repeated, severe urinary tract infections, which can harm the child's kidneys. This treatment should not be used in patients who have one kidney that does not work normally, an abnormal pouch in the bladder wall, an extra ureter, a urinary tract infection, or abnormal urination. Deflux should never be injected into blood vessels.

Healon GV (Abbott Medical Optics, Inc, Abbott Park, IL) is indicated for use in anterior segment ophthalmic surgical procedures.[83] Healon GV creates and maintains a deep anterior chamber to facilitate manipulation inside the eye with reduced trauma to the corneal endothelium and other ocular tissues. Healon GV also can be used to efficiently maneuver, separate, and control ocular tissues.

Synvisc (Genzyme Corporation, Cambridge, MA) and Nuflexxa (Savient Pharmaceuticals, Inc., East Brunswick, NJ) are indicated for the treatment of pain in osteoarthritis of the knee in patients who have failed to respond adequately to conservative nonpharmacological therapy and simple analgesics, for example, acetaminophen.[83,84]

Restylane is used in cosmetic surgery for the correction of wrinkles and facial sulci, including perioral and periorbital wrinkles, wrinkles in the genal region, lower lip elevation, and drooping of the nasogenian sulcus. It is also used in lip contour and augmentation as well as correcting depressed scars. It is contraindicated in patients with severe allergies manifested by a history of anaphylaxis, history or presence of multiple severe allergies, or allergies to gram-positive bacterial proteins.[85]

Hyaluronic acid's use in correction of glabella wrinkles (ie, between the eyebrows, above the nose) is controversial because this region is crisscrossed with relatively large blood vessels near the skin surface and can lead to the possibility of severe complications, including blindness.[86] Current and potential noncosmetic uses for hyaluronic acid are listed in Table 4.

General Biology

Natural Occurrence

Hyaluronic acid naturally occurs in the human body in the avascular body compartments like the synovial fluid and vitreous humor.[67] It is also abundant in tendon sheaths and bursae and found in the small amounts of fluid in the "serous" cavities (pleura, pericardium, and peritoneum) and in the less well-

Table 4. Current and Potential Noncosmetic Applications of Hyaluronic Acid

Application	Reference
Ophthalmic	
Comfort eye drops	Tan et al[6]
Cataract surgery to protect endothelium and maintain anterior chamber dome during surgery	Tan et al[6]
Vitreous substitute - Healon	Tan et al[6]
Penetrating keratoplasty (corneal transplantation)	Goa and Benfield[279]
Trabeculectomy in glaucoma management	Goa and Benfield[279]
Retinal reattachment	Goa and Benefield[279]
Trauma surgery	Goa and Benefield[279]
Orthopedic	
Joint mobilization during bone setting	Tan et al[6]
Arthroscopic surgery	Tan et al[6]
Surgical	
Ear surgery (eg, provides support to allow hearing of perforated ear drums)	Tan et al[6]
Nose and throat surgery	Tan et al[6]
Antiadhesive applications after abdominal surgery and in hand tendon surgery	Tan et al[6]
Spacing applications (eg, fallopian tube surgery)	Tan et al[6]
Encapsulate cells for implantation	Laurent[280]
Osteoarthritis	
Possible human use based on proven effects in horses	Tan et al[6]
Rheumatoid arthritis	Goa and Benfield[279]
Wound healing	
Silver hyaluronate provides slow release of Ag^+ (microbiocidal) into wounds	Tan et al[6]
Hyaluronic acid applied topically or in dressing	Goa and Benfield[279]
Other	
Drug delivery vehicle - skin patch	Tan et al[6]
Drug delivery vehicle - topical	Brown et al[145]; Brown and Jones[277]
Drug delivery vehicle - nasal spray	Surendrakumar et al[153]
Allergy response prevention	Scuri[152]
Vocal cord implants	Laurent[280]

defined planes of tissue movement such as those between muscle bodies and skin. Hyaluronic acid persists between the individual fibers, spindles, and septa in skeletal and cardiac muscle.[87]

The largest amount of hyaluronic acid (7-8 g per average adult human, ~50% of the total in the body) resides in skin tissue, where it is present in both the dermis (~0.5 mg/g wet tissue) and the epidermis (~0.1 mg/g wet tissue).[4] The actual concentrations of hyaluronic acid in the matrix around the cells in the epidermis (estimated to be 2-4 mg/mL) are an order of magnitude higher than in the dermis (estimated to be ~0.5 mg/mL).

Based on this information, the CIR Expert Panel calculated that approximately 0.6 mg/g hyaluronic acid resides in the skin. Approximately 15% of a 60 kg (132 lb) woman is skin (CTFA 2006), yielding a total of approximately 9000 g of skin. The average woman has a total surface area of 16 900 cm^2.[88] Dividing the weight of skin by the area of skin yields a weight per area of skin: 9000 g / 16 900 cm^2 = 0.53 g skin/cm^2. By using the estimate of hyaluronic acid in skin by weight of approximately 0.6 mg/g, the amount of hyaluronic acid in skin by area is estimated to be the following: 0.6 mg hyaluronic acid/g × 0.53 g/cm^2/1 g skin = 0.318 mg hyaluronic acid/cm^2.

HMW hyaluronic acid, in the range of millions of daltons, is present in cartilage, in the vitreous of the eye, and in synovial fluid joints.[89] These authors noted that HMW hyaluronic acid inhibits the growth of blood vessels, while LMW hyaluronic acid fragments are highly angiogenic and are potent stimulators of blood vessel growth.

Biosynthesis

Synthesis of hyaluronic acid increased during mitosis.[90] When synthesis was blocked by an inhibitor, the cells were arrested in mitosis. The authors concluded that hyaluronic acid was required for the

detachment of the cells from their substrate before they could divide. Hyaluronic acid is biosynthesized in the outer cell membranes.[19]

In the skin, hyaluronic acid is synthesized primarily by dermal fibroblasts and by epidermal keratinocytes.[91] In fibroblast cultures, the rate of hyaluronic acid biosynthesis is regulated in part by cell density. At low cell densities, biosynthesis is high, and cell motility and cell proliferation are also high. At high cell densities, cell proliferation is low, and hyaluronic acid biosynthesis is shut down.[92]

Biological Functions

Biological functions involving hyaluronic acid and its salts include water retention in the matrix, tissue hydration, water homeostasis, lubrication, solute transport, cell migration, neutrophil adhesion, cell interaction, cell division, bone resorption, fetal wound healing, wound healing, development, and red blood cell aggregation and adhesion.[11,12,74,93-115]

Chondrocytes are dependent on a hyaluronic acid coat for the deposition of the cartilage matrix.[116] In egg fertilization, a layer of hyaluronic acid must be penetrated by the sperm, accomplished by virtue of hyaluronidase on the sperm head.[117] Sliwa suggested that hyaluronic acid plays a part in the attraction of sperm to the ovum.[118]

Hyaluronic acid in the extracellular matrix binds to cells through specific cell-surface receptors, including CD44 and the receptor for hyaluronan-mediated motility (RHAMM), leading to intracellular signaling and modification of cell behavior.[119]

Role in Development

Hyaluronic acid plays a role in mammalian development. For example, the concentration of hyaluronic acid is high during the morphogenetic phase of development and removed by hyaluronidase during differentiation.[120,121]

Possible functions for hyaluronic acid during development include promoting the detachment of cells from a substrate, disruption of intercellular junctions permitting cell migration, reduction of the effect of fibronectin on spreading and motility of neural crest cells, increased cell motility, vascularization control, cell protection, and embryo implantation.[122-129] Hyaluronic acid has been shown to be important to the development of the cornea, neural tube, mammary ducts, placenta, and epidermis.[130-134]

Immunological Effects

There are divergent reports on the effect of hyaluronic acid on macrophages.[2] Relatively high concentrations of HMW hyaluronic acid inhibit the movement and phagocytosis of macrophages, presumably due to the viscosity of the medium, while lower concentration enhance their phagocytosis and pinocytosis.[101,135-137]

Suppression of the formation of humoral precipitating antibodies to certain major classes of proteins present in the peritoneal fluid from a patient with Wilm's tumor was reported.[138] Their findings were interpreted to suggest that hyaluronic acid can interfere with both the elicitation of a complete antibody response and the formation of "normal" patterns of antigen-antibody precipitates in laboratory tests. The authors stated that their results support the possibility that hyaluronic acid may play an immunoregulatory role by masking potential immunogens.

Hyaluronic acid functions in U937 macrophage proliferation and death.[139] Hyaluronic acid inhibited U937 macorphage proliferation by impeding cell proliferation and inducing apoptosis.

Hyaluronic acid plays an important role in mucosal host defense in the lungs.[140] Hyaluronic acid retains and regulates enzymes important for homeostasis at the apical mucosal surface of the lungs, releasing them and activating some of them into the airway lumen at times of insult to the epithelium when hyaluronic acid is degraded. Because LMW hyaluronic acid increases ciliary action in the lungs, simultaneous with the release of enzymes, smaller hyaluronic acid fragments stimulate ciliary beating (through its interaction with RHAMM) and hence the clearance of foreign material from mucosal surfaces.

Infection Control

Pericellular hyaluronic acid of human synovial cells can interfere with infection by the Newcastle disease virus.[141] The authors hypothesized that cell-bound hyaluronic acid occurs as a dense gel and would be expected to impede the access of viruses to the plasma membrane.

Table 5. Compositions of Skin Models Used by Trommer et al[143]

System	Lipids	Concentration (μM)	Manufacturing Process
Simple system (A)	α-Linolenic acid	100	Shaking for 120 min
Complex system (B)	α-Linolenic acid	100	Liposomes prepared by the thin layer method
	Dipalmitoyl Phosphatidylcholine	200	
	Cholesterol	100	
Complex system with ceramide III (C)	α-Linolenic acid	100	Liposomes prepared by the thin layer method
	Dipalmitoyl Phosphatidylcholine	200	
	Cholesterol	100	
	Ceramide III	100	
Complex system with ceramide IV (D)	α-Linolenic acid	100	Liposomes prepared by the thin layer method
	Dipalmitoyl Phosphatidylcholine	200	
	Cholesterol	100	
	Ceramide IV	100	

Cytotoxicity

The cytotoxicity of hyaluronan-based hydrogels to vascular smooth muscle cells harvested from the thoracic aorta of Sprague-Dawley rats were assessed.[142] Sodium hyaluronate was dissolved in Dulbecco's Modified Eagle Medium (DMEM) to form a 1% viscous mixture to form HyA. Dextran-tetramethyl-rhodamine and dextran were mixed in a 0.04% (w/w) ratio (dex-rhod/dextran) to form Dex 15-rhod. HyA was incorporated into a 20% solution of methacryloyl-dex in DMEM with photoinitiators to form Dex 15-HyA. HyA was combined with methacryloyl in an aqueous environment then glycidyl methacrylate (GMA) was added to the solution for a theoretical substitution of HyA60. The resulting powder was dissolved in DMEM to form a 1% (w/v) solution that was free-radical photopolymerized to form HyA60. The solutions/gels were placed in indirect contact with rat cells with nutrient supplement for 48 hours. Cells exposed to HyA60 were significantly less viable than cells from the control groups. None of the other solutions/gels had toxic effects.

Photoreactivity

The effects of hyaluronic acid and its degradation products on irradiation-induced lipid peroxidation were tested.[143] Liposomal skin lipid models of increasing complexity were used to quantify the effects of hyaluronic acid and hyaluronic acid fragments (from *S zooepidemicus*) under ultraviolet exposure in the presence of iron ions. The molecular weights of the 5 batches of hyaluronic acid, determined by laser light scattering, were 1×10^6 g/mol and 6, 3.3, 3.2, and 2.2×10^5 g/mol. The 4 model skin preparations were produced with the composition shown in Table 5. Ten millimolars ferrous sulfate was added to the samples as an electron donator and catalyst of the Haber-Weiss reaction to initiate reactive oxygen species (ROS) generation via a Fenton type reaction. Samples of each composition were irradiated with a UV-B dose of 0.25 J/cm^2, which corresponds to 2 to 3 times the human minimal erythemal dose with and without hyaluronic acid. A thiobarbituric acid (TBA) assay was used to determine the amount of malondialdehyde (MDA) in each exposed sample. The concentration of the TBA reaction products was lower for all of the samples treated with hyaluronic acid preparations ($P < .05$) than the untreated samples for all skin model systems. The authors reported that the mass spectrometry ion signals demonstrated photodegradation of hyaluronic acid. Electron paramagnetic resonance (EPR) spectroscopy was used to determine the effects of hyaluronic acid on ROS and on stable free radicals. There was no difference in the EPR signal intensities between the samples with the hyaluronic acid or its fragments and the reference. The authors concluded that neither hyaluronic acid nor its enzymatically degraded fragments are able to reduce stable free radicals. The authors suggested that exclusive topical administration of hyaluronic acid in cosmetic and pharmaceutical semisolid formulations could be protective for lipids within the skin.

Absorption, Distribution, Metabolism, and Excretion

Absorption/Dermal Penetration

Hyaluronic acid from human umbilical vein (1350-4500 Da) was applied in a citric buffer to a 1 × 6 cm area of the shaved backs of 11 male Sprague-Dawley rats.[144] The citric buffer was applied to a similar area elsewhere on the back as a control. The rats were treated twice daily for 5 days and killed 3 days after the final treatment. The skin was excised and processed for histology, and the number of blood vessels per unit length was calculated. The addition of hyaluronic acid to rat skin had no adverse effect on the morphology of the skin. The overall mean blood vessel number per millimeter was significantly higher in treated skin than untreated skin. The hyaluronic acid penetrated to a maximum depth of 136 μm beneath the epidermis. These authors also applied radiolabeled hyaluronic acid (^{3}H-glucosamin; 10 μCi/mL) in a citrate buffer to a 1 cm^2 circle shaved on the back of one young adult Spraque-Dawley rat under general anesthesia. After 4 hours, the shaved area was excised to the depth of muscle and processed for frozen sectioning. Sections, 40-μm thick, were cut from the fatty side upwards, dissolved in NaOH, and counted. Radiolabeled hyaluronic acid was found to penetrate rat skin to a maximum depth of approximately 800 μm. The highest values, in excess of 400 dpm, were evident at a depth of 500 to 700 μm, deeper that the maximum depth examined in the blood vessel experiment above.

Autoradiography was used to detect the dermal penetration of hyaluronic acid, in the form of [^{3}H]hyaluronan, using mouse skin. SKh/1 hairless mice, aged 3 to 6 months, were used.[145] One group of 4 was treated with radioactive gel and the other with nonradioactive gel. In the first part of the experiment, the mice were treated every 12 hours for 3 or 12 applications of approximately 50 mg (range, 44.7-63.1 mg) that was gently rubbed on a marked area of 5 to 6 cm^2 on the dorsum of the trunk. Twelve to 16 hours after the last application, the mice were killed and the skin fixed and examined autoradiographically. Radiolabel was found mainly in the dermis, from the outermost layer down to the lymphatic and blood vessels just above the platysma muscle. Counterstaining showed that silver grains were clearly confined between collagen bundles. There were grains aggregated at hair follicles and the keratinous layers of the epidermis. The authors stated that these findings were an indication that there was failure to penetrate these areas, more rapid transit through them, or degradation within these areas.

In a second experiment using 10 mice, radiolabel was found in the same distribution within the dermis at the 2-hour time point, with the radiolabel concentrated within cell boundaries in the basal epidermis, the dermal matrix, and in the lining cells of the lymphatic sinuses.[145] Grains were also found in the keratinized layer of the skin and in the rudimentary hair follicles. The authors stated that in both of the mouse studies the fraction of hyaluronic acid recovered from the skin surface was small from 8 hours onward. Because the quantity of hyaluronic acid recovered from within the skin was high after 30 minutes but was similar to control after 1 to 8 hours, the authors suggested that levels equilibrated at approximately 1 hour. In the bloodstream, there was a significant macromolecular content as early as 30 minutes after gel application, which the authors identified as hyaluronic acid. Because the molecular weight profile of hyaluronic acid in the bloodstream (3.6×10^5 Da) was only slightly lower than that applied to the skin (4×10^5 Da), the authors concluded that passage through the skin was not restricted to smaller polymers. From 4 to 8 hours after application, increased amounts of radioactivity were found, but mainly as the metabolites of the labeled acetyl group of hyaluronic acid. As in serum, relatively little hyaluronic acid was found intact after 4 hours, but the authors stated that there was clear evidence that hyaluronic acid had been absorbed and its metabolic degradation had begun within 1 to 2 hours after application. Mice receiving one or several applications did demonstrate lower proportion of [^{3}H] in the skin after 12 to 16 hours.

Distribution

The transfer of radioactivity into the fetuses of pregnant rats injected intravenously with ^{14}C-SL-1010 sodium hyaluronate was examined.[146] SD series pregnant rats were injected on day 17 of pregnancy (number of rats not provided). Autoradiograms of the entire body were taken at 1, 4, 24, 48, and 72 hours after the injection. The dams were killed by freezing. The brain, Harder's gland, lungs, heart, liver, spleen, kidneys, adrenal gland, placenta, and amniotic fluid were collected from the dams. Three to 5 whole

Table 6. Tissue Concentration of Radioactivity After Intravenous Administration of ^{14}C-SL-1010 (A Formulation of Sodium Hyaluronate) to Rats on Day 18 of Pregnancy[146]

	Radioactivity (μg equivalent of SL-1010/g wet tissue)		
Sample	1 h (n = 2)	4 h (n = 3 or 4)	24 h (n = 3 or 4)
		Dam	
Plasma	211.791	117.343 ± 29.934	3.082 ± 0.209
Blood	126.571	90.098 ± 19.890	2.071 ± 0.098
Brain	2.797	2.314 ± 0.212	0.633 ± 0.141
Harder gland	6.839	20.462 ± 3.530	29.072 ± 6.048
Heart	12.217	7.966 ± 2.749	1.624 ± 0.108
Lung	26.396	16.915 ± 2.910	3.135 ± 0.121
Liver	31.667	35.159 ± 2.208	13.256 ± 1.190
Kidney	20.529	29.369 ± 4.214	3.932 ± 0.184
Spleen	21.166	24.712 ± 1.687	17.103 ± 2.048
Adrenal	14.753	13.970 ± 0.552	6.500 ± 1.108
Placenta	22.195	18.271 ± 2.962	3.267 ± 0.173
Amniotic fluid	0.219	0.870 ± 0.438	0.185 ± 0.008
		Fetus	
Whole body	0.608	2.141 ± 0.443	3.432 ± 0.499
Fetal liver	0.913	4.347 ± 1.339	6.427 ± 1.028

fetuses per dam were homogenized, and 3 to 5 livers were collected from other fetuses in the litters. The radioactivity of the fetuses increased over time until 24 hours after the injection and was maintained until 48 hours then reduced after 72 hours. Radioactivity was distributed throughout the body of the dams 1 to 4 hours after administration and decreased in the tissues of the entire body over time.

The authors repeated the above injection to pregnant rats on day 18 of pregnancy.[146] At 1, 4, and 24 hours after the injection, the dams were killed, and the plasma was obtained. The distribution of radioactivity in the tissues is shown in Table 6 and follows the same general pattern seen in the first experiment. Lactating rats (n = 3) were mildly ether-anesthetized on the 15th to 17th days after giving birth 1, 4, and 24 hours after administration of ^{14}C-SL-1010 sodium hyaluronate (10 mg/kg) to the caudal vein. Milk was collected (16-200 μL), and blood from the ocular fundus vein was collected at the same time. Radioactivity in the milk increased with time until 24 hours. The radioactivity in the plasma was highest at 1 hour and decreased with time. The authors stated that sodium hyaluronate was nontoxic to the dams and offspring.

In another experiment, the authors collected milk from 2 lactating rats and divided the fractions into total casein, casein fraction, calcium (Ca) binding casein fraction, hyaluronic acid fraction, and supernatant fraction after binding with cetylpyridinium chloride, and measured the radioactivity of each.[146] Total casein contained 77% to 84% of the radiolabel. A subset of that, the casein fraction, contained 56% to 63% of the radiolabel and the Ca binding casein fraction of that contained around 21% of the radiolabel. The hyaluronic acid fraction was 0.2% to 0.5% (almost background level). Approximately 20% of the radioactivity was in the supernatant fraction.

Metabolism

The turnover of hyaluronic acid was tested by injecting it into the anterior chamber of the eyes of female New Zealand White rabbits, weighing 2.7 to 3.4 kg.[147] The test material was purified; [^{3}H]hyaluronic acid was dissolved in phosphate-buffered saline containing 1% dimethyl sulfoxide. The weight-average molecular weight was 920 000 and a number average of 80 000. [^{3}H]Hyaluronic acid (3.8 mL) was mixed with 1.93 g of commercial hyaluronic acid (Healon), then concentrated until the original volume of commercial hyaluronic acid was reached. The hyaluronic acid concentration of the mixture was determined by radioassay to be 10 mg/mL. Two rabbits had 0.055 mL of aqueous humor removed and replaced by the labeled hyaluronic acid mixture, and 2 more had 0.2 mL replaced. In addition, 1 rabbit was injected in the anterior chamber with a trace amount of [^{3}H]hyaluronic

acid that had not been mixed with the commercial hyaluronic acid; 1 rabbit was injected intramuscularly in the thigh with 0.1 mL with the mixture, and 1 other rabbit was injected subcutaneously in the back of the neck with 0.1 mL of the mixture. Two-milliliter blood samples were collected before the injections and approximately 1, 2, 4, 8, and 24 hours after the injections. Blood samples were then collected daily for 4 days then every other day for 9 to 13 days. ^{3}H radiolabel was detectable 2 to 3 hours in blood after injection into the eye. It reached its maximum within 2 days and then decreased exponentially. The half-life of the [^{3}H]hyaluronic acid with the 0.2 mL injections was approximately 14 hours and, with the 0.055 mL injections, was approximately 8 hours. The subcutaneously injected [^{3}H]hyaluronic acid had a half-life of approximately 50 hours, and the intramuscularly injected material had a half-life of approximately 30 hours. The authors stated that these times should be adjusted for an approximate 45-minute delay due to metabolism of hyaluronic acid in the liver.

