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(54) Title: AMNIOTIC MEMBRANE PREPARATIONS AND PURIFIED COMPOSITIONS AND METHODS OF USE

(57) Abstract: Compositions having a combination of specific biological components have been found to exert a number of useful effects in mammalian cells, including modulating TGF-β signaling, apoptosis, and proliferation of mammalian cells, as well as decreasing inflammation in mice. These components can be obtained commercially, or can be prepared from biological tissues such as placental tissues. Placental amniotic membrane (AM) preparations described herein include AM pieces, AM extracts, AM jelly, AM stroma, and mixtures of these compositions with additional components. The compositions can be used to treat various diseases, such as wound healing, inflammation and angiogenesis-related diseases.

AMNIOTIC MEMBRANE PREPARATIONS AND PURIFIED COMPOSITIONS AND METHODS OF USE

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. Provisional Application No. 60/720,760, filed September 27, 2005, which is incorporated herein by reference in its entirety.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

[0002] The invention was made with United States government support under grant number RO1 EY06819 awarded by the National Institutes of Health. The United States government may have certain rights in the invention.

FIELD OF THE INVENTION

[0003] The invention relates generally to the fields of biology and pharmaceuticals. More particularly, the invention relates to compositions and methods for modulating cellular physiology and pathological processing using a combination of compounds that can be found in amniotic membrane preparations.

BACKGROUND OF THE INVENTION

[0004] The placenta is a temporary organ that surrounds the fetus during gestation. The placenta allows for transport of gases and nutrients, and also provides other metabolic and endocrine functions. The placenta is composed of several tissue types. The umbilical cord connects the placenta to the fetus, and transports oxygen to the fetus. The umbilical cord has two arteries and a vein. Wharton's jelly, a specialized gelatinous connective tissue material, surrounds the umbilical cord to protect it from damage during fetal movement and development. The outer "shell" of the placenta is known as the "chorion." Much of the placental disc is composed of chorionic villi, which are extensions of the chorionic villous tree. Through these structures, fetal nutrition exchange occurs. The amniotic membrane (AM) is an avascular membranous sac that is filled with amniotic fluid. This membrane is the innermost membrane surrounding a fetus in the amniotic cavity. This tissue consists of an epithelial layer and a subadjacent avascular stromal layer.

SUMMARY OF THE INVENTION

[0005] Described herein are purified compositions and amniotic membrane preparations (that is, compositions that are prepared from amniotic membrane materials, including the amniotic membrane, amniotic stroma and amniotic jelly). In some embodiments, at least one component of the purified compositions are obtained from amniotic membrane preparations. Also described herein are purified compositions in which at least one component of the purified composition is obtained from human placenta and chorion. Also described herein are methods for preparing any of the aforementioned purified compositions and preparations. Also described herein are methods for storing and preserving any of the aforementioned purified compositions and preparations. Also described herein are methods for using any of the aforementioned purified compositions and preparations, including preservative methods, cell culture methods, tissue culture methods, therapeutic methods, prophylactic methods and cosmetic methods.

[0006] In one aspect are purified compositions comprising:

- Cross-linked high molecular weight hyaluronan (HA);
- Tumor necrosis factor-stimulated gene 6 (TSG-6);
- Pentraxin (PTX-3); and

PCT/US Thrombospondin (TSP-1).

[0007] In a further embodiment, at least portion of the components of the purified compositions are prepared from a human amniotic material selected from a human amniotic membrane, a human amniotic jelly, a human amniotic stroma, or a combination thereof. In another or further embodiment, the purified compositions further comprise Smad7. In a further or alternative embodiment of the purified compositions, the cross-linking of the HA comprises a covalent bond to a heavy chain of inter-α-trypsin inhibitor. In a further or alternative embodiment of the purified compositions, the ratio of protein to HA is less than about 100.

[0008] In a further or alternative embodiment of the purified compositions, the preparation procedure for the purified composition includes

- Obtaining a frozen or previously-frozen human placenta;
- Thawing the placenta and isolating the human amniotic material from the thawed placenta; and
- homogenizing the human amniotic material in a suitable buffer.

[0009] In a further or alternative embodiment, the preparation procedure further includes:

- Freezing the human amniotic material; and
- Grinding the frozen amniotic material.

[0010] In a further or alternative embodiment, the preparation procedure further includes:

- Lyophilizing the homogenate; or
- Centrifuging the homogenate and isolating the supernatant from the centrifuged homogenate.

[0011] In a further or alternative embodiment, the preparation procedure further includes:

- Lyophilizing the supernatant into powder.

[0012] In a further or alternative embodiment, the purified compositions further comprise a pharmaceutically acceptable carrier for a non-solid dosage form or an extended release solid dosage form.

[0013] In another aspect described herein are methods for inhibiting scar formation in a subject comprising the step of providing an effective amount of a scar formation inhibition composition to a subject in need of scar formation inhibition; wherein the scar formation inhibition composition comprises at least one component prepared from a human amniotic material selected from a human amniotic membrane, a human amniotic jelly, a human amniotic stroma, or a combination thereof extracted from an amniotic membrane.

[0014] In a further or alternative embodiment of such methods, at least one component was extracted from the human amniotic material. In a further or alternative embodiment of such methods, the human amniotic material is the human amniotic stroma.

[0015] In a further or alternative embodiment of such methods, the extraction procedure comprises:

- Obtaining a frozen or previously-frozen human placenta;
- Thawing the placenta and isolating the human amniotic material from the thawed placenta;
- Homogenizing the human amniotic material in a suitable buffer;
- Optionally lyophilizing the homogenate to a powder; and
- Admixing the homogenate or the powder with a pharmaceutically acceptable carrier for a non-solid dosage form or an extended release solid dosage form.

[0016] In a further or alternative embodiment, the preparation procedure substitutes the step of lyophilizing the homogenate with the step of:

- Centrifuging the homogenate, isolating the supernatant from the centrifuged homogenate, and optionally lyophilizing the supernatant to a powder.

[0017] In another aspect are methods for reversing scar formation in a subject comprising the step of providing an effective amount of a scar reversal composition to a scarred subject; wherein the scar reversal composition comprises at least one component prepared from a human amniotic material selected from a human amniotic membrane, a human amniotic jelly, a human amniotic stroma, or a combination thereof extracted from an amniotic membrane. In another or further embodiment of these methods, at least one component was extracted from the human amniotic material. In another or further embodiment of these methods, the human amniotic material is the human amniotic stroma.

[0018] In another or further embodiment of these methods, the extraction procedure comprises:

- Obtaining a frozen or previously-frozen human placenta;
- Thawing the placenta and isolating the human amniotic material from the thawed placenta;
- Homogenizing the human amniotic material in a suitable buffer;
- Optionally lyophilizing the homogenate to a powder; and
- Admixing the homogenate or the powder with a pharmaceutically acceptable carrier for a non-solid dosage form or an extended release solid dosage form.

[0019] In another or further embodiment, the preparation procedure substitutes the step of lyophilizing the homogenate with the step of:

- Centrifuging the homogenate, isolating the supernatant from the centrifuged homogenate, and optionally lyophilizing the supernatant to a powder.

[0020] Another aspect described herein are methods for inhibiting angiogenesis in a subject comprising the step of providing an effective amount of an angiogenesis inhibition composition to a subject in need of angiogenesis inhibition; wherein the angiogenesis inhibition composition comprises at least one component prepared from a human amniotic material selected from a human amniotic membrane, a human amniotic jelly, a human amniotic stroma, or a combination thereof extracted from an amniotic membrane.

[0021] In another or further embodiment of these methods, at least one component was extracted from the human amniotic material. In another or further embodiment of these methods, the human amniotic material is the human amniotic stroma.

[0022] In another or further embodiment of these methods, the composition comprises:

- Cross-linked high molecular weight hyaluronan (HA);
- Tumor necrosis factor-stimulated gene 6 (TSG-6);
- Pentraxin (PTX-3); and
- Thrombospondin (TSP-1).