The time it takes for excess hyaluronic acid to clear the blood of sheep at normal levels and at increased levels was investigated.[148] Ten Merino ewes, weighing between 20 and 38 kg, were fitted with venous and arterial catheters for infusion and blood sampling in the right jugular vein and the right carotid artery, respectively. A baseline blood sample was taken; then a 20 μg intravenous tracer dose of [^{3}H]hyaluronic acid was given. Arterial blood samples were drawn at 2, 4, 6, 8, 10, 15, 20, and 30 minutes after injection to determine half-life. Three to 4 hours later, a 2 mg intravenous bolus dose of unlabeled hyaluronic acid was given immediately followed by a 40-minute intravenous infusion of additional hyaluronic acid at a rate of approximately 125 μg/min (2 mL/min). This dosage was estimated to give a steady-state plasma concentration of approximately 1 μg/mL to 2 μg/mL; the normal level is 0.12 $\pm$ 0.05 μg/mL. Arterial blood samples were taken at baseline, 5, 10, and 15 minutes after the bolus dose. Twenty micrograms of [^{3}H]hyaluronic acid were then given, and arterial blood samples were taken 2, 5, 20, and 40 minutes later. The hyaluronic acid infusion was stopped, and blood samples were taken 1, 5, 10, 20, and 40 minutes postinfusion. [^{3}H]Hyaluronic acid half-life was 5.3 $\pm$ 1.1 minutes (range, 3.3-6.5 minutes). The authors stated that both elimination curves obtained fitted well to a linear, 1-compartment, kinetics model. After bolus injection, the mean plasma concentration of hyaluronic acid was 2.42 $\pm$ 0.48 μg/mL. The maximum metabolic rate was 0.062 $\pm$ 0.009 μg/mL/min or 3.32 $\pm$ 0.30 μg/min/kg body weight. The elimination of [^{3}H]hyaluronic acid at the plateau level occurred with a half-life of 26.9 $\pm$ 7.0 minutes (range, 18.2-43.5 minutes). The authors reported that there were no adverse effects or raised hyaluronic acid levels in the bloodstream of the sheep.

Laurent and Fraser repeated the experiment of Laurent et al on cynomolgus monkeys.[149] Hyaluronic acid labeled with ^{3}H in the acetyl group of the N-acetyl-glucosamine was produced and purified. The weight-average molecular weight was 4.2 $\times$ 10^{6}, which was reproducible after 7 months showing stability. Unlabeled hyaluronic acid with a concentration of 10 mg/mL was mixed as in the previously mentioned experiment. Twelve cynomolgus monkeys of both sexes, weighing between 2.3 and 4.9 kg, were used. Injections were through the peripheral cornea into the anterior chamber. Aqueous humor (100 μL) was withdrawn, and 50 μL of [^{3}H]hyaluronic acid was injected into the eye of 5 monkeys. The procedure was repeated using 75 μL on 3 monkeys, and 4 additional monkeys were treated with 50 μL of [^{3}H]hyaluronic acid with the addition of pilocarpine, 10 μL at 4%, dropped into the eye at 1, 8, and 24 hours after injection. The authors reported that ^{3}H in the blood could be detected 2 to 3 hours after injection. It reached a maximum at 2 to 3 days in the animals not given pilocarpine and at 1 day in the monkeys treated with pilocarpine. After reaching maximum concentration, the [^{3}H] in plasma decreased exponentially. The authors stated that the mean half-life for the 8 monkeys without pilocarpine treatment was 21 hours and 9.5 hours for the pilocarpine-treated animals. The researchers found about the same time for the half-life from the eye when both volumes were used (average of 21 $\pm$ 3 hours) but showed a lag phase of a few hours followed by a more rapid turnover during the following 24 hours and often a slower rate later.

[^{3}H]Hyaluronic acid was injected into the pleural space of 6 adult New Zealand White rabbits weighing 2.2 to 2.7 kg. Injections ranged from 21.1 to 46.3 μg, and the molecular weight was 6 $\times$ 10^{6}.[150] Blood samples were taken from an ear vein at 0, 2, 4, 6, 8, 12, and 24 hours after injection. Blood samples were also collected every day after 4 days and every other day for the next week. The authors stated that when metabolized, the ^{3}H from the hyaluronic acid

appears as [^{3}H]H_2O in the blood and is proportional to the loss of hyaluronic acid from the pleural space. The half-life for pleural hyaluronic acid varied with the amount of [^{3}H]hyaluronic acid injected and was in the 8- to 15-hour range.

Excretion

A polyethylene tube was inserted into the bile duct of male rats under ether anesthesia.[146] After confirming the excretion of bile approximately 1 hour after treatment, ^{14}C-sodium hyaluronate (SL-1010) was administered at 10 mg/kg into the caudal vein. Bile was collected every 2 hours for 10 hours and then at 24 hours. The cumulative excretion rate into the bile up to 24 hours was 0.4% of the administered dose. The bile was analyzed using gel filtration column chromatography and the hyaluronidase digestion method. A major peak eluted at a LMW region centered on the fraction number 51. The lower peak that eluted in the others from the fraction numbers 20 to 45 was confirmed to be hyaluronic acid because it disappeared with the addition of hyaluronidase.

Effect on Penetration of Other Chemicals

Brown and Jones, in a review article, compared dermal penetration studies of hyaluronic acid and proposed that hyaluronic acid's influence as a drug delivery system may depend on the species of animal.[277] They noted that in mice, the drug reservoir forms in the deeper layers of the skin (dermis) compared with humans where it forms in more shallow layers (epidermis).

Hyaluronic Acid Produced During Inflammation

The effect of intraperitoneal infection on hyaluronic acid presence and turnover in the intraperitoneal cavity was investigated.[151] Nineteen New Zealand White rabbits, male and female, with an average weight of 2.9 kg, were injected with irritating agents to induce peritonitis. The irritant was made up of suspensions or solutions of 0.1% latex beads, 3% thioglycolate, and 2% starch in phosphate-buffered saline (PBS). The authors used a peritoneal lavage with 100 mL of PBS at 1, 2, 3, 4, or 5 days. An additional 5 control rabbits, male and female, average weight of 2.9 kg, were lavaged without previous injection. The sham lavage was made up of PBS. One rabbit died within 24 hours after the injection, but the remaining animals had no clinical signs of distress. All the injected irritants caused a 10- to 1000-fold increase in hyaluronic acid concentrations in the lavage fluid on days 2 and 3, with large individual variations.

Animal Toxicology

Acute Toxicity

Oral

No deaths in ICR mice orally administered >1200 mg/kg hyaluronic acid were produced by fermentation.[31] No further details were provided.

Peritoneal

Denlinger and Balazs injected 0.5 mL of buffer, sodium hyaluronate (n = 12), or highly purified sodium hyaluronate (n = 10) into the peritoneal space of random-bred white male rats.[21] Animals were killed and blood collected at 12, 24, 48, 72, and 96 hours after injection. The peritoneal cavity was injected with 10 mL of buffer. The abdominal wall was then gently massaged, and after the skin was removed from a large part of the abdominal wall, the injected fluid was extracted. The leukocyte count in the peritoneal wash reached its peak at 24 hours after sodium hyaluronate injection ($55 \pm 4 \times 10^6$ cell/animal). The count decreased at 48 hours (count not provided). The count returned to control level in the sodium hyaluronate at 96 hours. The highly purified sodium hyaluronate leukocyte count peaked at $36 \pm 3 \times 10^6$ cell/animal and was not significantly different from the control level. The authors concluded that highly purified sodium hyaluronate can be used as a surgical tool in the eye without any adverse effects.

Pleural Space

Allen et al injected 21.1 μg to 46.3 μg of [^{3}H]hyaluronic acid into the pleural space of 6 adult New Zealand White rabbits weighing between 2.2 kg and 2.7 kg.[150] After 15 days, the animals were killed. All 6 rabbits tolerated the pleural injections well and manifested no evidence of pneumothorax, such as respiratory stress. No toxicity was noted by the researchers.

Inhalation

Sheep allergic to human neutrophil elastase (HNE) were used to test hyaluronic acid as a way to block bronchoconstriction.[152] Using a disposable medical nebulizer, the sheep inhaled a placebo then the HNE (dissolved in PBS) 30 minutes later to establish a baseline. The sheep later inhaled 3 or 15 mg LMW hyaluronic acid (150 kD) or HMW hyaluronic acid (300 kD) dissolved in PBS. After either 0.5, 4, or 8 hours, the sheep were then challenged with HNE. Inhaled HNE in untreated sheep caused a short-lived bronchoconstriction reaching its peak quickly (0-5 minutes) after challenge and resolved within 30 minutes. Aerosolized LMW hyaluronic acid blocked the HNE-induced airway response for the 0.5- and 4-hour time period. The 8-hour time period was only partially effective at the 3 mg dose but was completely effective at 15 mg. There were no ill effects reported from the hyaluronic acid. In another set of trials, 6, 7.5, or 15 mg HMW hyaluronic acid was administered 8 hours before the HNE challenge. The 15 mg of HMW hyaluronic acid was completely effective while the 6- and 7.5-mg doses were less effective in blocking bronchoconstriction. There were no toxicities reported.

Short-Term Toxicity

Inhalation

Male Beagle dogs were used to test the effectiveness of using a mixture of hyaluronic acid and recombinant human insulin in dried powder form as an inhaled drug delivery system for insulin.[153] Hyaluronic acid formulations containing insulin (10% w/w) were found to extend the mean residence time in blood and terminal half-life when compared to spray dried pure insulin. There were no toxicities reported.

Implantation

Four samples of Restylane (20 mg/mL nonanimal stabilized hyaluronic acid) and 4 samples of high density polyethylene reference standard were implanted into the paravertebral muscles in each of 3 anesthetized rabbits.[154] After the rabbits were observed for 4 weeks, they were killed, and implant sites were examined macroscopically and microscopically. A white focus (possibly a deposit of hyaluronic acid) was seen at 3 of the 12 Restylane implant sites. Encapsulation, with a thickness of 1 mm, was seen around one of the 12 control implants. Microscopic examination revealed that there was minimal to slight chronic inflammatory cell infiltration at both types of implant sites. The fibrous membrane was graded minimal to slight around the control and minimal to marked around the Restylane implants.

Chronic Toxicity

Implantation

A study conducted by Q-Med AB of Sweden on Deflux was reported.[155] The product is composed of microspheres of crosslinked dextan suspended in a carrier gel of nonanimal, stabilized hyaluronic acid. A 2-year implant study of Defulx was conducted using 22 rabbits. The objectives of this study were to determine the biocompatibility and migration potential of Deflux when implanted into the rabbit bladder submucosa. In each rabbit, 1 g of the test article was injected submucosally into each of the following bladder sites: right and left bladder neck and right and left bladder wall. Follow-up evaluations were conducted at 1 week and 1, 3, 6, 12, and 24 months. The urinary bladder, pancreas, kidney, liver, lung, draining lymph nodes, and brain were removed and examined histologically for inflammation, infection, irritation, foreign body responses, tissue necrosis, and scarring. Other organs were examined for gross abnormalities. The microspheres induced a fibrous tissue reaction around each microsphere without any adverse inflammatory reaction.

Another 2-year implant study of Deflux was reported.[155] Twelve dogs were implanted with 2.5 g of the test article in the following sites: right and left urinary bladder neck and right and left urinary bladder wall. The follow-up evaluations were conducted at 2 weeks and 3, 6, 12, and 24 months. In each animal, the urinary bladder, pancreas, kidney, liver, lung, draining lymph nodes, and brain were removed and examined histologically for inflammation, infection, irritation, foreign body responses, tissue necrosis, and scarring. Other organs were examined for gross abnormalities. The microspheres remained in the tissue in all injection sites for at least 2 years without causing any adverse foreign body reaction. There were no inflammatory reactions.

Ocular Toxicity

A highly purified special fraction of sodium hyaluronate was used in an experiment testing the amount of

inflamation caused when using hyaluronic acid to replace the liquid vitreous in the eyes of owl monkeys (*Aotus trivirgatus*) and rhesus monkeys (*Macaca mulatta*).[21] Sodium hyaluronate from 2 sources of rooster combs and from human umbilical cord were also tested. The molecular weight varied between 1.2 and 3.0×10^6, and the concentration was 10.0 ± 2.0 mg/mL. Sodium hyaluronate was dissolved in a physiological balanced salt solution that was also used as the control substance. Sixty-seven owl monkeys and 15 rhesus monkeys were used in this experiment. In owl monkeys, 1 mL of the liquid vitreous, representing approximately half of the total, was slowly withdrawn from 1 eye and replaced with the test solution. The same was done with the rhesus monkeys except that only 0.5 mL vitreous liquid was replaced. Evaluation for inflammation by visual examination and leukocyte count was carried out 48 hours after surgery and at day 7. The leukocyte count in the aqueous humor varied between 0 and 115 cells/mm^3 in owl monkey eyes and between 0 and 85 cells/mm^3 in rhesus monkey eyes. The mean values were 28 ± 7 and 38 ± 13 cells/mm^3, respectively. The difference between these values was not significant ($P \approx .4$). Only 2 eyes had slight turbidity and slight haziness in the vitreous in the owl monkeys. Only 2 rhesus monkeys had slight turbidity and haze. In both species, these reactions completely disappeared within 72 to 96 hours after injection.

These authors also injected the highly purified sodium hyaluronate into the eyes of an undisclosed number of owl monkeys that had previous reactions to concanavalin A, endotoxin, and a less purified sodium hyaluronate after the eyes had been clear of the reactions for 1 month.[21] The highly purified sodium hyaluronate implantations did not cause any significant inflammatory reaction in the previously inflamed eyes. The leukocyte counts after a first and second implantation of sodium hyaluronate were not significantly different from normal owl monkey aqueous after highly purified sodium hyaluronate was injected 1 or 2 times.

These authors repeatedly, up to 6 times, injected sodium hyaluronate into owl monkey eyes.[21] The experiment started with 76 eyes and finished with 11. The average time between each injection was 5.5 months. The leukocyte count 48 hours after the first injection in 102 eyes varied between 0 and 200 cells/mm^3. The mean and standard error of the mean were 20 ± 3 cells/mm^3. In 71% of these implantations, the cell count was between 0 and 24. Repeated implantations of sodium hyaluronate did not increase the severity of the haze and flare or any immunogenic response.

In a continuation of the above studies, highly purified HMW factions of sodium hyaluronate were injected into 6 eyes of 5 owl monkeys.[21] Each received 2 to 4 injections over 5.5 years in 2 eyes, 6.5 years in 2 eyes, and 9 years in 2 eyes. All eyes were completely normal, having no pathology in the anterior segment, lens, vitreous, retina, or choroid.

Akasaka et al reported a "negative" result of an eye stimulus test based on the standard Draize test of hyaluronic acid produced by the fermentation.[31] No further details were provided.

Skin Irritation

Hyaluronic acid did not cause irritation in a single-stimulus skin test using Japanese rabbits and Hartley guinea pigs.[31] No further details were provided.

Skin Sensitization

Potassium hyaluronate prepared from human umbilical cords was tested for antigenicity using rabbits.[156] Potassium hyaluronate was purified by repeated extraction with 90% phenol solution and by repeated precipitation from aqueous solution at pH 9 to 10 by 1.25 vol of ethanol saturated with potassium acetate. Potassium hyaluronate was then centrifuged after treatment with *p*-nitrobezyl bromide and acidified to hyaluronic acid. The aminobenzyl ether of the hyaluronic acid was coupled to horse-serum albumin and whole rabbit serum (3.6 g protein to 1 g compound). Three rabbits were immunized with the horse serum–hyaluronic acid preparation by injection at 30 mg, 60 mg, and 120 mg subcutaneously at weekly intervals for the first course. The rabbits were bled 1 week postinjection. The second course consisted of 4 intravenous injections of 20 mg at weekly intervals. These animals were bled (second time) 10 days postinjection. Precipitin tests were carried out in bulk. Complement fixation was carried out using 2 minimal hemolytic doses of complement. Agglutination was tested on a washed suspension of cells from a young culture of a capsulated group C *Streptococcus* whose capsules were known to be dissolved by hyaluronidase. A positive agglutination reaction was reported with the horse serum albumin but not to test substances that contained rabbit serum. The presence or

absence of hyaluronic acid did not change the reaction. No adverse effects from the injections were reported.

The possibility of an antigenic response to hyaluronic acid derived from different sources was tested.[157] Hyaluronic acid was prepared from 2 different extractions from human umbilical cords and NY 5 strain of *Streptococcus*. The Streptococcal-derived hyaluronic acid was free of protein. The bacterial antigens were derived from 3 strains of group A beta hemolytic streptococci belonging to serological types 1, 4, and 14 and a strain of hemolytic *Streptococcus aureus*. Young adult rabbits weighting between 5.5 and 6.5 kg were inoculated with the antigens either by injection intravenously in the marginal ear vein, intramuscularly, or subcutaneously (n = 3 for each route). For the intravenous injections, each rabbit received 1 injection of 0.5 mL of antigen daily for 3 days during the first week (1 mg umbilical cord hyaluronic acids or 1.3 mg streptococcal hyaluronic acid adjusted to 1 mg after the first week). They were allowed to rest 4 days, then received 1 mL daily for 3 days then allowed to rest for 4 days. This continued for 7 more weeks for a total of 27 injections except that the doses of the seventh week were administered subcutaneously in a dose of 0.5 mL. Each rabbit was bled from the ear before treatment and after the injections of the third, sixth, and eighth day after the ninth week of injections. The serum was separated, merthiolate added, and stored at 4°C to 8°C. For the intramuscular and subcutaneous injections, the hyaluronic acid content was 1 mg/mL; the dose was 0.5 mL 3 times per week for an average of 23 injections. Each rabbit was bled before treatment, at 2-week intervals and 10 days after the last injection. The sera from the rabbits in each treatment were tested for the presence of antibodies against the group-specific C carbohydrate and for antitype-specific M protein. None of the rabbits had any noticeable physical reactions following injections of antigens. None of the rabbits developed precipitating antibodies against either umbilical cord or streptococcal hyaluronic acid.

These authors performed skin sensitization tests on rabbits with the same hyaluronic acid preparations as above.[157] Each rabbit was skin-tested with 0.1 mg in 0.1 mL of the antigen that it received: 2 types of umbilical cord hyaluronic acid or the streptococcal hyaluronic acid. Injection sites were observed immediately and at 24, 48, and 72 hours for erythema and induration. A test for nonprecipitating, skin-sensitizing antibodies was performed with the sera of rabbits that had received streptococcal or umbilical cord hyaluronic acid. Test sites were injected with 0.1 mL of each rabbit's serum intradermally into a normal rabbit. Forty-eight hours later, each prepared site was injected with 0.01 mL of the same hyaluronic acid, which had been employed to vaccinate the animal supplying the serum. The results were read at 30, 45, and 60 minutes. The skin test revealed slight erythema lasting for 2 days about the injections site in one rabbit, which had received *Streptococcus* strain 1, and in one, which had received hyaluronic acid from S *aureus*. A large area of erythema, fading gradually in 3 days, was observed about the injection site in one rabbit, which had received umbilical cord hyaluronic acid, and a small area of erythema lasting 1 day was in another rabbit, which had received the same antigen. A small raised area of induration, lasting for 2 days, and a similar reaction, plus a small amount of erythema, was observed in 2 rabbits, which received streptococcal hyaluronic acid. Erythema fading in 3 days was observed in one rabbit, which received streptococcus A-1 plus hyaluronic acid, and in one, which received S *aureus* plus hyaluronic acid. Erythema was observed at 1 hour in 2 rabbits, which received crude extract plus streptococcus A-4. At 24 hours, the area of erythema was reduced, and there were small areas of induration. The induration remained for several days. The results of the tests for nonprecipitating, skin-sensitizing antibodies were entirely negative during the 1-hour observation. The authors did not report any findings for the 24-, 48-, and 72-hour observations.

The antigenicity of streptococcal-derived hyaluronic acid was tested on rabbits.[158] Streptococcal-derived hyaluronic acid was from 5 sources (isolated by other researchers). Each rabbit was immunized with 1 mL (2 mg/mL isotonic saline) of streptococcal-derived hyaluronic acid emulsified with 1 mL Freund's complete adjuvant. Injections were given in multiple sites (intramuscular, subcutaneous, and intradermal). One month later, the rabbits were injected again with 1 mL (1 mg/mL) hyaluronic acid without adjuvant. Preimmunization and postimmunization sera were compared. The sera were analyzed by double diffusion studies using antiserum to streptococcal-derived hyaluronic acid. The authors stated that the findings indicated that the rabbits formed precipitating antibodies to the streptococcal hyaluronic acid, which, they speculated,

may indicate that the streptococcal hyaluronic acid crossreacts with proteoglycan from cartilage.

The immunogenicity of purified hyaluronic acid was evaluated using rabbits.[159] Hyaluronic acid derived from rooster combs and human umbilical cords was used. Hyaluronic acid preparations contained 0.28% to 0.57% of protein or peptides. Rabbits received 4 intramuscular injections at 4-weekly intervals using 1 of the following formulas: human umbilical cord hyaluronic acid, 500 μg/dose; human umbilical cord hyaluronic acid, precipitated with cetylpyridinium chloride (CPC), 500 μg/dose; human umbilical cord hyaluronic acid–albumin conjugate, precipitated with CPC, 500 μg/dose; and rooster comb hyaluronic acid at dose levels of 5, 50, and 500 μg. Rabbit sera were tested before, during, and after immunization for passive cutaneous anaphylaxis (PCA) reactive antibodies in guinea pigs. No formation of PCA reactive antibodies against any of the 4 hyaluronic acid preparations was observed during or after immunization.