[0023] In another or further embodiment of these methods, the extraction procedure comprises:

- Obtaining a frozen or previously-frozen human placenta;
- Thawing the placenta and isolating the human amniotic material from the thawed placenta;
- Homogenizing the human amniotic material in a suitable buffer;
- Optionally lyophilizing the homogenate to a powder; and
- Admixing the homogenate or the powder with a pharmaceutically acceptable carrier for a non-solid dosage form or an extended release solid dosage form.

[0024] In another or further embodiment of these methods, the preparation procedure substitutes the step of lyophilizing the homogenate with the step of:

- Centrifuging the homogenate, isolating the supernatant from the centrifuged homogenate, and optionally lyophilizing the supernatant to a powder.

[0025] In another or further embodiment of these methods, the subject in need is a human with cancer. In another or further embodiment of these methods, the subject in need is a human with age-related macular degeneration.

[0026] In another or further embodiment of these methods, the angiogenesis inhibition composition is provided in the form of a non-solid dosage form or an extended release solid dosage form.

[0027] In another or further embodiment of these methods, the angiogenesis inhibition composition has the following properties:

- induces apoptosis of endothelial cells involved in vascular formation;
- prevents migration of endothelial cells involved in vascular formation; and
- prevents tube formation of endothelial cells involved in vascular formation.

[0028] A method for reducing or preventing inflammation in a subject comprising the step of providing an effective amount of an inflammation inhibition composition to a subject in need of inflammation inhibition or prevention; wherein the inflammation inhibition composition comprises at least one component prepared from a human amniotic material selected from a human amniotic membrane, a human amniotic jelly, a human amniotic stroma, or a combination thereof extracted from an amniotic membrane..

[0029] In another or further embodiment of these methods, at least one component was extracted from the human amniotic material. In another or further embodiment of these methods, the human amniotic material is the human amniotic membrane.

[0030] In another or further embodiment of these methods, the composition comprises:

- Cross-linked high molecular weight hyaluronan (HA);
- Tumor necrosis factor-stimulated gene 6 (TSG-6);
- Pentraxin (PTX-3); and
- Thrombospondin (TSP-1).

[0031] In another or further embodiment of these methods, the extraction procedure comprises:

- Obtaining a frozen or previously-frozen human placenta;
- Thawing the placenta and isolating the human amniotic material from the thawed placenta;
- Homogenizing the human amniotic material in a suitable buffer;
- Optionally lyophilizing the homogenate to a powder; and
- Admixing the homogenate or the powder with a pharmaceutically acceptable carrier for a non-solid dosage form or an extended release solid dosage form.

[0032] In another or further embodiment of these methods, the preparation procedure substitutes the step of lyophilizing the homogenate with the step of:

- Centrifuging the homogenate, isolating the supernatant from the centrifuged homogenate, and optionally lyophilizing the supernatant to a powder.

[0033] In another or further embodiment of these methods, the human has arthritis. In another or further embodiment of these methods, the human has inflammation in at least one eye. In another or further embodiment of these methods, the inflammation inhibition composition is provided as a non-solid dosage form or an extended release solid dosage form.

[0034] In another or further embodiment of these methods, the inflammation inhibition composition has at least two of the following properties:

- induces apoptosis of macrophages at the site of inflammation;
- increases the ratio of prostaglandin D2 to prostaglandin E1 at the site of inflammation;

PCT/US suppresses TGF-β activity at the site of inflammation; or

- inhibits interferon-gamma signal transduction at the site of inflammation.

[0035] Various AM preparations exert a number of physiologically significant effects in mammalian cells and intact mammalian tissues. Such effects include suppressing TGF-β signaling, increasing apoptosis of macrophages, decreasing cellular proliferation of, decreasing cellular migration of, and increasing apoptosis of vascular endothelial cells, protecting corneal and limbal epithelial cells and keratocytes from apoptosis induced by storage or by dispase treatment, and decreasing inflammation in tissues. In addition to pieces of intact AM, other preparations described herein include pieces of AM stroma, processed (e.g., ground or pulverized) AM or AM stroma, and various extracts of intact AM and AM stroma. AM extracts can be in liquid or lyophilized powder form.

[0036] The compositions also include thickened or gel forms of AM extracts which can be made by mixing the AM extracts with a thickener such as one or more extra cellular matrix components (ECM). A large number of ECM components are known such as collagen, hyaluronic acid (HA), and fibrin.