The effect of hyaluronic acid on antibody responses to egg albumen, dog albumin, and birch pollen proteins was tested.[160] Hyaluronic acid derived from rooster combs (10 mg/mL) was used. In the first experiment, 1 μg egg albumen in Freund's complete adjuvant was subcutaneously injected into hooded Lister and BNW/FU rats of both sexes. Three weeks later, egg albumen was injected in the solutions (1) coprecipitated with hyaluronic acid (n = 8), (2) adsorbed to $Al(OH)_3$ (n = 9), (3) admixed with hyaluronic acid (n = 9), and (4) denatured with ethanol (n = 8). Passive hemaglutination testing was used for estimation of antibodies. Egg albumen adsorbed to $Al(OH)_3$ and coprecipitated with hyaluronic acid produced comparable secondary antibody responses, which were stronger ($P < .001$) than those obtained by egg albumen admixed with hyaluronic acid or by ethanol denatured egg albumen. An enhanced secondary response was also obtained ($P < .01$) by coprecipitation of dog albumin and hyaluronic acid. Enhanced, although less pronounced, antibody responses were obtained by an admixture of hyaluronic acid to dog albumin. In the same experiment with birch pollen as antigen, there was no enhancement of hyaluronic acid compared with saline. Adsorption to $Al(OH)_3$ resulted in moderately enhanced secondary antibody responses in the experiments with birch pollen protein.

In a second experiment, the authors tested the effect of systemic hyaluronic acid on antibody response in the rats.[160] Hyaluronic acid (1.1 mg/kg) or saline was injected 1 or 3 days before the first injection of dog albumin or 1 day before a second antigen injections. Hyaluronic acid administered 1 to 3 days prior to the priming injection of dog albumin enhanced the secondary response markedly compared with that seen using dog albumin suspended in saline ($P < .01$), independent of the time of administration.

In a third experiment, hyaluronic acid was simultaneously injected with the first dose of dog albumin (0.044 mg/kg) at doses of 0.044 mg/kg, 0.44 mg/kg, and 4.440 mg/kg at separate skin sites.[160] A booster dose of dog albumin was given 14 days later without additional hyaluronic acid. The various doses of hyaluronic acid induced an enhanced immune response to dog albumin. This was independent of the hyaluronic dose level within the range of 0.044 to 4.44 mg/kg. The enhancing effect was discernible ($P < .05$) both in the primary and the secondary response. There were no reactions in a maximization test.[31] No details were provided.

Rabbits were used to investigate severe acute inflammatory reactions to Hylan G-F 20.[31] Three groups of rabbits (n = 4) were immunized subcutaneously at 4 separate sites per rabbit at weeks 0, 1, 4, 12, 18, and 24. They were immunized with either sodium hyaluronate, Hylan G-F 20, or a crude rooster comb preparation. Serum samples were collected before each immunization and before the rabbits were killed at week 29. A heterogeneous chicken comb protein preparation was prepared and purified. The resultant chicken protein preparation and a purchased purified hyaluronic acid were used as antigens to coat enzyme-linked immunoassay plates for a direct binding assay to detect antibodies. None of the preparations elicited a significant hyaluronic acid–specific antibody response. All 4 rabbits immunized with the positive control, crude rooster protein, and 3 of the 4 rabbits immunized with Hylan G-F 20 exhibited an anti-chicken protein response. Two of 4 of this group responded after only 3 injections, and the anti-chicken protein titer was sustained over a period of several weeks. The authors stated that the lower maximum optical density readings obtained with sera from the hylan-immunized rabbits, compared with those obtained with sera from the rabbits immunized with the crude rooster protein, probably represented reactivity to only a subset of proteins contained in the heterogeneous crude rooster protein. None of the rabbits immunized with sodium hyaluronate had a detectable response to chicken protein.[31]

Table 7. Treatment Groups of Guinea Pigs to Compare the Sensitization of Noncrosslinked (SupArtz) and Crosslinked (Synvisc) Hyaluronic Acid[161]

Group	Test Substance	Dosage (mg/kg)	Concentration (mg/mL)	Volume (mL/kg)
1	Synvisc	0.5	0.5	1
2	Synvisc	2.5	2.5	1
3	Synvisc + CFA[a]	2.5	2.5	2a
4	SupArtz	2.5	2.5	1
5	SupArtz + CFAa	2.5	2.5	2a
6	No injection	NA	NA	NA
7	Egg albumen + CFA	1 mg/animal	2	1 mL/animal

NA, not applicable.
[a] Equal amount of complete Freund's adjuvant (CFA) and test substance.

Hartley guinea pigs were used to compare the sensitization by noncrosslinked (SupArtz, Smith and Nephew, London, England) and crosslinked (Synvisc, Genzyme Corporation, Cambridge, MA) hyaluronic acid.[161] In the first part of the experiment, the guinea pigs (n = 8) were anesthetized with isoflurane, weighed, and subcutaneously injected with hyaluronic acid or egg albumin according to the doses in Table 7. Injections were administered on days 0, 7, and 14. Controls were not given injections. Sera were prepared from blood collected from the medial saphenous vein 12 days after the final sensitization injection. A skin allergy test was performed on the sensitized guinea pigs. Fourteen days after the last sensitizing injection, each abdomen was shaved and 2 injections of phosphate buffered saline (PBS) were given. This was followed by 2 injections of 0.1 mg hyaluronic acid sensitizing agent that the animal was sensitized to or 0.01 mg egg albumen if the animal was sensitized to egg albumen. The guinea pigs were observed at 3, 24, and 48 hours after the injections. Changes in skin conditions were measured and reactions recorded as follows: <1 mm, –; 1.0 to 5.0 mm, +; 5.1 to 10.0 mm, ++; 10.1 to 15.0 mm, +++; and >15.0 mm, ++++. At 3 hours, 7 of the 8 animals in the group treated with 0.5 mg/mL crosslinked (Synvisc) hyaluronic acid had reactions of ++ grade and 1 animal had a reaction of +. Six of the 8 animals treated with 2.5 mg/mL crosslinked (Synvisc) hyaluronic acid had reactions of ++ and 2 had a reaction of +. Three animals treated with 2.5 mg/mL noncrosslinked (SupArtz) hyaluronic acid had – grades, 3 with + grades, and 2 animals with ++ grade reactions. Six of the animals receiving 2.5 mg/mL noncrosslinked (SupArtz) hyaluronic acid + CFA had + grades, and 2 of these animals had ++ grade reactions. Two of the nonsensitized animals were treated with SupArtz and both had + grade reactions. At 24 hours, 6 of the animals treated with 0.5 mg/mL SupArtz had + grade reaction and 1 had a +++ grade reaction. Four of the animals treated with 2.5 mg/mL Synvisc had a + grade, 1 had a ++ grade, and 3 had – grade reactions. All 8 of the animals in the 2.5 mg/mL noncrosslinked (SupArtz) hyaluronic acid had – grade reactions. Seven of the animals in the group receiving 2.5 mg/mL noncrosslinked (SupArtz) hyaluronic acid + CFA had – grade reactions, and 1 had a + grade reaction. Of the animals treated with egg albumen, 6 had a ++ grade reaction, and 2 had a +++ grade reaction. Two control animals were treated with crosslinked (Synvisc) hyaluronic acid, and both had ++ grade reactions. Two other control animals treated with noncrosslinked (SupArtz) hyaluronic acid had – grade reactions. Forty-eight hours after the injections, 4 animals treated with 0.5 mg/mL noncrosslinked (SupArtz) hyaluronic acid had – grade reactions, 3 had a +, and 1 had a ++ grade reaction. Five animals treated with 2.5 mg/mL crosslinked (Synvisc) hyaluronic acid had + grade reactions, 1 had a –, and 2 had ++ grade reactions. Four of the animals treated with 2.5 mg/mL crosslinked (Synvisc) hyaluronic acid + CFA had ++ grade reactions, 2 had +, and 2 had – grade reactions. All of the SupArtz treated group had – grade reactions. Seven animals treated with egg albumen had +++ grade reactions, and 1 had a ++++ grade reaction. Two of the nonsensitized animals were treated with crosslinked (Synvisc) hyaluronic acid; 1 had a + grade reaction, and the other had a ++ grade reaction. Two nonsensitized animals treated with noncrosslinked (SupArtz) hyaluronic acid had – grade reactions. Eighteen days after the final sensitizing injection, the animals were anesthetized, weighed, and given an

intravenous injection into the medial saphenous veins of 3 mL/kg crosslinked (Synvisc) hyaluronic acid, 3 mL/kg noncrosslinked (SupArtz) hyaluronic acid, or 1 mL/kg egg albumen, matching the substance to what the animals were sensitized (without the CFA). The animals were observed for 3 hours for anaphylactic symptoms then observed again at 24 hours for additional symptoms.

Twenty addtional guinea pigs were used to perform an antibody titer on the sera from the sensitized animals.[161] The guinea pigs received 8 intradermal injections each: (1) 0.1 mL saline; (2) undiluted sera of a nonsensitized guinea pig; (3) and (4) sera in a 5-fold dilution; (5) and (6) sera in a 25-fold dilution; and (7) and (8) sera in a 125-fold dilution. Eighteen hours after the intradermal injections, the guinea pigs were anesthetized, weighed, and injected intravenously in the medial saphenous vein with 0.4 mL/kg Evans Blue (1% solution with saline) and 5 mg/kg crosslinked (Synvisc) hyaluronic acid for animals that received sera from Synvisc-sensitized animals or 5 mg/kg noncrosslinked (SupArtz) hyaluronic acid for animals that received sera from SupArtz-sensitized guinea pigs. Animals that received sera from egg albumen + CFA-sensitized animals received an intravenous injection of 0.4 mL/kg Evans Blue and 1 mg/animal egg albumen. Thirty minutes after the intravenous injections, the guinea pigs were killed, and the diameter of the blue spots on their skin was measured and photographed. The guinea pigs that received sera that were diluted 5-, 25-, and 125-fold also received the same intravenous injection. Hyaluronic acid IgG production in the guinea pig serum was determined by competitive ELISAs. Neither noncrosslinked (SupArtz) hyaluronic acid or crosslinked (Synvisc) hyaluronic acid caused a passive cutaneous anaphylactic response in guinea pigs at any concentration tested. The injected egg albumen did elicit a strong passive cutaneous anaphylactic response. Synvisc caused the production of higher numbers of hyaluronic acid–specific immunoglobins ($P = .0005$) when compared with the "native" form.

Ototoxicity

Twenty adult pigmented Sprague-Dawley rats were used to test the ototoxicity of hyaluronic acid.[162] Hearing tests were performed on the animals then the bulla tympanica was completely filled with 1.9% hyaluronic acid. This application was repeated twice at 2-day intervals. Five days after the last application, a new hearing test was performed. Fifteen of these animals were killed, and their middle ears were evaluated microscopically for the presence of hyaluronic acid. Hearing tests were given to the last 5 animals at 1 and 3 months after the last application. Five days after the last application of hyaluronic acid, various amounts of hyaluronic acid were observed in the middle ear. The round window niche was filled with viscous material. All perforations of the tympanic membrane were closed. One month after the application, there was some viscous material under the tympanic membrane. Two of the 5 middle ears examined still exhibited viscous material in the round window niche after 3 months. After application of hyaluronic acid, the auditory brainstem response thresholds dropped, but all thresholds had returned to normal 3 months after the last application. The authors concluded that hyaluronic acid was not ototoxic to the inner ear.

The toxicity of hyaluronic acid administered into the ear cavity of guinea pigs was examined.[163] Twenty (10 of each sex) young healthy albino Guinea pigs of the Duncin-Hartley strain, weighing between 450 and 800 g, were anesthetized. Each animal was shaved behind the right ear, and the tympanic bulla was opened by drilling a hole through its wall. The middle ear was filled with hyaluronic acid (average molecular weight of 3.4×10^6; 19 mg/mL), giving 200 to 300 μL as a single dose. The left ear was left intact. In a control group of another 20 animals, a similar experimental procedure was performed for access to the right middle ear, but nothing was administered in the tympanic bulla. The wounds were sutured, and after 14 days, the animals were killed by an overdose of pentothal sodium. In the control group, 18 animals had a slight amount of exudate in the right middle ear cavity, 1 had none, and 1 had a large quantity. In the test group, 3 animals had a small quantity of exudate, three had a large amount of colorless or yellow viscous exudate, and 14 had the right ear filled with viscous exudate. There was no exudate in any of the animal's left ears of either the test or control groups. There was no sign of missing outer hair cells caused by the hyaluronic acid except in one animal where the area of outer hair cell loss was greater in the treated ear. The authors concluded that hyaluronic acid administered into the middle ear of the guinea pig did not cause destruction of the cochlear sensory cells, strongly suggesting that it may not be harmful to administer hyaluronic acid in the middle ear of humans.

The hearing of 20 (10 of each sex) young, healthy albino Duncan-Hartley strain guinea pigs was tested while under anesthesia.[164] They weighed between 340 and 730 g. After half of the animals were tested, their middle ear was slowly filled from the bottom via a thin catheter with 200 to 300 μL of hyaluronic acid (average molecular weight of 3.4×10^6; 19 mg/mL). The hearing test was readministered immediately and again after 28 days. The other half, the control group, was also tested again after 28 days. The mean auditory thresholds shifted for the animals that were administered hyaluronic acid in their middle ear. Histologically, 3 of the control animals had a small amount of brownish material at the stapes and/or on the mucosal membrane of the right middle ear cavity, which was interpreted to a sequella of the surgery. Six of the test group animals had a small amount of brown viscous fluid in a recess in the right middle ear cavity. One of these also had a small amount of a similar fluid at the footplate of the stapes. Macroscopic examination of the middle ear cavity revealed that hyaluronic acid was almost completely eliminated from the cavity 28 days after its administration. Scanning electron microscopy revealed no morphological alterations of the hair cells that could be related to the hyaluronic acid.

Sodium hyaluronate was used as a lubricant in the implantation of Silastic silicone rubber (Dow Corning Corp, Midland, Michigan) electrodes with 4 platinum bands into the ears of cats.[72] One ear had one drop of sodium hyaluronate instilled through the round window, and a drop was spread on the probes before insertion. This was repeated in the other ear with sodium chloride solution as a control. There were no ill effects of the procedure or of sodium hyaluronate noted over the next 4 months. The effect of sodium hyaluronate on the hearing threshold was inconclusive in that 2 cats had higher hearing thresholds in the treatment ear, 3 cats had higher thresholds in the control ear, and no difference was detected in the sixth cat.

Neurotoxicity

The acute neurotoxicity of hyaluronic acid was tested on 20 New Zealand White male rabbits weighing between 2.0 and 3.0 kg.[165] An epidural injection of 0.2 mL/kg normal saline or hyaluronic acid (Hyruan, LG Life Sciences, Seoul, Korea) (10 mg/mL; molecular weight, 1100 kD; pH, 6.3-8.3) was administered ($n = 10$ per group). No signs of any motor or sensory change or any behavioral change were noted during the 3-week period after the epidural injection. One saline injected rabbit had decreased appetite, activity, and body weight. This animal also had wound inflammation at necropsy. The hyaluronic acid group had no pathological abnormalities by light microscopy, whereas 2 rabbits in the saline group had abnormal findings (localized or diffuse meningeal inflammation, focal inflammation, and degenerative myelopathy in the white matter). No structural changes of neurons or glial cells were detected by electron microscopy. The blood–brain barrier, nuclei of neurons, nucleoplasm, cytoplasm, astrocytes, oligodendrocytes, and basal lamina of capillaries were normal. The authors state that hyaluronic acid administered epidurally to rabbits was not found to cause any sensory-motor dysfunction, behavioral change, or neurotoxicity by either light or electron microscopy.

Reproductive and Developmental Toxicity

Rats

Several similar reproductive and developmental toxicity studies on hyaluronic acid and its salts were performed using rats. One study[166] is described in detail in this section and summarized in Table 8 with 10 other studies.

A multigenerational study of the effects of sodium hyaluronate derived from cockscombs on pregnant Sprague-Dawley rats and their offspring was performed.[166] In all cases, a 1% solution of hyaluronic acid in physiological saline solution was used. After weighing, male and female 12-week-old rats were housed together in pairs. From day 17 of pregnancy to day 20 after parturition, sodium hyaluronate was administered by subcutaneous injection in the back area to the dams at 7 mg/kg (0.7 mL/kg), 20 mg/kg (2 mL/kg), or 60 mg/kg (6 mL/kg). The control group was administered 6 mL/kg physiological saline solution ($n = 21$ or 22). Body weights were determined daily during pregnancy, and dams were examined on delivery day. Food consumption was measured on days 3, 6, 9, 12, 15, and 19 after parturition. Females nursed the pups for 21 days during which the pups were observed for abnormalities (physical and behavioral) and weighed 3 times per week. The dams were killed on day 21, bled, and dissected so to observe the organs. Implantation marks in the uteri were also counted to calculate the live

Table 8. Summary of Reproductive and Developmental Studies of Subcutaneously Injected Hyaluronic Acid Using Rats

Study	Source/ Treatment	Notes on F_0	Notes on F_1 – Notes on F_2	NOAEL
Furuhasi et al[166]	Rooster combs/subcutaneous injection from day 17 of pregnancy to day 20 after parturition	Unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility	No neonatal abnormalities found[a]	60 mg/kg d^{-1}
Ota et al[281]	Rooster combs/subcutaenous injection to females from day 17 of pregnancy to day 21 after delivery	Unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility; no effect found at necropsy after delivery	Neonatal development normal; unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility; no effect found at necropsy after delivery; no neonatal abnormalities found	50 mL/kg d^{-1}
Tanaka et al[282]	Bacteria/subcutaneous injection 2 weeks before mating and 1 week after mating	Unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility; no effect found at necropsy after delivery	No neonatal abnormalities found[a]	50 mL/kg d^{-1}
Tanaka et al[283]	Bacteria/subcutaneous injection to females on days 7 to 17 of pregnancy	Unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility; no effect found at necropsy after delivery	Neonatal development normal; unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility; no effect found at necropsy after delivery; no neonatal abnormalities found	50 mL/kg d^{-1}
Ono et al[284]	Rooster combs/subcutaneous injection to females from day 7 to day 17 of pregnancy	Unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility; no effect found at necropsy after delivery	Neonatal development normal; unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility; no effect found at necropsy after delivery[a]	50 mL/kg d^{-1}
Ono et al[285]	Rooster combs/subcutaneous injection to females 9 weeks prior to copulation and first week of primary copulation	Unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility; no effect found at necropsy after delivery	No neonatal abnormalities found[a]	50 mL/kg d^{-1}
Ono et al[286]	Rooster combs/subcutaneous injection to females from day 17 of pregnancy to day 21 after delivery	Unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility; no effect found at necropsy after delivery	Neonatal development normal; reproductive ability normal; neonatal development normal	50 mL/kg d^{-1}
Matsuura et al[287]	Bacteria/intraperitoneal injection to females day 7 to day 17 of pregnancy	Unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility; no effect found at necropsy after delivery	No neonatal abnormalities found; neonatal development normal	64 mg/kg d^{-1}
Matsuura et al[288]	Bacteria/intraperitoneal injection to females day 7 to day 17 of pregnancy	Unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility; no effect found at necropsy after delivery	No neonatal abnormalities found; reproduction ability normal; neonatal development normal	64 mg/kg d^{-1}

(continued)

Table 8. (continued)

Study	Source/ Treatment	Notes on F_0	Notes on F_1 – Notes on F_2	NOAEL
Hattori et al[289]	Not stated/daily subcutaneous injection to males and females for 8 weeks during 2 weeks of mating	Unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility; no effect found at necropsy after delivery	No neonatal abnormalities found; reproduction ability normal[a]	40 mg/kg d^{-1}
Kumada et al[290]	Not stated/subcutaneous injection to females from day 7 to day 17 of pregnancy	Unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility; no effect found at necropsy after delivery	Neonatal development normal; unabsorbed residue of hyaluronic acid at injection sites; no effect on weight or food and water consumption; no effect on fertility; no effect found at necropsy after delivery; no neonatal abnormalities found	40 mg/kg d^{-1}

[a] F_2 observation not reported.

birth index. The newborn survivor and morbidity indexes were determined at hour 16 for the F_1 generation. Weight, sex, and malformations were noted. On day 4, the F_1 newborn pups were divided into litters of 10 (5 male, 5 female). Individual body weights were measured on days 4, 7, 14, and 21. Morbidities and general symptoms were recorded every day. Other measurements included auricle separations (days 2, 3, and 4), fur growth (days 8 through 12), dentition (days 10 through 14), and opening of eyes (days 16 and 17).

During week 3, the pups culled from the litters were killed and treated with 70% ethanol and their skeletal structures examined. On day 21, the F_1 generation was subjected to motor function test (corneal reflex, righting reflex, and avoidance reaction). Three males and 3 females from each group were killed and dissected. The remaining pups were weighed weekly and kept until 10 weeks of age. Sexual development was noted at week 3 and 4 for males and weeks 5 and 6 for females. A motor test (rotating rod, vertical, and diagonal board) was performed at week 4. At week 7, the electrical shock avoidance aptitude test was administered. At week 10, the remaining pups were mated.

Two-thirds of the dams were killed on the 20th day of pregnancy, weighed, measured, and dissected. The number of surviving fetuses was counted. The remaining third of the dams was allowed to give birth. The litters were grouped into 10 each as before. After 21 days of nursing, the live birth index, viability, and nursing indexes were determined.

There was no morbidity among the control or experimental dams. The weights of the dams in the 60 mg/kg group were higher than the control group, and the relative weights of the heart and lungs were lower on the 20th day of pregnancy. The 7 mg/kg group increased food consumption on day 4 after delivery. There were no other macroscopic differences observed during pregnancy or nursing. A gelatinous residue at the site where the 60 mg/kg injections were made was noted histologically. Nodular hyperplasia of reticular zone cells was present in the adrenal gland for 1 of 3 of the 7 mg/kg group, for 2 of 3 in the 20 mg/kg group, and 3 of 3 in the 60 mg/kg group. The severity of adrenal gland effects ranged from slightly sporadic and reduced nodular foci (most cases) to one case of severe pervasive nodular foci in the 60 mg/kg group. No other abnormalities were found.

There were no differences between treated and controls for the number of implantations, mean gestation length, number of newborns, sex ratio, live birth index, viability index, or external malformations compared with controls. There were no differences in the timing of separation of auricle, appearance of abdominal hair, odontiasis, eye opening, descent of testes, or vaginal opening in treated when compared with controls.

There was no difference in the change of body weight through day 70 for male and female F_1 pups compared with controls. There were no differences in absolute or relative organ weights at day 21 compared with controls. The relative weight of the epididymis in the male 7.0 mg/kg group was lower than the control group at day 70 ($P < .05$). At 70 days, the thymus and uterus weights of the 7.0 mg/kg group were less than the control group ($P < .05$); the relative weight of the bled carcass was less than control ($P < .05$), and the relative and absolute weights of the ovaries were less than the control group ($P < .05$).

The skeletal examinations of the F_1 pups showed no malformations nor differences between the groups with dams administered sodium hyaluronate and the control group. The motor function tests of day 21 showed no abnormalities. Balance on the diagonal board was maintained at a steeper angle for all the male experimental groups and the female 20.0 mg/kg group, compared with the control group ($P < .05$). Of the F_1 offspring, there was no difference in the copulation and fertility index between treated and control animals.

When examining the F_1 generation and their fetuses (F_2), the authors reported no difference in the number of corpora lutea, number of implantations, number of live fetuses, percentage of resorptions, percentage of macerated fetuses, percentage of dead fetuses, fetus body length or weight, or adhesion of placenta compared to controls. The 7.0 mg/kg group had a lower male/female sex ratio than the control, and the female placenta weight was higher ($P < .05$). The 60.0 mg/kg group had a longer male tail length ($P < .05$). When comparing the newborns of the F_2 generation, the authors stated that the mean gestation length was longer for all treatment groups. There were no differences in the number of implantations, number of newborns, sex ratio, live birth index, viability index, lactation index, body weight at birth on day 21, or external malformations compared with controls. In the postnatal development of the F_2 generation, there were a higher number of pups that had separation of auricle on day 3 in the 7.0 mg/kg and the 20.0 mg/kg groups when compared with the control group ($P < .05$); there

were no differences on day 2 or 4 for these 2 groups and no difference at all for the 60.0 mg/kg group compared with the control group. There were a higher number of pups with the appearance of abdominal hair on day 10 in the 20.0 mg/kg group ($P < .05$) compared with the control group but not with any other group or on any other day. There were more pups with odontiasis in the 20.0 mg/kg group on day 11 when compared with the control group ($P < .05$); there was no difference on any other day for all 3 groups. On day 17, a higher number of pups had opened their eyes in the 60.0 mg/kg group when compared with the control ($P < .05$). There were no differences in the other groups. The authors concluded that there were no adverse prenatal or postnatal effects due to hyaluronic acid in rats; the NOAEL was reported to be 60 mg/kg. As noted in Table 8, there was a slightly higher NOAEL in rats of 64 mg/kg d^{-1} and a lower NOAEL in rats of 40 mg/kg d^{-1}.

Rabbits

A reproductive and developmental toxicity study was conducted using KBL: Japanese white rabbits to determine the effects of SL-1010 sodium hyaluronate (average molecular weight of 1.78 million, concentration not reported) in isotonic sodium chloride solution.[167] After 3 weeks of acclimation when the females were 4 months old and the males were over 5 months old, the nulliparous females and males were mated. When a pair had been observed to mate twice, that day was considered day 0 of pregnancy. Four groups of pregnant rabbits (n = 13 or 14) were grouped in a stratified random sampling method by body weight based on the weights on day 0 of pregnancy. Sodium hyaluronic acid solution, 0.5, 15, and 50 mg/kg d^{-1}, was subcutaneously administered to the dams once per day from day 6 to day 18 of pregnancy. The locations of the injections were rotated between 6 sites: the left and right sides of the neck, chest, and lumbar. The doses were control (isotonic sodium chloride solution), 5 mg/kg d^{-1}, 15 mg/kg d^{-1}, and 50 mg/kg d^{-1}. The dams were observed for general condition and health before and after each injection. Body weight was measured on day 0, days 6 to 19, day 23, and day 28 of pregnancy. Food consumption was measured every other day from day 1. The dams were killed on day 28 of pregnancy. The uteri and ovaries were removed and the main organs observed macroscopically. The corpora lutea were counted. The number of implantations, dead embryos and fetuses, and viable fetuses were recorded. Body weight and placental weight of the live fetuses were measured. Then they were sexed and examined for external anomalies and intrathoracic and interperitoneal abnomalities. The intrathroracic and interperitoneal organs of the fetuses were fixed, stained, and microscopically examined for anomalies, including dilation of the ventricle system. The skeletons were stained and examined for skeletal anomalies or variations. The number of sacral and caudal vertebra with ossification was counted as an indicator of progression of ossification. There were no observed changes in health or general condition of the treated dams during the pregnancy period. No miscarriages were observed. There was no body weight difference between the control group and the 5 mL/kg d^{-1} group throughout the pregnancy. From day 15 to day 17, the mean weight of the 15 mL/kg d^{-1} group was higher than the control group ($P < .05$). The mean body weight gain for day 6 to day 19 was also more than the control group ($P < .05$). There was no difference in mean body weights for this group for the remainder of the pregnancy period. On day 12, the mean body weights of the 50 mL/kg d^{-1} group was higher than the control group ($P < .05$); this continued through day 19 ($P < .01$). The weight gain for day 6 to day 19 was higher ($P < .01$) and lower from day 19 to day 28 ($P < .01$). There were no differences between the treated and control groups during the remainder of the pregnancy period. The increased body weight was attributed to the unabsorbed sodium hyaluronate accumulating in each dam; there were no differences in food consumption for the pregnancy period. There were no differences in any of the measured reproduction parameters or external anomalies in their fetuses. There were no differences in skeletal abnormalities found. There were no differences in the visceral observations of fetuses.

A developmental toxicity study was performed of sodium hyaluronate using New Zealand White rabbits.[168] The rabbits were grouped into control, 8 mg/kg d^{-1}, 20 mg/kg d^{-1}, and 50 mg/kg d^{-1} and mated (n = 16 of each sex). In all groups but the 50 mg/kg d^{-1} group, 13 females became pregnant. In the highest dose group, 15 females became pregnant. The injections were made on the 6th through the 18th day of gestation. Body weights of the dams were measured on day 0, 6, 7, 8, 9, 12, 15, 24, and 29 of gestation. Food intake was measured every day of pregnancy. The dams were killed on the 29th day

of gestation with pentobarbital sodium and necropsied. The ovaries and the uterus were removed from each animal, and the number of corpora lutea, implantations, live fetuses, and dead fetuses were counted. Body weight and placental weight of the fetuses were measured. The live fetuses were sexed and observed for external and visceral abnormalities. Skeletal specimens were processed and examined for abnormalities. There were no deaths and no changes in the general condition of the animals. In the 20 mg/kg d^{-1} and the 50 mg/kg d^{-1} groups, there was protrusion around the periphery of the injection site containing a gelatinous/foamy material retention, which the authors interpreted to be unabsorbed sodium hyaluronate solution. The 50 mg/kg d^{-1} group had increased body weights on the 15th through the 24th day of pregnancy compared with controls, which the authors suggested was related to retention of sodium hyaluronate, but was not an indication of toxicity. This was also true of the 19th day of pregnancy for the 8 mg/kg d^{-1} group. The 50 mg/kg d^{-1} group had increased placental weights compared with controls. Otherwise, there were no differences observed between the experimental groups and the control group. There were occasional external, visceral, and skeletal anomalies in each group, but there was no statistical pattern, and they were thought to be due to spontaneous generation by the authors. The NOAEL was 50 mg/kg d^{-1}, the highest dose tested.

The effects of HMW sodium hyaluronate (NRD101; molecular weight, 1.9 million) were studied on organogenesis using Japanese white rabbits (SPF).[169] After 4 weeks of acclimation, 12-week-old males were paired with 25- to 26-week-old females or 56- to 57-week-old females (the 2 age groups were not separated in the results of this experiment). The pregnant females were placed into 1 of 4 groups (n = 17) and housed individually. Based on a subacute toxicity test on rats, the test dosages were set at 40 mg/kg d^{-1}, 20 mg/kg d^{-1}, and 10 mg/kg d^{-1}, delivered subcutaneously on days 6 to 18 of pregnancy. The general conditions of the dams were observed daily. Body weights were measured on days 6 to 18 and on days 20, 22, 24, 26, and 28 of pregnancy. Food consumption was measured on day 2 of pregnancy before treatment and on days 6 to 18, 20, 22, 24, 26, and 28 of pregnancy. The dams were killed, exsanguinated, and necropsied. The brain, pituitary, thymus gland, lungs, heart, liver, spleen, adrenal gland, kidneys, uterus, and ovaries were weighed. The uterus and ovaries were removed from each animal. The corpea lutea, absorbed embryos, dead fetuses, and surviving fetuses were counted. The surviving fetuses were examined for external abnormalities, and body weights and weights of placenta were measured. One-third of the fetuses were fixed and necropsied. The remainder was used for skeletal examination. There was no maternal toxicity in any treatment group. There were no body weight or food consumption differences that could be attributed to the sodium hyaluronate, and none of the measurements taken at necropsy demonstrated an adverse effect. There were no teratological effects demonstrated. Sodium hyaluronate did not affect survival, sex ratios, or any of the other parameters examined in the fetuses.

Genotoxicity

Hyaluronic acid produced by the bacterial fermentation method was inactive in mutagenicity tests. No details were provided.[31]

A reverse mutagenicity test of bacterial sodium hyaluronate (1%) on *Salmonella typhimurium* (TA98, TA100, TA1535, and TA1537) and *Escherichia coli* (WP2uvr A) was performed.[170] The preincubation method was used with and without metabolic activation (S9 derived from phenobarbital and 5,6-benzoflavone induced Sprague-Dawley rat livers). Sodium hyaluronate was tested at 31.5 μg/plate, 62.5 μg/plate, 125 μg/plate, 250 μg/plate, 500 μg/plate, and 1000 μg/plate at 2 plates/test concentration. The positive controls were 2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide, N-ethyl-N'-nitro-N-nitrosoguanidine, and 1,3-propanediamine-N-(2-chloroethyl)-N'-(6-chloro-2-methoxy-9-acridinyl)-dihydrochrolide in the absence of S9 Mix and 2-aminoanthracene in the presence of S9 Mix. The negative control was purified water. There was no increase in the mutation frequency in any test strain at any concentration.

The genotoxicity of sodium hyaluronate was in an in vivo micronucleus test using CD-1 (ICR) male mice (8 weeks of age).[171] The test solution of sodium hyaluronate (molecular weight of 2.4 million) was 1% in phosphate buffer. Phosphate buffer was used as the negative control, and mitomycin C was used as the positive control. The experimental groups were injected twice into the abdominal cavity (24 hours apart) with 0, 75 mg/kg (7.5 mL/kg), 150 mg/kg (15.0 mL/kg), or 300 mg/kg (30 mL/kg)

of the sodium hyaluronate solution. The positive control group received 10 mL/kg mitomycin C in distilled water injected once. Bone marrow cells were sampled from 5 mice from each treatment group 24 hours, 48 hours, and 72 hours after the last injection. Mice were killed by cervical dislocation and marrow extracted from the thigh bone using fetal calf serum. The samples were fixed and stained. The number of blood cells with micronuclei among 1000 polychromatic erythrocytes per subject and the number of polychromatic erythrocytes in 1000 blood cells were counted. No signs of toxicity were noted while the mice were alive, and there were no deaths during the treatment period. The positive control produced an increase in micronuclei. There was no difference found between the treatment groups and the control groups in regard to the number of polychromatic erythrocytes.

A reverse mutation test using 1% sodium hyaluronate in phosphoric acid (2 lots with molecular weights averaging 2.12 million and 2 million) was conducted.[172] Histidine-requiring strains of *S typhimurium* (TA98, TA100, TA1535, and TA1537) and 5 tryptophan-requiring strains of *E coli* WP2*uvr*A were used. S9, fractionated from rodent livers, was used for metabolic activation. Hyaluronic acid doses were 31.3 μg/plate, 62.5 μg/plate, 125 μg/plate, 250 μg/plate, 500 μg/plate, and 1000 μg/plate. Isotonic sodium chloride solution was used as the negative control. The positive control for TA98 and TA100 was 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide; *N*-ethyl-*N*'-nitro-*N*-nitrosoguanidine was used for the positive control for TA1535 and WP2*uvr*A, and 9-aminoacridine was used for TA1537. The tests were run for both 24 hours and 48 hours. There was no difference in the number of reverse mutations between any of the test groups and the negative controls. These authors also performed an in vitro chromosomal aberration test using cultured Chinese hamster fibroblast cells with and without metabolic activation. The dosages were 250 μg/mL, 500 μg/mL, and 1000 μg/mL of 1% sodium hyaluronate. Isotonic sodium chloride solution was used as the negative control, and 5 μg/mL of *N*-ethyl-*N*'-nitro-*N*-nitrosoguanidine and 20 μg/mL of benzo[a]pyrene were used as positive controls. Petri dishes received 5 mL of medium in which 4×10^3/mL cells were dispersed. On the third day, the test substances, positive controls, or negative controls were added. Incubation continued for either 24 hours or 48 hours. Positive control groups produced expected results, but there was no difference in chromosomal aberrations between treatment and negative control groups.

Multiple mutagenicity tests were performed on HMW sodium hyaluronate (trade name NRD101; molecular weight, 1.9 million).[173] An Ames test used *S aureus* strains TA100, TA98, TA1535, and TA1537 and *E coli*. Sodium hyaluronate was provided by the manufacturer (0.25%, 0.5%, and 1% aqueous solutions) and diluted with distilled water to yield 312.5, 625, 1250, 2500, and 5000 μg/plate. Distilled water was the negative control. The positive controls were 2-aminoantracene, 9-aminoacridine hydrochloride, sodium azide, and N-ethyl-N'-nitro-N-nitrosoguanidine disolved in dimethylsulfoxide. The test solutions were tested with and without metabolic activation. Positive control groups produced expected results, but there were no differences in the number of reverse mutation colonies between any test culture and the negative control.

In an in vitro chromosomal aberration test using Chinese hamster lung fibroblasts, sodium hyaluronate was tested at 62.5 μg/mL, 125 μg/mL, 250 μg/mL, 500 μg/mL, and 1000 μg/mL for a treatment time of 24 and 48 hours with and without metabolic activation.[173] The controls were the same as above. Two hundred metaphase cells were observed for each slide for gaps, breaks, exchanges, and other chromosomal abnormalities. Positive control groups produced expected results, but there were no differences between the treatment groups and the negative controls.

Sodium hyaluronate was tested in a micronucleus assay using 8-week-old ICR (Crj: CD-1) mice, treated with 90, 180, and 360 mg/kg once a day for 1 day or for 4 consecutive days.[173] Bone marrow specimens were collected 24, 48, and 72 hours after the final injection or the single injection after killing the mice by cervical dislocation. The number of polychromatic erythrocytes with micronuclei among 1000 erythrocytes was counted. There were no differences between any of the treatment groups and the negative controls.

Carcinogenicity

Tumor Cells

McBride and Bard used several types of cultured fibrosarcoma cells, lymphoblastoid cells, mammary carcinoma cells, VERO and BHK-21 cells, and

Table 9. Metatstic Potential of HA-L and HA-H Cells Following Intravenous Injection of B16-F1 Mouse Melanoma Cell Lines Into C57BL/6 Mice[176]

Cell Type	No. of Cells Injected($\times 10^5$)	Lung Nodules/Lung	
		Mean ± SD	Range
HA-L	2.5	3 ± 1.6	0-5
	10	115 ± 35	80-150
HA-H	2.5	172 ± 66.5[a]	90->200
	10	>200[b]	>200

[a] $P < .01$ compared with HA-L.
[b] $P < .05$ compared with HA-L.

fibroblasts from 14-day-old mouse embryos and human skin to study the hyaluronic acid halo produced by these cells.[174] Cells of each type were placed on cover slips or in 96-well microtiter plates at a density of 10^4 cells/cm^2. C2Hf/Bu fibrosarcoma (FSA) cells displayed translucent halos ranging in diameter up to 17 μm, averaging 8.8 μm. The halos were not penetrated by spleen cells added to the culture. Motile cells had very prominent barriers around their trailing edge, but the halo tended to be much less pronounced at their leading edge. The exclusion phenomenon was seen with particles other than normal spleen cells. Halos also excluded immune spleen cells, lymph node cells, thymocytes, normal peritoneal exudate cells, and erythrocytes. When fixed with 1% formal saline or 2.5% glutaraldehyde, the halos remained. When dehydrated, the halos were no longer visible.

The same results were achieved with 8 types of FSA cells from mice; 3 types of mammary carcinoma cells from mice; adenocarcinomas 1, IM, and 2 from mice and rats; 3T3 G and 3T3 S cells from mice, BHK-21 hamster cells; mouse embryo fibroblasts and adult human skin fibroblasts.[174] The exception was the 7 lymphoblastoid cells lines tested. Vero cells also appeared not to have the protective halo. The authors note that halos <2 μm could not be detected. When bovine or ovine testicular hyaluronidase (10 IU/mL) was introduced, the barrier was removed, and the spleen cells could approach the cell membrane. This was also the result with fungal (0.1-10 IU/mL) hyaluronidase. After removal of the enzyme, the cells regenerated their ability to repel spleen cells within 2 hours.

The production of hyaluronic acid by 3 human malignant mesothelioma cell lines (Mero-25, Mero-14, Mero-82) and 9 primary human mesothelial cell types were measured.[175] The mesothelioma cell lines produced small amounts of hyaluronic acid (<0.1 μg/10^6 cells/48 hours) compared with mesothelial cells (10 to 72 μg/10^6 cells/48 hours). When placed in conditioned media from the mesothelioma cells, fibroblast and mesothelial cell production of hyaluronic acid increased; a concentration of 50% of 10-fold concentrated conditioned medium in relation to culture medium (v/v) induced a near maximal effect in mesothelial cells and 70% of the maximal effect in fibroblasts.

The metastatic potential of tumor cells expressing different levels of cell surface hyaluronic acid were examined.[176] Flow cytometry was used to isolate B16-F1 mouse melanoma cell lines expressing either high (HA-H) or low (HA-L) hyaluronic acid on their surfaces. HA-H had approximately 32 times more cell surface hyaluronic acid than HA-L cells. After removal of hyaluronic acid from the cell surfaces with testicular hyaluronidase, it was found that HA-H produced hyaluronic acid approximately 15 times faster than HA-L; hyaluronic acid levels were restored within 20 hours in both cell lines. The 2 cell lines had similar growth rates in vitro; when injected into syngeneic mice, the 2 cell lines gave rise to tumors that grew at similar rates. HA-H or HA-L cells were injected into the tail veins of syngeneic C57BL/6 mice (2.5×10^5 or 1×10^6 cells /mouse; $n = 10$; experiment was run 3 times). Mice were killed 14 days later, and the lungs were fixed. Visible lung tumor nodules were assessed under a dissecting microscope. Another set of mice were allowed to live to determine the mortality from HA-H and HA-L. The HA-H cells caused a greater number of lung metastases that were larger than the HA-L cells as shown in Table 9. In general, the nodules of the HA-L cells were smaller (0.1-0.5 mm) than the HA-H cells (0.1-8 mm). The mortality rate was 50% at day 25 and day 32 for the HA-H and HA-L cells, respectively.

Human mesothelioma cells (Mero-25) with and without transfected HAS2 gene (the gene controlling the ability to produce hyaluronic acid) were compared.[177] The cells were plated (5×10^4 cell/dish), incubated for 24 hours, and then overlaid with 1×10^7 formalin-fixed erythrocytes. The cells were observed through an inverted microscope and the size of the hyaluronic acid halos measured. Halos around the HAS2 transfected cells were larger than the halos around the unmodified cells ($P < .05$). The HAS2 transfected cells produced 59.6, 103.8, and 187.2 ng/10^4 cells/24 hours hyaluronic acid compared with 14 ng/10^4 cells/24 hours from unmodified cells. The peaks in the size range of hyaluronic acid was 3.9×10^6 kD. Proliferation of both types of cells was compared by counting the cell number at 1, 3, 6, and 9 days after subculturing. The modified cells had about a 2-fold higher proliferative capacity than the unmodified cells.

These authors analyzed the cell cycle profiles by placing both types of cells in culture in starvation medium, centrifuged, and subjected to DNA content analysis and cell cycle kinetics using a flow cytometer for cell cycle analysis.[177] After serum stimulation, HAS2-transfected cells had a higher proportion of cells in S phase (26.6%) compared with unmodified cells (18.9%). The cells were grown in soft agar for 4 weeks, and colonies larger than 0.5 arbitrary units were counted by microscope and their area measured for a growth assay. The hyaluronic acid–producing cells formed 2- to 3-fold larger colonies in soft agar than unmodified cells. The shape of the colonies formed by the modified cells was more irregular than unmodified cells. Increased synthesis of hyaluronic acid was found to lead to an increased proliferation rate and to anchorage-independent growth in soft agar.

The HAS2 gene was inserted into a murine astrocytoma cell line (SMA560) using the murine mHAS2/pCIneo plasmid.[178] SMA560 cells and HAS2-modified SMA560 cells (3 strains) were suspended in PBS (10^6 cells/100 μL) and injected subcutaneously into the flanks of syngeneic VM/Dk mice (n = 10). When a tumor became palpable, it was measured in 3 dimensions using calipers. When the tumors reached 1 cm^3, the mice were killed and the tumors excised and frozen in embedding medium. Overexpression of HAS2 caused a reduction in tumor growth rate; the onset of tumor formation was similar to unmodified cells. One strain of modified cells formed cysts with few tumor cells surrounding necrotic tissue, while the other 2 strains formed distinct tumors that were histologically similar to the controls.

The authors injected each of the above cell lines (10^5 cells in 10 μL PBS) into the caudate nucleus of syngeneic mice (n = 10) to induce intracranial tumors.[178] The mice were killed when they became moribund or after 1 month; the brains were excised and frozen in embedding medium. The tumors and brains were sectioned for microscopic examination. The experiment was repeated and extended to 2 months. Unmodified SMA560 cells formed large intracranial tumors within 18 days. The mice injected with the modified cells did not result in any obvious disease within 8 weeks. Upon examination, it was observed that individual tumor cells were observed at the injection site but no tumors. Northern blot analysis was performed on human glioma cell lines (D54, D270, D645, U373 MG, and U251 MG). It was observed that U251 MG and D270 expressed HAS2 at high levels; U373 MG, U87 MG, and D645 expressed HAS2 at lower levels; and D54 cell did not express HAS2 at all. The size of the hyaluronic acid halo, in general, corresponded with the level of HAS2 gene expression in D270 cells. However, U251 MG cells had only a small hyaluronic acid halo.