[0037] Although preparations, materials, and methods similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable preparations, methods and materials are described herein. All publications mentioned herein are incorporated by reference in their entirety. In the case of conflict, the present specification, including definitions will control. In addition, the particular embodiments discussed below are illustrative only and not intended to be limiting.

CERTAIN DEFINITIONS

[0038] The term "acceptable" with respect to a formulation, composition or ingredient, as used herein, means having no persistent detrimental effect on the general health of the subject being treated.

[0039] "Antioxidants" include, for example, butylated hydroxytoluene (BHT), sodium ascorbate, ascorbic acid, sodium metabisulfite and tocopherol. In certain embodiments, antioxidants enhance chemical stability where required.

[0040] "Binders" impart cohesive qualities and include, e.g., alginic acid and salts thereof; cellulose derivatives such as carboxymethylcellulose, methylcellulose (e.g., Methocel®), hydroxypropylmethylcellulose, hydroxyethylcellulose, hydroxypropylcellulose (e.g., Klucel®), ethylcellulose (e.g., Ethocel®), and microcrystalline cellulose (e.g., Avicel®); microcrystalline dextrose; amylose; magnesium aluminum silicate; polysaccharide acids; bentonites; gelatin; polyvinylpyrrolidone/vinyl acetate copolymer; crosspovidone; povidone; starch; pregelatinized starch; tragacanth, dextrin, a sugar, such as sucrose (e.g., Dipac®), glucose, dextrose, molasses, mannitol, sorbitol, xylitol (e.g., Xylitab®), and lactose; a natural or synthetic gum such as acacia, tragacanth, ghatti gum, mucilage of isapol husks, polyvinylpyrrolidone (e.g., Polyvidone® CL, Kollidon® CL, Polyplasdone® XL-10), larch arabogalactan, Veegum®, polyethylene glycol, waxes, sodium alginate, and the like.

[0041] The term "carrier," as used herein, refers to relatively nontoxic chemical compounds or agents that facilitate the incorporation of a compound into cells or tissues.

[0042] "Carrier materials" include any commonly used excipients in pharmaceutics and should be selected on the basis of compatibility with compounds disclosed herein, and the release profile properties of the desired dosage form. Exemplary carrier materials include, e.g., binders, suspending agents, disintegration agents, filling agents, surfactants, solubilizers, stabilizers, lubricants, wetting agents, diluents, and the like. "Pharmaceutically compatible carrier materials" may include, but are not limited to, acacia, gelatin, colloidal silicon dioxide, calcium glycerophosphate, calcium lactate, maltodextrin, glycerine, magnesium silicate, polyvinylpyrrollidone (PVP), cholesterol, cholesterol esters, sodium caseinate, soy lecithin, taurocholic acid, phosphotidylcholine, sodium chloride, tricalcium phosphate, dipotassium phosphate, cellulose and cellulose conjugates, sugars sodium stearoyl

lactylate, carrageenan, monoglyceride, diglyceride, pregelatinized starch, and the like. See, e.g., *Remington: The Science and Practice of Pharmacy*, Nineteenth Ed (Easton, Pa.: Mack Publishing Company, 1995); Hoover, John E., *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, Pennsylvania 1975; Liberman, H.A. and Lachman, L., Eds., *Pharmaceutical Dosage Forms*, Marcel Decker, New York, N.Y., 1980; and *Pharmaceutical Dosage Forms and* Drug Delivery Systems, Seventh Ed. (Lippincott Williams & Wilkins1999).

[0043] The terms "co-administration" or the like, as used herein, are meant to encompass administration of the selected therapeutic agents to a single patient, and are intended to include treatment regimens in which the agents are administered by the same or different route of administration or at the same or different time.

[0044] The term "delayed release" as used herein refers to the delivery so that the release can be accomplished at some generally predictable location more distal to that which would have been accomplished if there had been no delayed release alterations.