Balb/c nu/nu male mice or A/Jax mice were used to test the effects of hyaluronic acid on the growth of injected LX-1 human lung carcinoma cells or TA3/St cells.[179] ALZET osmotic pumps were inserted under the skin in the dorsal region of the mice loaded with either PBS or hyaluronic acid (approximate molecular weight, 2.5×10^3; n = 5). Pumps delivered the treatments at approximately 0.5 μg/0.05 μL/h. On the day after insertion, 0.5 to 1.0×10^6 of LX-1 or TA3/St tumor cells (in PBS) were injected in front of the pump. After 7 or 14 days of treatment, the mice were killed and the tumor's growth was measured by weight. Hyaluronic acid inhibited LX-1 tumor growth by approximately 50% to 80% and TA3/St tumor growth by approximately 60% to 65%. In additional experiments, animals were injected with LX-1 cells and were untreated for 7 days. They were then treated with hyaluronic acid for 14 days. This experiment was run twice. The growth inhibition was between 40% and 75%. When the treatment regime was reversed, treatment for 14 days and no treatment for 7 days, inhibition was 68%. When treated for 7 days followed by no treatment for 14 days, inhibition was 52%. In a soft agar

Table 10. Growth Characteristics of the Transplantable Tumors[180]

		Tumor Grafts		
Transplantable Clone	n	Weeks of Growth	Wet Weight (g)	Tumor Growth[a]
Mock	4	14.0 ± 0.8	4.2 ± 0.6	0.30
Has2-b	4	16.5 ± 2.0	4.2 ± 0.7	0.25
Has2-d	11	7.9 ± 0.5	3.4 ± 0.4	0.43
Hyal1-f	3	17.3 ± 3.0	2.5 ± 1.0	0.14
Hyal1-h	4	16.5 ± 2.5	2.6 ± 0.3	0.16

[a] Wet weight/weeks of growth.

assay, inclusion of 100 μg/mL hyaluronic acid in the agar inhibited colony formation by LX-1 human lung carcinoma, HCT116 human colon carcinoma, and TA3/St murine mammary carcinoma cells by 80%, 68%, and 72%, respectively.

The effects of inserting hyaluronan synthase 2 (Has2) and hyaluronidase 1 (Has1) genes into non-hyaluronic acid–producing rat colon carcinoma cells (PROb, a subclone of the cell line DHD-K12) were tested.[180] The genes for the synthase and the hyaluronidase were inserted separately into the cancer cells using pCl-neo producing the Has2-b, Has2-d, Hyal1-f, and Hal1-h cell lines. Has2-b and Has2-d synthesized about 2.5 and 12 μg hyaluronic acid per 1×10^6 cells/24 hours; Hyal1-f and Hal1-h exhibited hyaluronidase activity of about 360 and 220 mU/mL, respectively. No hyaluronic acid or hyaluronidase activity was detected in the wild-type PROb cells or in mock transfected cells. Transfected cells (5×10^6 cells in 50 μL PBS) were injected subcutaneously into the right shoulder of BD-IX rats. The subsequent tumors were measured with a caliper and allowed to grow to approximately 1 cm in diameter. The rats were killed and tumors were excised, cleaned of nontumor tissue, and the wet weight was determined. The tumors were then snap-frozen in liquid nitrogen. Tumor growth was determined by dividing the wet weight by the number of weeks of growth. Tumor production rates were different for each group as shown in Table 10. The tumors of mock transfected cells grew to 4.2 g in 14 weeks, similar to the low hyaluronic acid–producing Has2-b–derived tumors. The higher hyaluronic acid–producing Has2-d tumors reached 3.4 g in 8 weeks. The growth rates of the Hyal1-f and Hyal-h cells were lower compared with tumors of mock transfected cells; a wet weight of 2.5 g was reached after 17 weeks. The growth rate of tumors from the Has2-d transfectants was higher ($P = .038$), and the growth rate of tumors from Hyal1-f and Hyal1-h transfectants was slower ($P = .029$) compared with mock transfectants. The authors stated that the expression of Has2 enhanced tumor growth, whereas expression of Hyal1 delayed tumor development. The amount of hyaluronic acid in the tumors was determined by microtiter-based assay. Tumors from the high hyaluronic acid–producing clone contained an average of 150% more extractable hyaluronic acid compared with tumors derived from mock and Hyal1 transfectants ($P = .006$). The mean vessel area, boundary length, and diameter did not differ among the tumor types examined, leading the authors to suggest a similar vascular phenotype. The authors concluded that Has2 overexpression suppresses vascularization of the viable tumor fraction.

The effects of the 3 different genes controlling hyaluronic acid synthesis on tumor cell metastasis were tested.[181] Aneuploid human breast adnocarcinoma cell line MDA-MB-231 was selected based on its production of HAS2. Nontransformed rat cells were transfected with HAS1, 2, and 3. In the MDA-MB-231 cell line, overexpression of each HAS isoform promoted the formation of a hyaluronic acid coat where HAS2 produced a larger matrix than HAS1 or HAS3. These authors investigated the importance of HAS2 expression in highly invasive breast cancer (MDA-MB-231) by characterization of the antisense inhibition of HAS2 (ASHAS2). ASHAS2 resulted in a 24-hour lag in proliferation that was concomitant to transient arrest of 79% of the cell population in G_0 to G_1. ASHAS2 did not alter the expression of the other HAS isoforms, whereas hyaluronidase and the hyaluronic acid receptor, CD44, were downregulated. The antisense inhibition of HAS2 cells accumulated greater amounts of HMW hyaluronic acid (>10 000 kDa) in the culture medium, whereas mock and parental cells liberated less hyaluronic acid of 3 distinct molecular weights (100, 400, and 3000 kDa).

Five-week-old CBA nude mice were used to generate parental, mock, and antisense inhibition of HAS2 tumors.[181] The tumor cells were harvested in the logarithmic growth phase by scraping, resuspended (final concentration, 2×10^6), and injected into the mammary fat pad of the same mice. Tumor growth was measured twice weekly. After 84 days, the mice were killed and the primary tumor, liver, kidneys, brain, and lungs removed at necropsy and examined. Mice inoculated with parental or mock-transfected MDA-MD-231 established primary tumors with comparable growth over the experiment. Mice inoculated with ASHAS2 tranfectants did not establish primary tumors. Metastasis in animals inoculated with parental and mock-tranfected cells was most prevalent in the brain and lung but also detected in kidney and liver transfectants. Mice injected with MDA-MB-231 ASHAS2 did not exhibit metastasis to any organs.

Additional mice were injected in the left ventricle with 1×10^5 cells.[181] Animals inoculated with parental and mock-transfected cells had prevalent spread of the cancer to the brain, liver, kidneys, lung, and bone. Mice injected with MDA-MD-231 ASHAS2 did not exhibit metastasis to any organs. Mice inoculated with parental or mock-transfected MDA-MB-231 cells had a shorter survival period (72 and 77 days, respectively), compared with ASHAS2 animals (124 days; $P = .0001$). The authors stated that, collectively, these results strongly implicated the central role of HAS2 in the initiation and progression of breast cancer, potentially highlighting the codependency between HAS2, CD44 (hyaluronic acid receptor), and hyaluronidase 2 expression.

The mechanism that human pancreatic carcinoma cells (MIA PaCa-2) use to create LMW ($\sim$10-40 polymers) hyaluronic acid that induce angiogenesis, enhance CD44 (hyaluronic acid receptor) cleavage, and promote the migration of tumor cells in a CD44-dependent manner was researched.[182] MIA PaCa-2 cells show CD44 cleavage in the absence of any exogenous stimulation at a readily detectable level; therefore the possibility that these tumor cells may generate CD44 cleavage inducible hyaluronic acid oligosaccharides by expressing hyaluronic acid–degrading enzymes was tested. To prepare the MIA-PaCa-2 culture supernatant, MIA PaCa-2 cells were cultivated in a flask (3×10^5 cells/flask) overnight. The culture medium was collected, centrifuged, and concentrated 50-fold. It was then filtered (0.22-μm pore filter). Reverse transcriptase-PCR was used to detect hyaluronidases HYAL1 and HYAL2 transcript expression. Enzyme-linked immunosorbent (ELISA)–like assay was used to detect hyaluronidase levels. Western blotting and SDS-PAGE was used to detect hyaluronidase proteins. Hyaluronic acid levels were measured by ELISA. Size profiling and purification of MIA PaCa-2 hyaluronic acid was done by gel filtration chromatography. For the CD44 cleavage assay, MIA PaCa-2 cells were plated (5×10^4) and cultured overnight, then incubated with 10 μM MG132 for 30 minutes to inhibit secondary cleavage of the CD44 intracellular domain. The cells were incubated with various samples of hyaluronic acid for 1 hour then lysed. Samples were separated by electrophoresis under reducing conditions and transferred to a polyvinylidene difluoride filter. The filter was incubated with HRP-conjugated anti-rabbit IgG to detect the anti-CD44cyto pAb, or with HRP-conjugated anti-mouse IgG to detect the anti-β-tubulin mAb. The secondary antibodies were detected using ECL Western blotting detection reagents. Immunofluorescence microscopy was performed. To perform a migration assay, 12-well Costar Transwell (Corning, Inc., Lowell, MA) chambers containing polycarbonate filters with a 12-μm port size were used. Both sides of the filter were coated with 500 μg/mL 1000-kD hyaluronic acid. MIA PaCa-2 cells (2×10^5 cells/mL) were added to the upper compartment and incubated for 3 hours with or without BRIC235 or mouse IgG. Hyaluronic acid was added to the upper compartment at a final concentration of 50 μg/mL and incubated for an additional 15 hours. The cells on the upper side of the filters were wiped off. The filters were fixed in methanol, stained, and mounted on glass slides. Migrated cells were counted under a light microscope. The MIA PaCa-2 culture supernatant was found to contain hyaluronic acid–degrading enzymes, which digested hyaluronic acid in a pH-dependent manner with the optimal pH of 4.0. The presence of hyaluronic acid–degrading activities in the culture supernatant was confirmed by a substrate-gel electrophoresis analysis. MIA PaCa-2 cells expressed 2 of the known hyaluronidases, Hyal-1 and Hyal-2, at both the mRNA and protein levels and secreted both of these proteins into the culture supernatant. The researchers stated that other studies noted that Hyal-1 and Hyal-2 expression was detected in a human prostate cancer cell line LNCaP and in a human breast cancer cell line,

MDA-MB231, respectively. In this study, high levels of hyaluronic acid were detected in the supernatant; the cells generated hyaluronic acid ranging from approximately 10 to 40 saccharide pairs, which are similar in size to those that have been shown to enhance Cdrr cleavage in CD44-expressing tumor cells. The addition of MIA PaCa-2 culture supernatant induced the upregulation of CD44 cleavage in MIA PaCa-2 cells, as evidenced by an increase in the membrane-bound 25-kD cleavage product in Western blotting analysis. This cleavage was strongly inhibited by the Fab fragment of the anti-CD44 neutralizing monoclonal antibody BRIC235, indicating the upregulated CD44 cleavage was because of the interaction between CD44 and its ligand. The culture supernatant also enhanced the migration of MIA PaCa-2 cells in the Transwell migration assay; this migration was almost completely inhibited by the anti-CD44 monoclonal antibody BRIC235 but not by mouse IgG, indicating that the enhanced tumor cell motility was also dependent on the CD44–hyaluronic acid interaction.

Tumor Treatment

The use of testicular hyaluronidase (PH-20) to reduce the presence/production of hyaluronic acid in cancer treatment was tested.[183] Homozygous, female ICR SCID mice were injected with 5×10^6 human breast carcinoma cells (MDA435) into the mammary foot pad. Hyaluronidase activity is expressed in relative turbidity reducing units (rTRUs). The mice were injected intravenously with 75 rTRU hyaluronidase on days 0, 2, 4, and 6 or were administered a single injection of 300 rTRU hyaluronidase (day not specified). Control animals were administered saline. Tumors were measured every other day. After 4 days in both treatment regimes, the tumor volume decreased by 50%; the tumors in the controls continued to grow. After 1 month without any further treatment, major differences continued to be observed between tumors in the treated and nontreated animals. There were no apparent toxic effects or changes in the behavior of the mice during the experiment.

The effectiveness of the application of hyaluronic acid to drug-resistant cancer cells was tested.[184] MCF-7/Adr drug (doxorubicin)–resistant human mammary carcinoma cells were grown in culture for 24 hours in 24-well plates. Controls were the nonresistant parental MCF-7 cells. Various concentrations of chemotherapeutic agents were added and the cells incubated for an additional 72 hours. At that point, 10 μg/mL hyaluronic acid (mixture of 3 to 8 repeating disaccharides in length) was either added or not to the medium, and the cells were incubated for another 24 hours. Cells were harvested, and viable cells were counted. The hyaluronic acid caused approximately 55-fold sensitization increase in the MCF-7Adr cells but had little effect on the non-drug-resistant cells.

The authors tested a range of concentrations for effectiveness and found that up to 250 μg/mL there was little or no effect of hyaluronic acid alone.[184] In combination with doxorubicin, concentrations of 10 μg/mL were effective on the drug-resistant cancer cells. The authors repeated this experiment using other drug-resistant cells and reported that hyaluronic acid increased the sensitivity of cells resistant to taxol by approximately 12-fold, 1,3-bis(2-chloroethyl)-1-nitrosurea (BCNU) by approximately 78-fold, and to vincristine by approximately 10-fold. Hyaluronic acid had little effect on the non-drug-resistant cancer cells. Hyaluronic acid treated MDA-MB231 human mammary carcinoma cells, resistant to the folate analog methotrexate, were 133-fold more sensitive compared with nontreated cells.

The authors stimulated hyaluronic acid production in drug-sensitive MCF-7 cells by infection with a recombinant adenovirus driving expression of HAS2.[184] In 3 runs, modified MCF-7 cells produced 2.5- to 4-fold more hyaluronic acid than untreated cells or control cells infected with recombinant β-galactosidase andenovirus. The increased hyaluronic acid production induced a 10- to 12-fold increase in resistance to doxorubicin, the opposite effect of continuous treatment with hyaluronic acid. This experiment was repeated with emmprin, a member of the immunoglobulin (Ig) superfamily that is enriched on the surface of most malignant cancer cells and promotes tumor progression and regulates hyaluronic acid production. MCF-7 cells infected with recombinant emmprin adenovirus were approximately 10-fold more resistant to doxorubicin treatment than controls. The effect of emmprin was reversed by treatment with hyaluronic acid oligomers. The authors stated that this finding confirmed that emmprin increases drug resistance with hyaluronic acid.

The authors tested the effects of hyaluronic acid oligomers on the phoshoinositide 3-kinase (PI 3-kinase)/Akt cell survival pathway in MCF-7/Adr

cells.[184] Hyaluronic acid suppressed phosphorylation of Akt (protein kinase B) and stimulated expression of protein tyrosine phosphatase (PTEN) in the presence of doxorubicin, taxol, vincristine, and BCNU. PI 3-kinase activity was also inhibited, but there were no effects on total levels of Akt. BAD is a member of the family of apoptosis-regulating proteins. The authors expected hyaluronic acid treatment to lead to phosphorylation of BAD at serine residue 136 (BAD136), the site of Akt-mediated phosphorylation. However, in MCF-7/Adr cells, there was little phosphorylation of BAD136 in the presence or absence of the drugs or hyaluronic acid.

The use of hyaluronic acid in the endoscopic mucosal resection (EMR) procedure to remove tumors was tested.[77] Mice (n = 20) were injected into the right back with hyaluronic acid (0.2 mL; 5%) or saline (0.2 mL). A wound was inflicted by using a surgical knife to remove a 5-mm circle of skin, which was sutured closed. The mice treated with hyaluronic acid were then injected with transplantable adenocarcinoma cell line colon 26 (1×10^6 tumor cells) in 0.1 mL 0.5% hyaluronic acid. The controls were injected with the same cells in 0.1 mL PBS. Tumors were measured every 3 days. After 2 weeks, the tumors were removed, weighed, measured, and examined histopathologically. The hyaluronic acid group had larger tumors than the controls on days 9, 12, and 14 ($P = .001$, $P = .0001$, $P = .0001$, respectively). The tumor weights were also greater for the hyaluronic acid group on day 14 ($P = .001$). On day 14, the proliferating cell nuclear antigen-labeled index in cancer cells was higher in the hyaluronic acid group than in the control group ($P = .0001$). CD44 expression on the surface of the cancer cells was enhanced in the hyaluronic acid group compared with the control group. Western blot analysis also revealed that CD44 protein expression was higher in the hyaluronic acid group compared with the control group.

The use of hyaluronic acid in conjunction with cisplatin for the treatment of cisplatin-resistant head and neck squamous cell carcinoma (HNSCC) was investigated.[185] Two cell lines were used: SCC-4 and HSC-3, both from primary oral tongue squamous cell carcinomas. Various doses of cisplatin along with 50 μg/mL hyaluronic acid were used to treat plated cells (3000/plate), and the IC_{50} was calculated. Cisplatin alone inhibited tumor cell growth; the addition of hyaluronic acid resulted in a 5-fold reduced ability of cisplatin to cause HNSCC cell death. Hyaluronic acid plus anti-CD44 antibody did not inhibit tumor cell growth. For HSC-3 cells, the IC_{50} for cisplatin alone was 4 μM and 20 μM with hyaluronic acid. For SCC-4 cells, the IC_{50} for cisplatin alone was 20 μM and 100 μM with hyaluronic acid.

Yin et al[186] explored the inhibitory effects of hyaluronic acid with paclitaxel using tumor metastasis and ascites formation. Female mice (strain no. 615) were inoculated IP with U14 cells cervical tumor cells (2.5×10^6 cells/mouse) then treated daily for 5 days with saline, 30 mg/kg IP hyaluronic acid alone, 10 mg/kg IP paclitaxel alone, 30 mg/kg hyaluronic acid plus 10 mg/kg paclitaxel, or 15 mg/kg hyaluronic acid plus 5 mg/kg paclitaxel (n = 10 each group). Survival was recorded for 40 days; all mice were killed after 40 days. This experiment was repeated except that chemotherapy was started on day 2 after inoculation of tumor cells and continued for 3 days. Nine days after IP implantation of tumor cells, the mice were killed to observe for ascites formation. Paclitaxel alone and both the lower and higher dose combinations of paclitaxel and hyaluronic acid improved survival up to 40 days compared with saline control by 108.8%, 135.3%, and 135.3%, respectively ($P < .01$). Both the combinations improved life span when compared with paclitaxel alone ($P < .05$). Hyaluronic acid alone did not improve life span. Paclitaxel alone and both the lower and higher dose combinations of paclitaxel and hyaluronic acid reduced ascites formation inoculated with U14 cervical tumor cells ($P < .05$).

These authors also implanted 7-week-old female C57BL/6 mice with Lewis lung carcinoma (LLC) cells in the footpad (5×10^6 cells/mouse).[186] After 12 days, the mice were anesthetized and the tumor-injected foot removed surgically. The mice were grouped and treated daily for 5 days as above with saline, paclitaxel, and/or hyaluronic acid (n = 10 per treatment group). On day 40, the mice were killed and the lungs removed and fixed. Lung metastases were counted and measured under microscopy. Tumor metastases were reduced with hyaluronic acid alone (23.1%) and paclitaxel and hyaluronic acid at 1:1 (19.2%) and 1:3 (36.3%; $P < .05$). Blood samples were taken and analyzed for gene expression. The combination treatment upregulated the expression of vitamin D_3 binding proteins, which is a macrophage-stimulating activator.

Clinical Assessment of Safety

Dermal Absorption

Autoradiography was used to detect the dermal penetration of hyaluronic acid, in the form of [^{3}H]hyaluronan, using human skin.[145] The authors made 2 applications of 56.3 mg and 56.4 mg of [^{3}H]hyaluronic acid gel in a 1.8-cm^2 area of 1 forearm (number of subjects not given). This was repeated 12 hours later. The same was done to the opposing forearm with nonradioactive hyaluronic acid gel. Seven hours after the second application, the skin was swabbed as previously described, and samples were taken from the treated and control areas with a diagnostic trephine of 3-mm diameter. Adherent subcutaneous fat was removed before fixation. ^{3}H activity removed before fixation was measured in each case. The density of the grains was less intense, but there was still similar aggregation of grains in the keratinized layer, epidermis, and clear penetration activity to the deeper dermis with concentration at the level and just beneath the epidermis when examined autoradiographically. The authors concluded that hyaluronic acid penetrates normal epidermis to accumulate at least briefly in the dermis before its disposal and degradation via known metabolic pathways. The transit is rapid, and because there is no inward movement of extracellular fluid at this point, its passage must be mediated by extracellular diffusion, active transport through the cells, or combinations thereof.

Laugier et al[187] studied the dermal penetration of hyaluronic acid and hyaluronidase using human and synthetic skin. Synthetic skin and skin excised from the cadaver of a 42-year-old woman were used. Hyaluronidase was obtained from bacterium *S hyaluroniliticus* and purified. Applications of 40 µL volumes of hyaluronidase and hyaluronic acid (at 0.001, 0.010, and 0.100 mg/mL for synthetic skin and 0.01, 0.10, and 1.00 mg/mL for human skin samples) were placed on the skin surface. Samples were incubated for 24 hours in a humidified 5% CO_2 atmosphere. A punch biopsy was sampled from each experimental area and immediately placed into an acid-alcohol-formalin solution. Samples were fixed for 2 hours for the synthetic skin and 12 hours for the cadaver skin. The amount of hyaluronic acid and CD44 receptors (the cell surface protein that recognizes hyaluronic acid) in human and synthetic skin decreased in those treated with hyaluronidase. The decrease was concentration dependent. There was no significant change in the amount of hyaluronic acid or CD44 expression in either type of skin at any concentration. The authors concluded that hyaluronic acid does not penetrate the skin.

Natural Occurrence/Distribution

Some hyaluronic acid accumulates in the spleen, lymph nodes, and bone marrow.[188] An adult human male has hyaluronic acid entering general circulation at 10 to 100 mg/24 hours.[189]

According to Fraser et al,[87] hyaluronic acid is naturally present throughout the human body, particularly in the eyes and connective tissues. It is produced in the peripheral tissues where most of the turnover takes place in situ. A small amount is carried by lymph to the lymph nodes where it is metabolized.[188,190] The kidneys extract about 10% but excrete only 1% to 2% in urine.[87,188,190]

Some cells, such as chondrocytes in cartilage, actively synthesize and catabolize hyaluronic acid throughout the lifetime of the tissue.[4] The authors estimated that almost a third of the total hyaluronic acid in the human body is metabolically removed and replaced during an average day.

Metabolism

In humans, hyaluronic acid has a half-life of 2.5 to 5.5 minutes in blood. The mean amount of hyaluronic acid in blood is 30 to 40 µg/L.[189,191,192] From the blood, it is mostly taken up by the liver where catabolism takes place in the endothelial cells in the sinusoids.[191,193-195]

Hyaluronic acid breaks down into acetate and lactate.[193] Hyaluronic acid is broken down by hyaluronidases, β-glucuronidase, and β-N-acetylglucosaminidase, or by exposure to oxygen-free radicals.[195-197] Reactive oxygen species produced by keratinocytes are probably involved in the catabolism of epidermal hyaluronic acid.[198]

The level of hyaluronic acid in the blood is increased in people with liver cirrhosis where uptake and degradation are impaired.[189,199-201] This is also seen in rheumatoid arthritis and scleroderma.[199,202]

The half-life of hyaluronic acid in cartilage is normally 1 to 3 weeks.[4] Reticuloendothelial cells lining the lymphatics actively remove almost 90% of the hyaluronic acid before the remainder reaches the vascular system. The half-life of hyaluronic acid is

12 hours in the skin. The cells in the dermis actively synthesize more hyaluronic acid than they catabolize, much of which escapes only to be rapidly captured by receptors on reticuloendothelial cells in lymph nodes and liver, which internalize them for subsequent catabolism in lysosomes.

Injected hyaluronic acid and its derivatives undergo local degradation.[203] The metabolites are then further catabolized by the liver into carbon dioxide and water.

Effect on Penetration of Other Chemicals

Hyaluronic acid facilitates penetration of other substances through the human stratum corneum because a hydrated epidermis is more permeable.[204] The effects of hyaluronic acid on the in vitro diffusion and deposition of diclofenac within the skin were tested.[205] Human skin samples were obtained directly after abdominoplasties and surgery from male and female donors aged 30 to 50 years. Split thickness or epidermal sheet sections were used rather than full-thickness skin. The fat layer was removed. The skin was placed on a cork dissection board, and the epidermis was gently teased off the dermis using forceps and mounted in a Franz cell.

The receptor compartment was filled with previously sonicated Sørensen's buffer (pH 7.0). The area of exposed skin was 2.27 cm^2. The applied solution was either ^{14}C-labeled diclofenac (0.75 MBq/mg) mixed with Sørensen's buffer or ^{14}C-labeled diclofenac (0.75 MBq/mg) mixed with [^3H]hyaluronic acid (4.9 Mbq/mg). As a function of time after application of the labeled solution, 0.5 mL samples were removed from the sampling port in the receptor chamber and replaced with pre-equilibrated buffer.

Diffusion of ^{14}C-labeled diclofenac in buffer reached 10% in 12 hours, while it took ^{14}C-labeled diclofenac in the hyaluronic acid formula over a week to reach the same level. The buffer solution permeated the epidermal sheet relatively rapidly in the first 100 hours so that approximately 30% was in the receptor chamber, then leveled off. The hyaluronic acid solution maintained a steadier rate over the week of the experiment, and at 100 hours, only about 3% of the diclofenac had diffused through to the receptor chamber. Approximately 20% of the solution had diffused through the epidermal sheet by the end of the week-long experiment.[206] Brown et al showed that hyaluronic acid minimized the percutaneous absorption of diclofenac, indicating the formation of a reservoir of drug in the epidermis, which was confirmed using autoradiography.[205]

Lin and Maibach demonstrated that hyaluronic acid delivered twice the diclofenac to the epidermis over 24 hours compared with an aqueous control and sodium carboxymethyl cellulose.[207] Similar effects have been found with ibuprofen, clindamycin phosphate, and cyclosporin.[208-212]

The development of Solaraze (PharmaDerm, Melville, NY), 3% diclofenac in 2.5% hyaluronic acid gel, is used for the treatment of actinic keratosis .[213] The authors stated that hyaluronic acid enhanced the partitioning of diclofenac into human skin and its retention and localization in the epidermis when compared with an aqueous control, other glycosaminoglycans (ie, chondroitin sulphate), and commonly used pharmaceutically acceptable gelling agents (ie, sodium carboxymethyl cellulose) at either molar or rheologically equivalent concentrations.

Dermal Irritation

A "negative" result of a closed skin patch test of hyaluronic acid produced by fermentation was reported. No details were provided.[31]

Immunogenicity

General

Hyaluronic acid binds to monocytes and lymphocytes in inflammatory diseases such as ulcerative colitis and reportedly participates in other inflammatory conditions as well such as rheumatoid arthritis, scleroderma, and psoriasis.[202,214-218] Increased blood levels of hyaluronic acid have been reported in patients with sepsis.[219] Physical activity, which enhances the lymph drainage, also leads to a temporary increase in the serum hyaluronic acid level.[217]

Hyaluronic Acid

In tests for the stimulatory function of hyaluronic acid on polymorphonuclear leukocytes (PMNs), 5 to 10 mg of hyaluronic acid was subcutaneously injected into 6 healthy subjects and 10 persons with decreased resistance to bacterial infections and impaired phagocytic activity.[220] Heparinized venous blood was collected every or every other day, and the phagocytic rate of PMN was measured. PMNs were stimulated in all healthy subjects. The stimulation was evident 1 day after injection, maximized after

2 to 4 days, and lasted about a week. Leukocytosis or fever was not observed, and there was no local reaction at the injection site. The neutrophils of all the immunocompromised subjects responded by increased rates of phagocytosis of both IgG- and serum-opsonized particles. Peak phagocytic activities were seen 2 to 6 days after injection. An increased intracellular content of ATP and enhanced chemiluminescence of isolated PMN were also found after the hyaluronic injections.

Small fragments of hyaluronic acid may stimulate an inflammatory response.[221] In skin pathology, for example, they suggest that the accumulation of fragmented hyaluronic acid molecules in dermal papillae supports the growth of psoriatic lesions by stimulating the growth of capillaries and attracting inflammatory cells.

Sodium Hyaluronate

Immunogenicity of sodium hyaluronate was tested.[222] Nine of 10 healthy subjects (25-44 years of age; 50-73 kg; 163-183 cm tall; 8 females, 2 males) completed the study. For the skin prick test, the volar side of the forearm was pricked with Coca's solution (negative control consisting of 5 g of NaCl and 2.5 g of $NaHCO_3$/L), histamine chloride 1:10 000 (positive control), or sodium hyaluronate (10 mg/mL in a 2-mL disposable syringe; pH 7.3). Examination took place at 15 minutes and 2, 6, and 24 hours after pricking. For the immunization test, 2 subcutaneous injections of 1 mL sodium hyaluronate were administered to the upper arm at an interval of 1 week. In a microprecipitation test, 2-mL samples were taken from the subjects before and after the subcutaneous injections. They were divided into 2 samples, and 0.5 mL of either 5 or 50 μg/mL sodium hyaluronate was added to each. The protein content was estimated by the Folin test. In a complement analysis, sera and EDTA-plasma samples were collected on 4 occasions: before immunization, 24 hours and 6 days after starting immunization, and 2 weeks after the second injection. Sera was tested for total hemolytic complement titer. Plasma samples were tested for conversion products of factor C3 by immunoelectrophoresis in agarose. No skin reactions were observed at sites challenged with sodium hyaluronate or Coca's solution at 2, 6, and 24 hours after skin pricking. There was no increase in protein measured in the microprecipitation test after immunization for either the 5 or 50 μg/mL sodium hyaluronate. None of the sera showed a significant decrease in total hemolytic activity. No conversion products of factor C3 were seen in plasma samples collected before and after immunization.

Osteoarthritis Treatment

Hyaluronic acid and sodium hyaluronate have been used in clinical studies to evaluate their effectiveness in treating osteoarthritis (OA). Three such trials (the most recent) are described in detail; other trials and their safety results are summarized in Table 11 and discussed below.

The effectiveness of an injection of non-animal-stabilized hyaluronic acid on sufferers of OA was tested.[223] Subjects received a subcutaneous injection of 3 mL of either 60 mg hyaluronic acid in buffered sodium chloride (0.9%; pH 7; n = 172) or the identical buffered sodium chloride vehicle (n = 174). Seventy-four subjects did not finish the study. After 26 weeks, there was no significant difference between the control and treatment groups in the response to the treatment; the pain, stiffness, and physical function scores in both groups decreased over the study period. The safety evaluation included all recruited patients (n = 347). A total of 513 adverse effects (AEs) were reported by 227 patients (65.4%) over the study period. The majority of AEs (79.3%) were classified as mild/moderate. The number of patients reporting treatment-related AEs was 22 (12.8%) in the hyaluronic acid group and 14 (8.0%) in the saline group. The most common treatment-related AE was arthralgia, reported by 11 patients (6.4%) and 5 patients (2.9%) in the hyaluronic acid and saline groups, respectively. The majority of treatment-related AEs (>70%) were reported within 2 days of injection in both treatment groups. Treatment withdrawal attributable to AEs occurred in 13 hyaluronic acid and 6 control patients in the 2 groups; 5 and 4 of these events were considered related to treatment, respectively. Of the 9 treatment-related AEs leading to withdrawal, 7 reported general knee pain, 1 reported worsening OA pain in the knee (hyaluronic acid group), and 1 reported knee synovitis (placebo group). Ten of the patients withdrawing from treatment (7 in the hyaluronic acid group and 3 in the saline group) reported serious AEs (not defined), all of which were assessed by the investigator as being unrelated to the study treatment.

Table 11. United States and International Placebo-Controlled Trials Evaluating the Safety of Hyalgan,[1] Snyvisc,[2] and Supartz[3]

Study	Country: Regimen	No. of Patients	Results
Hyalgan[a] studies			
Carrabba et al[291]	Italy: placebo- and arthrocentesis-controlled, 1, 3, or 5 weekly injections with a 6-month follow-up	100	All regimens well tolerated; AEs mild and transient; no serious AEs
Bragantini et al[292]	Italy: saline-controlled, 3 weekly injections	55	Well tolerated; no serious AEs
Grecomoro et al[293]	Italy: vehicle-controlled, 3 weekly injections	36	Well tolerated; no reported AEs
Dixon et al[294]	UK: 0.2 mg sodium hyaluronate–controlled, up to 11 injections given at 1, 2, 3, 5, 7, 9, 11, 15, 19, and 23 weeks	63	Well tolerated; 3 reports of local joint reaction in Hyalgan patients
Dougados et al[295]	France: vehicle-controlled, 4 weekly injections	110	Well tolerated; no serious AEs; = control
Henderson et al[296]	UK: saline-controlled, 5 weekly injections	91	More poorly tolerated than placebo; local pain/swelling in 47% Hyalgan vs 22% placebo patients
Corrado et al[297]	Italy: buffered saline-controlled, 5 weekly injections	40	Decreased inflammatory effusion in Hyalgan patients
Listrat et al[298]	France: no injection-controlled, 3 courses of 3 weekly injections over 1 year	39	Well tolerated; 8 reports of injection pain limited to moment/few moment of injection
Altman and Moskowitz[299]	US: saline- and nonsteroidal anti-inflammatory drug–controlled, 5 weekly Hyalgan injections	456	Gastrointestinal AEs higher in naproxen group; local injection site pain higher in Hyalgan vs control
Huskisson and Donnelly[300]	UK: saline-controlled, 5 weekly injections	100	Well tolerated; = control; injection site reactions similar in placebo and active groups
Synvisc[b] studies			
Wobig et al[301]	Germany: placebo-controlled, 3 weekly injections	110	No serious AEs; only local AEs
FDA[83] Premarket Approval Application Study #5	US: arthrocentesis-controlled, 5 weekly injections	94	No significant differences in numbers or types of AEs between Synvisc and control
Supartz[c] studies			
Puhl et al[302]	Germany: vehicle-controlled, 5 weekly injections	195	Well tolerated; no clinical abnormalities
Dahlberg et al[303]	Sweden: vehicle-controlled, 5 weekly injections	52	Well tolerated; no serious AEs; similar injection-site pain with Supartz and control
Lohmander et al[304]	Sweden: vehicle-controlled, 5 weekly injections	240	Well tolerated; no serious AEs; greater severity of injection-site AEs in control patients ($P = .041$)
Wu et al[305]	Republic of China: saline-controlled, 5 weekly injections	90	Well tolerated; no clinical abnormalities

AE, adverse event.

[a] Hyalgan - sodium hyaluronate. Fidia Pharmaceutical Corporation, Washington, DC. Approved for marketing in US in May 1997. Molecular weight, 500-730 kDa; 1% protein.

[b] Synvisc - Hylan G-F 20. Sodium hyaluronate chemically crosslinked with formaldehyde and vinylsulfone to increase molecular weight. Biomatrix Inc, Ridgefield, New Jersey. Approved for marketing in US in August 1997. 80% Molecular weight, 6000 kDa; 20% molecular weight, indeterminate; 1% protein.

[c] Supartz - sodium hyaluronate. Seikagaku Corporation, Tokyo, Japan. Approved for marketing in US in January 2001. Molecular weight, 620-1710 kDa; 1% protein.

Hyaluronic acid was tested for effectiveness and safety as a treatment for OA of the knee. Seventy-six patients (92 knees) with moderate to severe OA received injections of 20 mg of sodium hyaluronate into the knee joint (intra-articular) at weekly intervals for 5 weeks.[224] There were no placebos. Clinical assessments were carried out at baseline and 6, 12, and 24 months. Monitoring for possible treatment-related undesirable or AEs was carried out at each clinical assessment. Seventy-two percent achieved a >50% improvement for 1 year or longer. No systemic effects were noted during the follow-up period. The AEs were minor and infrequent. They included brief

postinjection pain, minor bruising at the injection site, rare headache, and nausea.

Another approach to the evaluation of hyaluronic acid and its derivatives was taken.[225] Six knees in 5 patients that received a series of 3 intra-articular injections of hylan G-F 20 viscosupplementation underwent surgical procedures because of persistent symptoms of OA. No patient had a history of ongoing evidence of infection, evidence of immunocompromise, or a history of long-term use of immunosuppressive medications. No patient had an allergy to chicken or egg products. Two patients had received prior injections of corticosteroids to the knee joint. Previous surgical procedures on the knee included open reduction and internal fixation of a patellar fracture in 1 patient and arthroscopic debridement in 2. Routine histological examination of arthroscopic shavings from the latter procedures revealed no abnormalities. Each patient had pain, swelling, and warmth in the knee following viscosupplementation that developed within 48 hours after injection. This peaked at 4 to 5 days and gradually resolved in approximately 1 to 2 weeks following final injection. No patient had a fever or erythematous reaction. All patients managed the pain with nonsteroidal anti-inflammatory medications, but relief was minimal. No patient had evidence of peripheral leukocytosis on routine preoperative evaluation, and all had a normal C-reactive protein level. Knee aspriation was performed in 4 knees; no frank purulence or cloudy aspirate was found, and no organisms were seen on Gram staining. All 4 aspirates had a white blood cell count $<10\,000/mm^3$ ($<10.0 \times 10^9/L$). There were fewer than 5 neutrophils per high-power field, and cultures were negative. Two knees underwent arthroscopic debridement, one at 2 months and one at 6 months after last injection of hyaluronic acid (hylan G-F 20). Specimens were collected with use of a specimen trap connected to the arthroscopic shaver and preserved in 10% buffered formalin. Four knees underwent total knee arthroplasty between 5 and 9 months after final injection of hylan G-F 20. This resulted in the collection of tibial plateau, femoral condyle, shaved bone fragments, meniscus, and capsular tissue. Soft-tissue sections from each case revealed similar histological findings. Chronically inflamed synovium and adipose tissue containing numerous areas of histiocytic and foreign-body giant-cell reaction surrounding acellular, amorphous, pink fluid-like material were noted. Use of special stains for microorganisms had negative results. No birefringent crystalline material was observed under polarized light microscopy. The acellular material was stained with alcian blue, a stain for hyaluronic acid, which disappeared after hyaluronidase digestion. None of the bone fragments revealed granulomatous inflammation.

Vesicoureteral Reflux Treatment

Q-Med AB research on their product Deflux Injectable Gel, which consists of microspheres of cross-linked dextran suspended in a gel of nonanimal, stabilized hyaluronic acid to be used for children with vesicoureteral reflux (VUR).[155] Deflux is injected submucosally in the urinary bladder in close proximity to the ureteral orifice. Dextranimer microspheres are gradually surrounded by the body's own connective tissue, which provided a bulking effect. Thirty-nine subjects were treated with Deflux, and 21 were treated with antibiotic prophylaxis. They also treated a total of 170 subjects in 2 nonrandomized studies and followed them for 12 months. There were 14 urinary tract infections total in the 3 studies. In the first study, 2 patients had nausea, vomiting, and abdominal pain following injection procedure. This complication resolved in both cases. None of these problems were attributed to the hyaluronic acid.

Tissue Augmentation

Duranti et al[24] treated 158 patients with facial intradermal implant of hyaluronic acid gel for augmentation therapy of wrinkles, folds, acne scars, as well as lip augmentation and recontouring. All patients were white women with a mean age of 36.8 years (range, 26-68). Patients requiring further implants ($n = 11$) or extensive follow-up ($n = 4$) were excluded from the study. Patients were examined at time 0 and 1, 2, 4, and 8 months after the injection. Hyaluronic acid (Restylane; derived from bacterial sources) was placed into the mid-dermis using 27- or 30-gauge needles. Common antiseptic solutions were used to prepare the skin. There were 12.5% (34 cases) who experienced immediate AEs that were localized and transient. The most commonly reported were bruising, tenderness, discomfort, edema, and erythema at the treatment site. Most events lasted less than 3 days and resolved spontaneously. One erythema and swelling case lasted 5 days. Thirteen patients complained, particularly after lip augmentation, of

an intermittent swelling of the implanted material. There were 7 cases of erythema, 5 of edema, 5 of discomfort, 3 of tenderness, 3 of bruising, 1 of itching, and 1 of pain. There was no clinical evidence of major systemic side effects nor of acute or chronic hypersensitivity. No blood chemistry data were available.

These authors carried out a histological study on 5 volunteers (aged 26-54 years) for 52 weeks.[24] Prior to inclusion in the study, they were examined for skin diseases and double-tested for possible sensitivity to hyaluronic acid gel and collagen. The treatment consisted of spot injections of 0.05 mL of each product at 4 sites alternating between the products on the volar surface of the left and right forearm. Each person received a total of 8 implants. Biopsies were taken at weeks 4, 12, 24, and 52 after a physical evaluation of the sites. The biopsies were examined blind by 2 experienced pathologists who reached a consensus on each biopsy with respect to inflammation, foreign body reaction, and fibrosis. Hyaluronic acid implants maintained their spot size between the 12th and 24th week. At week 52, 4 of the implants were still clearly visible under the skin. Staining for hyaluronic acid revealed the presence of such material but with a significantly more watery appearance than at earlier biopsies. All of the specimens were free of fibrosis and severe foreign body reaction but often presented a slight inflammatory reaction. There were no differences in the presence of cells around each of the implants over time throughout the 52 weeks of observation.

The use of hyaluronic acid (Restylane; stabilized hyaluronic acid), for tissue augmentation, was tested.[226] One hundred thirteen patients (106 females and 7 males) were recruited, each receiving treatment in up to 3 sites, including glabellar lines, nasolabial folds, mouth angle wrinkles, and other facial lines. A total of 285 sites were treated. All patients were monitored for at least 30 minutes after treatment for erythema, swelling, local pain, redness, itching, and tenderness. All patients were evaluated at weeks 0, 1, 12, and 26. Twenty patients were randomly chosen to come back after 52 weeks for additional assessment. Additional injections were given to 66% of the 113 patients who were deemed to be in need of a "touch up." Nineteen (6.6%) of the sites showed redness, red spots, and/or swelling. Four sites (1.4%) developed dark areas. Three of these developed at week 1 and one at week 2. One patient reported slight pain at week 2. All of these events were resolved within 1 week.

A test on the use of stabilized hyaluronic acid for dermal augmentation was reported.[41] This was an open-label, 12-month study conducted at 6 sites. Investigators were all experienced plastic surgeons and dermatologists. A total of 216 patients were enrolled in the study, 191 female and 25 male, between 25 and 76 years old. All patients received at least 1 treatment, 30% received a second injection, and 17% received a third. The mean total volume of hyaluronic acid injected was 0.32 mL, ranging up to 1.60 mL. A total of 177 patients completed the 1-year study. Two of those who did not complete the study left because of dissatisfaction with their treatment and 3 because of localized discomfort or reactions associated with treatments. The authors stated that reactions were as expected and included transient and mild erythema, itching, swelling, and pain. Related or probably related adverse reactions occurred in less than 2% of all treatments and included persistent erythema, acne papule formation, and ecchymotic changes (blood from ruptured blood vessels leaking into subcutaneous tissue). No antigenic or immunogenic responses were observed.

A prospective study on the use of hyaluronic acid (Restylane) for lip tissue augmentation was reported.[154] This hyaluronic acid product was injected into the upper lips of 192 women aged 24 to 77 years (average age, 46 years). Of these patients, 88% had previously received collagen treatments. Two percent of the patients were allergic or had some adverse reactions to collagen. One patient had Hashimoto's thyroid disease, and 1 had rheumatoid arthritis. All patients received an initial treatment. Second and third treatments were at the mutual discretion of the patient and the investigator. Treatments were spread over 4 to 6 weeks. All patients were anesthetized with lidocaine cream 5%, and endobuccal anesthesia of the troncular type was administered to the most sensitive patients (2% adrenaline/lidocaine). Each patient received between 0.7 and 1.1 mL of hyaluronic acid. Swelling was noted in 86% of the patients during the first 24 hours and was noticeable at 5 days in 14% and 1% at 10 days. Redness was noted during the first 24 hours in 52% of the patients and in 36% of the patients the following day, and 12% on the third day. There was 1 case of a delayed effect. At the fifth week, 1 woman experienced inflammation that disappeared within 10 days with no treatment. The authors speculated that her trip to Africa with extra exposure to the sun in the fourth week may have been the cause.