[0045] "Dispersing agents," and/or "viscosity modulating agents" include materials that control the diffusion and homogeneity of a drug through liquid media or a granulation method or blend method. In some embodiments, these agents also facilitate the effectiveness of a coating or eroding matrix. Exemplary diffusion facilitators/dispersing agents include, e.g., hydrophilic polymers, electrolytes, Tween ® 60 or 80, PEG, polyvinylpyrrolidone (PVP; commercially known as Plasdone®), and the carbohydrate-based dispersing agents such as, for example, hydroxypropyl celluloses (e.g., HPC, HPC-SL, and HPC-L), hydroxypropyl methylcelluloses (e.g., HPMC K100, HPMC K4M, HPMC K15M, and HPMC K100M), carboxymethylcellulose sodium, methylcellulose, hydroxyethylcellulose, hydroxypropylcellulose, hydroxypropylmethylcellulose phthalate, hydroxypropylmethylcellulose acetate stearate (HPMCAS), noncrystalline cellulose, magnesium aluminum silicate, triethanolamine, polyvinyl alcohol (PVA), vinyl pyrrolidone/vinyl acetate copolymer (S630), 4-(1,1,3,3-tetramethylbutyl)-phenol polymer with ethylene oxide and formaldehyde (also known as tyloxapol), poloxamers (e.g., Pluronics F68®, F88®, and F108®, which are block copolymers of ethylene oxide and propylene oxide); and poloxamines (e.g., Tetronic 908®, also known as Poloxamine 908®, which is a tetrafunctional block copolymer derived from sequential addition of propylene oxide and ethylene oxide to ethylenediamine (BASF Corporation, Parsippany, N.J.)), polyvinylpyrrolidone K12, polyvinylpyrrolidone K17, polyvinylpyrrolidone K25, or polyvinylpyrrolidone K30, polyvinylpyrrolidone/vinyl acetate copolymer (S-630), polyethylene glycol, e.g., the polyethylene glycol can have a molecular weight of about 300 to about 6000, or about 3350 to about 4000, or about 7000 to about 5400, sodium carboxymethylcellulose, methylcellulose, polysorbate-80, sodium alginate, gums, such as, e.g., gum tragacanth and gum acacia, guar gum, xanthans, including xanthan gum, sugars, cellulosics, such as, e.g., sodium carboxymethylcellulose, methylcellulose, sodium carboxymethylcellulose, polysorbate-80, sodium alginate, polyethoxylated sorbitan monolaurate, polyethoxylated sorbitan monolaurate, povidone, carbomers, polyvinyl alcohol (PVA), alginates, chitosans and combinations thereof. Plasticizcers such as cellulose or triethyl cellulose can also be used as dispersing agents. Dispersing agents particularly useful in liposomal dispersions and self-emulsifying dispersions are dimyristoyl phosphatidyl choline, natural phosphatidyl choline from eggs, natural phosphatidyl glycerol from eggs, cholesterol and isopropyl myristate.

[0046] The term "diluent" refers to chemical compounds that are used to dilute the compound of interest prior to delivery. Diluents can also be used to stabilize compounds because they can provide a more stable environment. Salts dissolved in buffered solutions (which also can provide pH control or maintenance) are utilized as diluents in the art, including, but not limited to a phosphate buffered saline solution. In certain embodiments, diluents increase bulk of the composition to facilitate compression or create sufficient bulk for homogenous blend for capsule filling. Such compounds include e.g., lactose, starch, mannitol, sorbitol, dextrose, microcrystalline cellulose such as

Avicel®; dibasic calcium phosphate, dicalcium phosphate dihydrate; tricalcium phosphate, calcium phosphate; anhydrous lactose, spray-dried lactose; pregelatinized starch, compressible sugar, such as Di-Pac® (Amstar); mannitol, hydroxypropylmethylcellulose, hydroxypropylmethylcellulose acetate stearate, sucrose-based diluents, confectioner's sugar; monobasic calcium sulfate monohydrate, calcium sulfate dihydrate; calcium lactate trihydrate, dextrates; hydrolyzed cereal solids, amylose; powdered cellulose, calcium carbonate; glycine, kaolin; mannitol, sodium chloride; inositol, bentonite, and the like.