A study of the use of hyaluronic acid from 2 sources for tissue augmentation were conducted: Restylane produced by microbiologic engineering techniques and Hylaform extracted from rooster combs.[227] The authors stated that both products contain varying amounts of hyaluronin-associated protein and therefore a theoretic risk for sensitivity reactions existed. Patients (677 women and 32 men) were treated, 438 with Hylaform and 271 with Restylane without prior skin testing. The patient's ages ranged from 25 to 75 years. The filler was administered with an intradermal, horizontal tunneling injection. Repeated treatments were performed on 180 of the Hylaform patients and 56 of the Restylane patients. No abnormal skin reactions were observed at the time of injection other than mild transient erythema. Delayed inflammatory reactions developed 6 to 8 weeks after the injection at some of the injection sites in 3 of the 709 patients (0.42%). These patients had not had tissue augmentation injections before this procedure. Also, 3 other patients from outside the study were referred for evaluation after injections of hyaluronic acid and included in this report. Four patients (3 with Hylaform and 1 with Restylane) developed induration and inflammation at the injection sites. They all developed an abscess in the nasolabial region. On palpation, these areas were firm, tender, edematous, and erythematous. Resolution was achieved in 6 to 24 weeks. Three patients required treatment to reduce the induration and inflammation.

These authors performed retrospective skin tests on 5 of the patients with delayed reactions.[227] Aliquots (0.1 mL) of both fillers were injected intradermally into the forearm skin. Four of the 5 patients tested developed discernable skin reactions ranging from mildly inflammatory nodules to inflammatory reactions with an abscess in 1 patient. Both Hylaform and Restylane caused reactions in 3 patients. Hylaform (extracted from rooster combs) reacted in 1 patient, and 1 patient had no reaction. These reactions appeared between 5 and 7 weeks. All reactions resolved in 2 to 12 weeks.

The authors also performed a 3-mm punch biopsy on one patient's nasolabial area about 6 weeks after the original inflammatory reaction.[227] The biopsy showed a normal epidermis with major changes confined to the dermis and subcutaneous fat. The upper dermis showed elastic degeneration, which was consistent with actinic damage. The mid and lower dermis showed a moderate infiltration of lymphocytes and plasma cells with few scattered macrophages containing hemosiderin pigment. Eosinophils were not seen, and there were no foreign body giant cells. Organizing fibrosis was most prominent in the lower dermis. All changes extended into the subcutaneous fat.

The data collected by Q-Med Esthetics, the manufacturer of a hyaluronic acid gel, for use in soft-tissue augmentation were examined.[228] They found that out of 144 000 possible treatments there were 222 adverse reactions reported, including localized hypersensitivity reactions, swelling, erythema, and induration at the transplant site, in 1999. There were no reports of systemic symptoms or anaphylaxes. In 2000, there were 144 reported adverse reactions out of 262 000 treatments. These included redness, edema, tenderness, injection site inflammation, erythema, swelling, pain, itching, discoloration, and temporary palpable lumpiness. Most of these problems resolved within 2 weeks. There were rare reports of localized granulomatous reactions, bacterial infection, and acneiform and cystic lesions. Two cases of injection site necrosis in the glabelar area a few days after injection were observed, which the authors considered likely secondary to compression of vascular supply from excessive use of product. The authors stated that the results may be skewed because the "treatment" number was deduced from the number of preloaded syringes sold, not by the number of patients; there is the possibility of doses not being used or multiple doses applied to the same person.

Physicians in European countries that use hyaluronic acid produced by Q-Medical for soft-tissue augmentation were surveyed.[229] A total of 12 344 syringes were sold by the company to these physicians. A total of 4320 patients were treated and evaluated in this survey. A total of 34 cases of hypersensitivity were reported between 1997 and 2001. Sixteen cases were immediate reactions. Fourteen of these resolved within 3 weeks, 1 lasted 6 weeks, and 1 lasted 2 months, resolving with the use of corticosteroid cream. One abscess was reported and was resolved with several evacuations. There were 18 delayed reactions. Most of these appeared a few weeks later, but 1 case appeared 6 months after injection with a streptococcal infection. There were 3 cases of delayed abscess. The author attributed 2 cases of reticular, livedoid reactions of the nose to an intravascular injection. These 2 cases lasted for 2 weeks before resolving without any sequela and/or scarring. Skin testing was not recommended by the author.

Cancer and Other Diseases

Elevated levels of hyaluronic acid are associated with tumor progression and cancer migration, an effect postulated to result from the influence of hyaluronic acid on cell division and attachment as well as its stimulation of angiogenesis.[230,231] Tumor cells have been found to increase production of hyaluronic acid; the cells in vitro are seen to have a halo of the substance around them in the growth medium.[232]

The levels of hyaluronic acid in neoplastic epithelial cells may be predictors of malignancy of gastric, breast, and ovarian cancer tumors.[231,233,234] This is also true of atherosclerotic cardiovascular disease.[235]

LMW hyaluronic acid (3-12 disaccharide units) has been shown to inhibit tumor growth.[236] Increased concentration of hyaluronic acid stimulates cell-survival signaling and reportedly stimulates drug resistance in drug-sensitive cancer cells.[184,237]

Increased hyaluronic acid levels in the blood interfere with platelet function, and the patients have disturbances in blood coagulation mimicking acquired von Willebrand's disease.[238] It has been shown that nephroblastomas produce platelet-derived growth factor, and it is known that this growth factor can activate hyaluronic acid synthesis, as did epidermal, basic fibroblast, and transforming growth factors.[239,240]

Increased amounts of hyaluronic acid have been found in the serum of patients suffering from Wilm's tumor.[241-245] Another factor, which they called "hyaluronic acid stimulating activity" (HASA) in serum and urine of Wilm's tumor patients, was reported.[246] This glycoprotein is synthesized by the fetal kidney and circulated in fetal blood. These authors suggested that Wilm's tumor occurs when the transformed rests of the fetal kidney retains the ability to produce HASA. Through HASA, the tumor cells can presumably induce other cells to produce hyaluronic acid.

If the hyaluronic acid in the serum is of high molecular weight (2×10^6 Da), as in Wilm's patients, there is hyperviscosity in the serum.[247,248] If the polysaccharide is of a low molecular weight, the angiogenic activity could be the underlying cause of the metastasizing power of patients with bone metastasizing renal tumors.[247,248]

Mesothelioma has been shown to have elevated hyaluronic acid.[249,250] High hyaluronic acid levels have been shown to be an indicator of a poor prognosis.[251,252] The patients who responded to treatment had decreased serum levels.[252] When studied in culture, mesothelioma cell lines produce negligible hyaluronic acid, in contrast to normal mesothelial cells. This is an analogous situation to HASA in Wilm's tumors.[175]

Hyaluronic acid significantly inhibited the active E rosette forming T lymphocytes in vitro.[253] In many cases, the level of hyaluronic acid that surrounds a cancer correlates with tumor aggressiveness.[254] Anttilla et al reported that stromal hyaluronan (hyaluronic acid) accumulation may be a powerful enhancer of tumor progression and, as such, provides a novel, independent prognostic marker and potential target for therapy.[234] Hyaluronic acid used to facilitate tumor excision surgery may stimulate the cell growth of any residual tumor cells after endoscopic mucosal resection.[77]

In discussing the relationship between tumors and hyaluronic acid, Laurent et al stated that, except for studies of Wilm's tumor and mesothelioma, there are several reports in which patients with various types of cancer have been screened for serum hyaluronic acid.[48,97,255-257] Increased levels have sometimes been noted in a few patients, while the majority of the cases have normal values; the high values have not been correlated with any particular tissue or metastatic involvement.[255]

Two studies have specifically assayed serum from patients with breast cancer and arrive at different conclusions. While Delpech et al[258] found a significant increase in serum hyaluronic acid especially in patients with metastases, Ponting et al[259] could not demonstrate any correlation with a number of prognostic factors. However, both groups concluded that serum hyaluronic acid is of no prognostic value.

An increase of collagen peptides and an increase of serum hyaluronic acid in patients with multiple myeloma were noticed, and researchers speculated that it was caused by myeloma activity in close proximity to periosteum or joints.[260] The same properties of hyaluronic acid that facilitate growth and motility during fetal development and tissue formation are utilized by cancers to promote their own growth.[176,261]

Cancer Patients

Serum hyaluronic acid was sampled from 57 women with breast cancer and 26 without and compared them to 50 patients with benign breast lesions

(controls).[258] Hyaluronic acid was increased in the sera of metastatic patients compared with nonmetastatic patients ($P < .0001$) as well as the control sera ($P < .01$). The difference was not related to the number of metastatic sites. Lower concentrations of hyaluronic acid were observed in patients after 3 months who were responding to chemotherapy. The initial concentrations of hyaluronic acid had no predictive value.

The level of hyaluronic acid in the sera of 238 women with breast cancer, measured by radiometric assay, showed no increase when compared to 120 healthy women (controls).[259] Predictive properties of hyaluronic acid were examined for stage of disease, lymph node involvement, tumor size, histology, and presence of estrogen or progesterone receptors in the tumors. There was no correlation with any of these parameters.

The cellular expression of hyaluronic acid in 215 stage I to IV gastric cancer patients was measured using a specific biotinylated probe.[233] Of the tumors, 93% were stained for hyaluronic acid in the parenchyma, and all had hyaluronic acid in the stroma inside and around the tumor. Hyaluronic acid expression was compared to clinical and histological features of the tumors. Strong staining intensity in the tumor parenchyma was associated with deep tumor invasion and with mixed types of classifications (diffuse, intestinal, mixed, unclassified). Hyaluronic acid–positive cells were associated with deep tumor invasion ($P < .0001$), nodal metastasis ($P < .07$; $F = 4.2$), positive lymphatic invasion ($P < .002$), poor differentiation grade ($P < .006$), and inferior prognosis in univariate survival analysis ($P < .0025$) (but not with multivariate analysis).

The relationship between hyaluronic acid and epithelial ovarian cancer tumors was examined.[234] Samples and histories were collected from 309 patients with adequate archival tumor material. A biotinylated affinity probe specific for hyaluronic acid was applied to histological sections of the samples and 45 matched metastatic lesions. The staining was scored for the percentage of area of strong hyaluronic acid signal (peritumoral and intratumoral stroma) as low (<35%), moderate (35%-75%), or high (>75%).

Levels of stromal hyaluronic acid were 95 (31%) low, 116 (37%) moderate, and 98 (32%) high. The high stromal hyaluronic acid level was associated with poor differentiation ($P < .0005$), serous histological type ($P < .05$), advanced stage ($P < .03$), and large primary residual tumor (>2 cm; $P < .03$) but not correlated with high CD44 expression. High amounts of hyaluronic acid in cancer cells were associated with poor differentiation of the tumor ($P < .002$). Low levels of stromal hyaluronic acid were associated with early FIGO stage ($P < .008$) and mucinous histological type ($P < .05$).

The 5-year survival of the disease decreased with increasing stromal hyaluronic acid levels for both overall (45% vs 39% vs 26%; $P = .002$) and recurrence-free (66% vs 56% vs 40%; $P = .008$) survival. High levels of stromal hyaluronic acid were more frequent ($P = .0001$) in metastatic lesions than in primary tumors.[234]

Samples were collected from 143 human breast carcinoma patients.[231] The localization and signal intensity of hyaluronic acid were analyzed in the paraffin-embedded tumor samples using a biotinylated hyaluronic acid–specific probe. In the immediate peritumoral stroma, hyaluronic acid signal was moderately or strongly increased in 39% and 56% of the cases, respectively. Normal ductal epithelium showed no hyaluronic acid, but in 57% of the tumors, at least some of the carcinoma cells were hyaluronic acid positive. Hyaluronic acid in the malignant cells was located on the plasma membrane in 77 of 143 (54%) cases, in the cytoplasm in 65 of 143 (45%) cases, and in the nucleus in 21 of 143 (15%) cases. The intensity of the stromal hyaluronic acid signal and the presence of cell-associated hyaluronic acid were both related to poor differentiation of the tumors ($P < .003$), axillary lymph node positivity ($P < .015$), and decreased survival of the patients.

The levels of CD44 (hyaluronic acid receptor) and hyaluronic acid associated with disease progression and survival of cutaneous melanoma were studied.[231] A series of 292 clinical stage I cutaneous melanomas were analyzed by immunohistochemistry using an anti-CD44H antibody (clone 2C5). Hyaluronic acid was demonstrated histochemically using a biotinylated hyaluronic acid–specific affinity probe (bHABC). CD44 was positively associated with cellular hyaluronic acid. Decreasing levels of cancer cell–associated CD44 and hyaluronic acid were related to increasing Breslow thickness ($P < .000\,05$ and $P < .001$, respectively), increasing Clark level ($P < .000\,05$ and $P < .000\,05$, respectively), and increasing pT category ($P < .000\,05$ and $P < .000\,05$, respectively); this trend was evenly distributed within all 3 categories. Decreasing CD44 and hyaluronic acid levels also associated with bleeding

($P = .024$ and $P = .011$, respectively) and recurrent disease ($P < .00005$ and $P < .01$, respectively). Stromal hyaluronic acid intensity was not correlated with CD44 or any of the clinicopathological variables. Stromal hyaluronic acid intensity was not related to overall survival or recurrence-free survival.

The uses of 3 serum markers (tissue polypeptide antigen (TPA), hyaluronic acid, and cancer antigen 125) were explored in following the progress of mesothelioma.[262] Historical blood profiles were examined for 11 patients over 1 to 63 months with 1 to 18 samples. Correspondence between initial TPA levels and survival were better than either hyaluronic acid or cancer antigen 125 markers. Five patients had increasing serum levels of all 3 markers as the mesothelioma progressed (according to CT scans). In 3 patients, stable disease was followed by a decrease in all 3 serum marker levels.

Paraffin-embedded sections of 114 basal cell carcinomas (BCC), 31 in situ carcinomas (ISC), and 35 squamous cell carcinomas (SCC) were stained for the presence of hyaluronic acid and CD44.[263] Compared with normal epidermis, ISC and well-differentiated SCC samples showed an enhanced hyaluronic acid signal on carcinoma cells ($P < .001$), while CD44 expression resembled normal skin. Less-differentiated SCC samples had reduced and irregular expression of hyaluronic acid and CD44 on carcinoma cells. In BCC samples, hyaluronic acid was frequently present on cell nuclei; this was not noted in the other types of samples. Hyaluronic acid in the connective tissue stroma around tumors was more frequent in SCCs than BCC. Varsican staining was positive around hair follicles and dermal blood vessels of normal skin. Peritumoral varsican signal was present in a part of the BCCs but not in other tumors.

The immunohistochemical expression of hyaluronic acid synthase (HAS) and serum levels of hyaluronic acid correlation with the clinicopathological manifestations of endometrial carcinoma were determined.[264] Sera and cancer tissue was collected from 59 endometrial cancer patients and sera from 22 healthy postmenopausal women. Hyaluronic acid concentration was determined by inhibitory ELISA. The cancer tissues were immunostained by the avidin-biotin-peroxidase complex method using anti-HAS1, 2, and 3 and anti-CD44 antibody. The expression of HAS1 was related to the depth of myometrial invasion, histological grade, and lymph-vascular space involvement, but not HAS 2 and 3. CD44 expression was more frequent in HAS2- and HAS3-positive groups than in the HAS2- ($P = .0094$) or HAS3-negative ($P = .0134$) groups. The expression of HAS1 was not related to CD44 expression. Serum hyaluronic acid levels were higher in the endometrial cancer patients (418.4 ± 210.6 ng/mL) than in the healthy control group (99.5 ± 48.2 ng/mL; $P < .0001$). The levels increased with depth of myometrial invasion, histological grade, and lymph-vascular space involvement. Serum hyaluronic acid levels were higher in the HAS1-positive group than the HAS1-negative group ($P < .0001$); the expression of HAS2 and HAS3 were unrelated to serum hyaluronic acid levels.

The amount of hyaluronic acid present in human tumors was measured.[265] A total of 256 samples were collected after surgery from cancer patients between 1995 and 2002. The samples were fixed and stained and the grade of tumors evaluated. Samples were selected for the presence of benign and malignant histology. Histochemical localization of hyaluronic acid was accomplished by a biotinylated hyaluronic acid–affinity probe. Twenty samples were graded as well-differentiated tumors (astrocytoma, salivary gland, thyroid, infiltrating breast, stomach, urinary bladder, and colon tumors), all of which had intense hyaluronic acid staining in the tumor cells, intratumoral, and in the associated surrounding stroma. In the poorly differentiated tumor samples (astrocytomas, infiltrating breast, stomach, gall bladder, pancreas, caecum, prostate, ovary), cells showed almost no hyaluronic acid stain; the intratumoral and stromal areas showed moderate hyaluronic acid stain. Irrespective of their origin, the poorly differentiated tumor samples showed negative staining for hyaluronic acid in the tumor epithelial or stromal area. With regard to the surrounding tissue, there was intense hyaluronic acid staining in intratumoral and peritumoral areas of all the well-differentiated tumors compared with benign areas. Carcinomas from astrocytomas, gangliogliomas, thyroid, breast, and salivary samples displayed intense accumulation of hyaluronic acid in intratumoral areas. Highly aggressive poorly differentiated tumors of different origins demonstrated moderate to low levels of stromal hyaluronic acid in the immediate vicinity of the tumor cells. Epithelial cells of benign areas were mostly hyaluronic acid negative. In the 20 tumors studied, epithelial cell surfaces were positive for hyaluronic acid in the well-differentiated tumors compared with the poorly differentiated tumors.

Hyaluronic acid levels were measured by immunoradiometric techniques in 850 patients with invasive breast cancer.[266] The mean follow-up time was 55.1 months. Cytosolic hyaluronic acid levels in tumors ranged from 4 to 59 767 mg/mg protein; the median was 4960 mg/mg protein. Hyaluronic acid levels were higher in younger patients ($P = .0001$); the levels were also higher in premenopausal women compared with postmenopausal women ($P = .001$). Hyaluronic acid levels were higher in ductal or lobular histological type than other histological types (colloid, medullary, papillary) ($P = .001$). No association was found between hyaluronic acid intratumoral levels and relapse-free survival and overall survival. High hyaluronic acid intratumoral levels were associated with longer relapse-free survival ($P = .01$) in the subgroup of patients with ductal histological type tumors and those ($P = .01$) without any type of systemic adjuvant treatment.

Case Reports

The case of a 74-year-old man who was referred for worsening OA of the right knee was reported.[267] Previous treatments included arthroscopic debridement, physical therapy, and intra-articular steroid injection. He declined total knee replacement. Radiographs showed advanced OA with faint linear calcifications suggestive of chondrocalcinosis. Physical examination showed a mild varus deformity and anterior and medial joint tenderness. There was no joint effusion. Three intra-articular injections of hyaluronic acid (Hylan G-F 20, Synvisc) were administered weekly for 3 weeks. The first 2 injections were uncomplicated. One week after the third injection, the patient developed a large, painful right knee effusion. Synovial fluid analysis revealed 5300 white blood cells and 680 red blood cells/μL. Gram stain and cultures were negative. Enzyme-linked immunosorbent assay did not detect antibodies to *Borrelia borgdorferi*. Microscopic analysis revealed multiple intracellular rhomboid crystals typical of pseudogout. The patient underwent joint aspiration and conservative treatment, which improved his symptoms.

Adverse effects of intra-articular hyaluronic acid (Hylan GF-20) injections to a 46-year-old male patient with moderate medial compartment OA were reported.[268] He was otherwise healthy and had no history of allergy to food or drugs, including eggs or chicken. Three injections 1 week apart were performed. The third injection was performed without difficulty and was uneventful. Within 2 hours, the patient developed rapidly progressive painful swelling of the knee. A large effusion developed in less than 24 hours and was evaluated in the emergency room. He had a fever of 38.9°C (102°F) and complained of severe knee pain. Arthrocentesis yielded 100 cc of clear yellow fluid. The cell count was 9600 leukocytes with 27% neutrophils, 38% monocytes, 25% lymphocytes, and 16% eosinophils. There were no crystals. Gram stain revealed leukocytes but no bacteria. Cultures of synovial fluid and peripheral blood were negative at 72 hours. He was first treated with naproxen but returned to the emergency room 24 hours later. He then received an intra-articular injection of triamcinolone hexacetonide. The swelling and pain improved gradually over the next 5 days and was controlled with ibuprofen. The authors stated that the temporal pattern, marked synovial fluid eosinophilia, and response to corticosteroid injection were all consistent with an allergic reaction.

A 54-year-old white woman underwent treatment for facial lines and wrinkles with modified hyaluronic acid gel.[269] She was treated in April 1998 and November 1998 with only mild bruising and erythema at the injection sites, which resolved in 1 to 2 days. In June 1999, she again had treatments. Two weeks later, she developed acute, multiple, tender red nodules within the treatment areas. Physical examination showed multiple, discrete nodules measuring 0.5 to 1 cm in diameter in various stages of development. Some nodules exuded a coagulated, yellow, stringy material and appeared to be secondarily infected with frank pus. Other lesions were more indurated, almost fibrotic, with significant erythema but still with intact overlying skin. A culture detected no pathogenic bacteria. The patient was treated with minocycline and methylprednisolone. Warm saline compresses were also applied. The most purulent and fibrotic nodules were injected with triamcinolone acetonide for symptomatic relief. The symptoms rapidly cleared. However, 2 weeks later, the patient returned with recurrent inflammation. The same treatment was repeated with resolution of her symptoms.

A 53-year-old woman was described with an exudative reaction that increasingly turned granulomatous 2 days after the injection of hyualuronic acid (Hylaform).[270] The peak of the eczematous reaction was reached after 4 to 6 days, and it healed after a further 4 to 5 days.

Two patients with reactions to intra-articular injections for OA were reported.[271] The first was a 73-year-old woman with a long history of left knee pain. She had been treated with nonsteroidal anti-inflammatory drugs, steroid intra-articular injections, and physical therapy. A right Baker's cyst had been surgically removed 6 years prior. Physical examination showed crepitus with reduced flexion, a left popliteal cyst, and a slight knee effusion. Radiographs showed osteoarthritic changes and bilateral genu valgum. She underwent intra-articular injections of hyaluronic acid (Hylan GF-20) into the left knee. Before the first injection, the knee was aspirated, yielding 5 mL of clear synovial fluid. White and red blood cell counts were 90×10^6/L and 50×10^6/L, respectively. No crystal was identified, and routine cultures were negative. The first injection was uneventful. One week later, a second injection was given. Before the injection, 10 mL of synovial fluid was aspirated with a white blood cell count of 310×10^6/L, sterile and no crystals. Four hours after the second injection, she developed severe left knee pain with local inflammation signs and a fever of 38°C (100.4°F). The synovial fluid contained $80\,000 \times 10^6$/L white cells with 82% polymorphonuclear cells and remained sterile after 5 days. No crystal was detected. Treatment with piroxicam, paracetamol, and ice resulted in partial recovery within 7 days. At the third injection, a small effusion persisted, and 20 mL of fluid was aspirated. The white cell count had reduced to $15\,750 \times 10^6$/L with 72% granulocytes and remained sterile without detectable crystals. She was completely recovered from the reaction in 2 weeks. The second patient was a 59-year-old woman who had right knee OA for 7 years. There was no effusion and no erythema. Knee flexion was limited. Radiographs showed osteoarthritic changes without meniscocalcinosis. Pain did not diminish with NSAIDs or steroid intra-articular injection. Before the first hyaluronic acid (Hylan GF-20) injection, less than 1 mL of synovial fluid was aspirated and examined. White blood cell count was 90×10^6/L. After the first injection, the knee had a small effusion and swelling within 4 days and improved spontaneously. Before the second injection, the white blood cell count of the synovial fluid was 90×10^6/L with sterile cultures and without crystals. Two hours after the injection, she presented with severe knee pain, swelling, and effusion. Knee aspiration yielded 50 mL of inflammatory synovial fluid. The white cell count was $32\,600 \times 10^6$/L. The fluid was sterile in culture, and no crystals were found. The arthritis resolved within 7 days after treatment with paracetamol alone. The patient declined a third injection.

Ten cases were reported with clinical allergic reactions after wrinkle treatment with injectable hyaluronic acid.[40] The manifestations look like nettle rash reactions or, more often, like delayed and long-lasting inflammatory cutaneous reactions. The author suggests that even though the percentage of allergy to hyaluronic acid ($\pm 3\%$) is lower than injectable bovine collagen ($\pm 4\%$), allergy tests may be in order before treatment.

A case was reported of a 45-year-old woman who developed erythematous swelling of treated areas and mildly tender nodules at injection sites 3 days after soft-tissue augmentation of the nasolabial lines and oral commissures.[272] The same treatment 90 days prior was uneventful. The right side was worse than the left. Symptoms cleared after 10 to 15 days with treatment with hydrocortisone cream 2.5%. There were no sequelae at 90 days' follow-up.

A 54-year-old woman who had hyaluronic acid gel injected in her nasolabial folds was described.[273] The gel was produced by crosslinking part of the glucosaminoglycan molecule of hyaluronic acid under controlled conditions to yield a gel. The resulting polymer resides in the intercellular matrix of the skin for about 6 to 12 months. The woman developed redness and intermittent swelling of the nasolabial folds, followed by the development of severe palpable and painful erythematous nodular papulocystic lesions, which evolved into severe abscesses on both folds. The complications were resolved with drainage and surgical removal of the papulonodular material. A biopsy showed granulomas with severe foreign body reactions.

A patient was reported who presented with a possible granulomatous reaction to hyaluronic acid (Restylane, filler from bacterial sources) or a bovine collagen filler.[274] The authors state that adverse effects of classic foreign-body granuloma are rare and estimate more frequent reactions in patients who have been sensitized to these products, although they can also occur in nonsensitized patients. Skin tests on this patient showed that she was not sensitized to these 2 fillers.

A case of a 40-year-old man who was injected with hyaluronic acid (Restylane) along a prominent horizontal forehead furrow line and the vermillion border of his upper lip from the same syringe was

reported.[275] Five months later, he showed an elevated, lumpy red line in the forehead that developed over a 24-hour period. There was no pain or tenderness, and the inflammatory reaction gradually disappeared over the next 3 weeks. No reaction was evident on the lip, and the author suggested this was possibly due to the small amount of hyaluronic acid injected or the reaction was camouflaged by the natural redness and indistinct outline of the patient's vermillion border.

A case was reported of a 65-year-old woman who had a negative skin test result and still developed a hypersensitivity reaction to hyaluronic acid.[276] A skin test was performed on the left forearm with 0.1 mL intradermal injection of Restylane obtained from a 0.7-mL syringe of Restylane from the manufacturer. The syringe was sealed in an envelope. There was no hypersensitivity reaction noted at the site for 4 weeks after the test. The patient underwent treatment with Restylane over a period of almost 1 year at the nasolabial folds, lips, perioral rhytides; corners of the mouth; nasolabial folds and perioral rhytids; and perioral areas. Approximately 0.7 mL of Restylane was used for each treatment. Six weeks after her last treatment, the patient presented with slight edema of the nasolabial folds with no erythema. Massage did not relieve the condition, and she returned in 6 weeks with extensive erythema, edema, and induration in the injected regions. She was started on a 3-week prednisone taper starting at 40 mg. The patient improved over the next few weeks; then in mid-April 2004, she presented with a flare in the previously affected areas, coinciding with the tapering of the prednisone. The recommended biopsy was refused. Intralesional 1.0 mL triamcinolone acetonide (2 mg/kmL) was injected resulting in no change after 1 week. The patient was restarted on a longer course of prednisone at 40 mg, which was tapered over 8 weeks with slight results at follow-up. She received several monthly intralesional triamcinolone acetonide injections (5 mg/mL) to the inflamed areas when the erythema had faded and the previously raised thickened areas were almost flat.

Summary

Hyaluronic acid, sodium hyaluronate, and potassium hyaluronate function in cosmetics as skin conditioning agents and viscosity increasing agents. These compounds are formed by the bonding of N-acetyl-D-glucosamine with glucuronic acid in a disaccharide chain that can grow as long as 10 000 pairs in length. As the chain lengthens and coils into a spherical shape, the molecule mechanically holds water, which in turn allows small molecules to pass through and excludes or slows the passing of larger molecules. Adjacent chains can interact with each other to form a network. The viscosity of these molecules increases with concentration. Hyaluronic acid salts are soluble in saline but almost insoluble in organic solvents. Hyaluronic acid can act as an antioxidant but is degraded. It can be degraded by UV radiation.

Hyaluronic acid and its salts may be derived from rooster combs, bovine tracheas and vitreous, bacterial fermentation, and human umbilical cords. In cosmetics, hyaluronic acid (5 and 1800 kD) sources are bacterial fermentation and rooster combs. Impurities include proteins, DNA, and chondroitin sulfate when derived from animal sources.

Hyaluronic acid reportedly is used in 27 cosmetic product categories, at concentrations up to 1%. Sodium hyaluronate reportedly is used in 32 cosmetic product categories, with a maximum concentration of 2%. Potassium hyaluronate reportedly is used in 8 cosmetic product categories, although no use concentration data were available.

Noncosmetic uses include multiple medical uses, including treatments for tissue augmentation, dry eye, osteoarthritis, and wounds. Hyaluronic acid also has multiple surgical and drug delivery applications.

Hyaluronic acid is found naturally in avascular body compartments. In humans, it is most abundant in the skin but also found in synovial fluid, vitreous humor, tendon sheaths, and bursae. Hyaluronic acid is synthesized primarily by dermal fibroblasts and epidermal keratinocytes. Functions in the body include tissue hydration, lubrication, solute transport, cell migration and function, wound healing, and red blood cell aggregation and adhesion.

Hyaluronic acid has been detected to a maximum depth of 800 μm after dermal application to rats. In mice and humans, hyaluronic acid penetrated to the dermis. Hyaluronic acid can moderate the penetration of other chemicals such as diclofenac, causing a slower absorption of that drug and preferential accumulation in the epidermis.

Radioactive hyaluronic acid was found to transfer to the fetuses of pregnant rats injected on day 17 of pregnancy after 4 hours. Hyaluronic acid was found in the skin, plasma, blood, Harder's gland,

kidney, spleen, and livers. Similarly treated lactating rats were found to have radioactivity in their milk after 24 hours. In humans, hyaluronic acid has a half-life of 1 to 3 weeks in the cartilage, 2.5 to 5.5 minutes in the blood, and 12 hours in the skin. Radiolabeled hyaluronic acid injected into the anterior chamber of rabbits had a half-life of 14 hours, 50 hours for subcutaneous injection, and 30 hours for intramuscular injection. For sheep, hyaluronic acid has a half-life of 5.3 $\pm$ 1.1 minutes in the blood. When injected in the eye, hyaluronic acid has a half-life of 21 hours in cynomolgus monkeys. Hyaluronic acid has a half-life of 8 to 15 hours in rabbits when injected into the pleural space. Inflammation causes an increase in hyaluronic acid levels and half-life in rabbits.

In an acute toxicity study, no deaths were reported of mice orally administered >1200 mg/kg hyaluronic acid. Hyaluronic acid had no ill effects to guinea pigs, rats, or cats when injected into the middle ear. Rabbits had no sign of neurotoxicity from hyaluronic acid when injected into the spinous process.

Hyaluronic acid or sodium hyaluronate caused no ill effects to rats or rabbits when injected peritoneally. There was no short-term ototoxicity to guinea pigs, rats, or cats when hyaluronic acid was injected into the middle ear. There was no evidence of cytotoxicity from sodium hyaluronate to mouse bone marrow cells; however, hyaluronan-based hydrogels were cytotoxic to smooth muscle cells from the thoracic aorta of rats. The respiration of hyaluronic acid had no ill effects in dogs and sheep.

Owl monkeys and rhesus monkeys had no ill effects from repeated injection of hyaluronic acid into the eyes. Repeated injections of sodium hyaluronate into the eyes of owl monkeys increased the leukocyte count up to 200 cells/mm^3 after 48 hours. The severity of haze and flare in the eyes after the injections did not increase over time, and there was no immunogenic response. This experiment was continued for up to 9 years in 2 eyes with no adverse effects. When implanted into the bladder submucosa of rabbits and dogs, there was no inflammation, infection, irritation, foreign body response, tissue necrosis, or scarring for up to 24 months.

Rabbits had no sensitization response to multiple injections of hyaluronic acid from human umbilical cords or streptococcal fermentation. Repeating the experiment resulted in erythema from umbilical cord hyaluronic acid in 2 rabbits and 2 that received streptococcal hyaluronic acid. Tests for nonprecipitation, skin-sensitizing antibodies were negative during the 1-hour observation. Injected streptococcal hyaluronic acid samples containing 0.1% to 0.3% protein caused rabbits to form precipitating antibodies to the hyaluronic acid. No passive cutaneous anaphylaxis reactive antibodies were formed in rabbits for hyaluronic acid. Antibody response by rooster comb–derived hyaluronic acid in rats caused an enhanced secondary antibody response to birch pollen, egg albumen, and dog albumin. Neither commercial sodium hyaluronate preparations nor a crude rooster comb sodium hyaluronate preparation elicited a hyaluronic acid–specific antibody response in rabbits. Crosslinked hyaluronic acid caused acute cutaneous anaphylaxis and delayed-type hypersensitivity in guinea pigs up to 48 hours after injections.

Hyaluronic acid did not cause reproductive or developmental toxicity in studies using rats or white rabbits. Hyaluronic acid was not genotoxic in reverse mutagenicity tests on *S typhimurium* and *E coli*, in vivo micronucleus tests using mice, or in vitro and in vivo chromosomal aberration tests using Chinese hamster lung fibroblast cells.

Hyaluronic acid levels have been found to be increased in tissues surrounding some breast cancer; gastric cancer; poorly differentiated, serous histological type, advanced stage, and large primary tumor epithelial ovarian cancer; endometrial cancer; ganglioma; thyroid cancer; and salivary gland cancer. Hyaluronic acid levels have been found to be normal or reduced in association with breast cancer, early FIGO stage and mucinous histological-type epithelial ovarian cancer, and murine astrocytoma.

Mouse melanoma cell lines with high hyaluronic acid production had increased lung metastasis and lower survival than melanoma cell lines with low hyaluronic acid production. Medium from mesotheliaoma cell line culture, transferred to mesothelial cells in culture, caused increased hyaluronic acid production. Increased hyaluronic acid intensity in breast cancer patients was related to axillary lymph node positivity and poor survival. Mesothelioma cells genetically enhanced to produce hyaluronic acid grew 2- to 3-fold larger colonies in soft agar than non–hyaluronic acid–producing mesothelioma cells. Hyaluronic acid reduced LX-1 lung carcinoma tumor growth. Using rat carcinoma cells, hyaluronic acid synthase (HAS2) enhances tumor growth, whereas expression of hyaluronidase activity of Hyal1 delays tumor development.

Aneuploid human breast adnocarcinoma cells modified with antisense inhibition of HAS2 expression produced more HMW hyaluronic acid. When these cells were injected into mice, no primary tumors were established, and there was no metastasis to any organs, and the mice had longer survival than those injected with nonmodified cells.

Well-differentiated tumors (astrocytoma, salivary gland, thyroid, infiltrating breast, stomach, urinary bladder, and colon tumors) had intense hyaluronic acid staining in the tumor cells, intratumoral, and in the associated surrounding stroma. Poorly differentiated tumor samples (astrocytomas, infiltrating breast, stomach, gallbladder, pancreas, caecum, prostate, and ovary) with carcinoma or sarcoma had almost no hyaluronic acid when stained. Enhanced motility of human pancreatic carcinoma cells was dependent on the CD44–hyaluronic acid interaction where LMW hyaluronic acid induced angiogenesis, enhanced CD44 cleavage, and promoted the migration of the tumor cells in a CD44-dependent manner.

Stromal hyaluronic acid was not related to survival or recurrence-free survival from cutaneous melanoma. Compared with normal epidermis, in situ carcinomas and well-differentiated squamous cell carcinomas showed an enhanced hyaluronic acid signal on carcinoma cells, while CD44 expression resembled normal skin. Less-differentiated squamous cell carcinoma samples had reduced and irregular expression of hyaluronic acid and CD44 on carcinoma cells. In basal cell carcinoma samples, hyaluronic acid was frequently present on cell nuclei but not in the other types of samples.

Hyaluronidase applied to tumors or tumor cells injected into the footpads of mice reduced growth rates in human breast carcinoma. The in vivo application of hyaluronic acid increased the effectiveness of chemotherapeutic agents when applied to drug-resistant mammary carcinoma cells but had little effect on nonresistant cells in mice. Increasing the expression of hyaluronic acid in these MCF-7 cells increased the resistance to doxorubicin. Hyaluronic acid reduced the effectiveness of cisplatin in treating cisplatin-resistant head and neck squamous cell carcinoma. Hyaluronic acid in combination with paclitaxel increased survival of mice over paclitaxel alone; tumor metastases were reduced by a combination of paclitaxel and hyaluronic acid over paclitaxel alone.

There were no skin reactions to an immunogenicity test of sodium hyaluronate on humans (10 mg/mL). Leukocytosis or fever were not observed in healthy subjects and persons with decreased resistance to bacterial infections and impaired phagocytic activity when subcutaneously injected with 5 to 10 mg of hyaluronic acid. Phagocytic rate of polymorphonuclear leukocytes measured with IgG-coated latex particles was stimulated in all healthy subjects; neutrophils of all the immunocompromised subjects responded by increased rates of phagocytosis in both IgG- and serum-opsonized particles.

In multiple studies on the use of injected hyaluronic acid as a treatment of osteoarthritis, most treatments were successful. Adverse effects such as minor discomfort, bruising, headache, nausea, and increased white blood cell count were reported, all treatable with nonsteroidal anti-inflammatory medications and/or aspiration of the swollen joint. Some patients had severe pain, swelling, and very high white blood cell counts ($>10.0 \times 10^9$/L) in the aspirated fluid, usually after multiple treatments with hyaluronic acid. No treatment-related effects were reported with the use of hyaluronic acid as a component of a treatment for vesicoureteral reflux.

When tested for tissue augmentation, hyaluronic acid was found to be safe and nonreactive in most cases. Most cases of bruising, tenderness, discomfort, edema, and erythema lasted less than 3 days and resolved spontaneously. There were cases of redness, red spots, dark areas, and/or swelling. Some adverse effects required treatment. One study found that less than 2% of all treatments had adverse reactions, including erythema, acne papule formation, and ecchymotic changes. No antigenic or immunogenic responses were observed. In another study, 86% had swelling, 52% had redness after injections to the upper lip.

There were 144 reported adverse reactions reported from the use of hyaluronic acid for soft-tissue augmentation out of a possible 262 000 uses, 222 out of 144 000 in 1999. Reactions included localized hypersensitivity, swelling, erythema, edema, tenderness, inflammation, itching, discoloration, temporary lumpiness, and induration. Most resolved within 2 weeks. Of a total of 12 344 applications sold by another company and used on a total of 4320 patients between 1997 and 2001, 34 cases of hypersensitivity were reported.

Many case reports of adverse effects from hyaluronic acid have appeared, most related to its use to treat osteoarthritis or for tissue augmentation; all these cases were from injections and not topical use.

Discussion

While hyaluronic acid has multiple sources, including rooster combs, bovine sources, and bacterial fermentation, in cosmetics, the only sources of hyaluronic acid used are bacterial fermentation and rooster combs. Because there is an avian source for these cosmetic ingredients, the matter of avian flu was considered. Because the heat from the manufacturing process reliably kills the avian flu virus, no safety concern exists in this regard. While there are no specific infectious agent concerns, the CIR Expert Panel is mindful of the need to derive these ingredients only from disease-free animals. Bacterial sources should be free of pyrogens.

The Expert Panel recognized that these ingredients can enhance the penetration of other ingredients through the skin (eg, HC Yellow No. 4, Disperse Yellow 3). The Panel cautioned that care should be taken in formulating cosmetic products that may contain these ingredients in combination with any ingredients whose safety was based on their lack of dermal absorption data or when dermal absorption was a concern.

After reviewing inhalation toxicity data on dogs and sheep, the CIR Expert Panel determined that hyaluronic acid, sodium hyaluronate, and potassium hyaluronate can be used safely in sprays because the ingredient particle size is not respirable. The Panel reasoned that, for example, the particle size of anhydrous hair sprays (60-80 μm) and pump hair sprays (>80 μm) is large compared with the median aerodynamic diameter of 4.25 $\pm$ 1.5 μm for a respirable particulate mass.

The CIR Expert Panel considered that the amount of hyaluronic acid present naturally in human skin was relevant to considering the effect of exogenous hyaluronic acid. The amount of hyaluronic acid in the skin is approximately 0.6 mg/g skin. The average woman has a total surface area of 16 900 cm^2; approximately 15% of a 60-kg woman is skin which is approximately 9000 g. Dividing the weight of skin by the area of skin on a woman (9000 g/16 900 cm^2), the figure of 0.53 g skin/cm^2 is reached. The CIR Expert Panel estimated the amount of hyaluronic acid in skin by area to be 0.318 mg hyaluronic acid/cm^2 skin.

The CIR Expert Panel compared the amount of hyaluronic acid found in the skin to the maximum amount of hyaluronic acid applied to the skin by cosmetic products, as noted in this report, of 0.02 mg/cm^2 by a product with the maximum concentration of 2%, and found the contribution via application of such a cosmetic product to be negligible. Acute, short-term, and chronic toxicological studies indicated low toxicity.

The CIR Expert Panel recognized that hyaluronic acid has been linked to metastatic cancer and sought to resolve whether the relationship was causal. In that regard, one seminal study reported a reduced level of hyaluronic acid associated with an unfavorable prognosis of clinical stage 1 cutaneous melanoma. These results suggest that, in melanoma, hyaluronic acid does not play a role in the metastatic process. In another pivotal study, hyaluronic acid was studied using the hyaluronic acid receptor, CD44 (a cell surface glycoprotein that is involved in cell/cell and cell/matrix interactions) on epidermal keratinocyte tumors, specifically, basal cell carcinomas and squamous cell carcinomas. In basal cell carcinomas, CD44 expression was quite low. In squamous cell carcinomas, CD44 expression was variable. As the malignancy became less differentiated and, therefore, would be expected to have a higher risk for metastasis, the expression of hyaluronic acid decreased.

These key findings suggest that hyaluronic acid likely does not play a causal role in metastasis and that increased expression of hyaluronic acid genes may be a consequence of metastatic growth not the converse. These results, together with the levels of hyaluronic acid that would be applied to the skin, would further insure the safety of this ingredient in cosmetic products.

The CIR Expert Panel discussed the possible need for additional dermal irritation and sensitization, UV absorption, and/or photosensitization and photoirritation data. Taking into consideration the above-mentioned calculation on the amount of hyaluronic acid in the skin compared to the amount that might be contributed by the application of cosmetics, the Expert Panel decided that the amount of hyaluronic acid in cosmetics would be negligible and not a concern in these areas.

Even though adverse reactions to injected hyaluronic acid used in the treatment of osteoarthritis and tissue augmentation are reported, these data do not raise safety concerns regarding the use of hyaluronic acid in cosmetics. There were no reported reactions to topically applied hyaluronic acid, further supporting that hyaluronic acid at levels currently

used in cosmetics applied to the skin should not be of concern.

The CIR Expert Panel recognizes that there are data gaps regarding use and concentration of these ingredients. However, the overall information available on the types of products in which these ingredients are used and at what concentrations indicate a pattern of use, which was considered by the Expert Panel in assessing safety.

Conclusion

The CIR Expert Panel concluded that hyaluronic acid, sodium hyaluronate, and potassium hyaluronate are safe as cosmetic ingredients in the practices of use and concentrations as described in this safety assessment.

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Conflicts of Interest

No potential conflict of interest relevant to this article was reported. F. Alan Andersen, PhD, and Lillian C. Becker are employed by the Cosmetic Ingredient Review.

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