[0047] An "enteric coating" is a substance that remains substantially intact in the stomach but dissolves and releases the drug in the small intestine or colon. Generally, the enteric coating comprises a polymeric material that prevents release in the low pH environment of the stomach but that ionizes at a higher pH, typically a pH of 6 to 7, and thus dissolves sufficiently in the small intestine or colon to release the active agent therein.

[0048] "Filling agents" include compounds such as lactose, calcium carbonate, calcium phosphate, dibasic calcium phosphate, calcium sulfate, microcrystalline cellulose, cellulose powder, dextrose, dextrates, dextran, starches, pregelatinized starch, sucrose, xylitol, lactitol, mannitol, sorbitol, sodium chloride, polyethylene glycol, and the like.

[0049] The terms "effective amount" or "therapeutically effective amount," as used herein, refer to a sufficient amount of an agent or a compound being administered which will relieve to some extent one or more of the symptoms of the disease or condition being treated. The result can be reduction and/or alleviation of the signs, symptoms, or causes of a disease, or any other desired alteration of a biological system. For example, an "effective amount" for therapeutic uses is the amount of the composition including a compound as disclosed herein required to provide a clinically significant decrease in disease symptoms without undue adverse side effects. An appropriate "effective amount" in any individual case may be determined using techniques, such as a dose escalation study. The term "therapeutically effective amount" includes, for example, a prophylactically effective amount. An "effective amount" of a compound disclosed herein, is an amount effective to achieve a desired pharmacologic effect or therapeutic improvement without undue adverse side effects. It is understood that "an effect amount" or "a therapeutically effective amount" can vary from subject to subject, due to variation in metabolism of the composition, age, weight, general condition of the subject, the condition being treated, the severity of the condition being treated, and the judgment of the prescribing physician.

[0050] The terms "enhance" or "enhancing," as used herein, means to increase or prolong either in potency or duration a desired effect. Thus, in regard to enhancing the effect of therapeutic agents, the term "enhancing" refers to the ability to increase or prolong, either in potency or duration, the effect of other therapeutic agents on a system. An "enhancing-effective amount," as used herein, refers to an amount adequate to enhance the effect of another therapeutic agent in a desired system.

[0051] The terms "kit" and "article of manufacture" are used as synonyms.

[0052] The term "modulate," as used herein, means to interact with a target either directly or indirectly so as to alter the activity of the target, including, by way of example only, to enhance the activity of the target, to inhibit the activity of the target, to limit the activity of the target, or to extend the activity of the target.

[0053] As used herein, the term "modulator" refers to a compound that alters an activity of a molecule. For example, a modulator can cause an increase or decrease in the magnitude of a certain activity of a molecule compared to the magnitude of the activity in the absence of the modulator. In certain embodiments, a modulator is an inhibitor, which decreases the magnitude of one or more activities of a molecule. In certain embodiments, an inhibitor completely prevents one or more activities of a molecule. In certain embodiments, a modulator is an

activator, which increases the magnitude of at least one activity of a molecule. In certain embodiments the presence of a modulator results in an activity that does not occur in the absence of the modulator.

[0054] The term "non water-soluble diluent" represents compounds typically used in the formulation of pharmaceuticals, such as calcium phosphate, calcium sulfate, starches, modified starches and microcrystalline cellulose, and microcellulose (e.g., having a density of about 0.45 g/cm^3, e.g. Avicel, powdered cellulose), and talc.

[0055] By "pharmaceutically acceptable," as used herein, refers to a material, such as a carrier or diluent, which does not abrogate the biological activity or properties of the compound, and is relatively nontoxic, i.e., the material may be administered to an individual without causing undesirable biological effects or interacting in a deleterious manner with any of the components of the composition in which it is contained.

[0056] The term "pharmaceutical combination" as used herein, means a product that results from the mixing or combining of more than one active ingredient and includes both fixed and non-fixed combinations of the active ingredients. The term "fixed combination" means that the active ingredients, e.g. the AM preparations and purified compositions described herein and a co-agent, are both administered to a patient simultaneously in the form of a single entity or dosage. The term "non-fixed combination" means that the active ingredients, e.g. the AM preparations and purified compositions described herein and a co-agent, are administered to a patient as separate entities either simultaneously, concurrently or sequentially with no specific intervening time limits, wherein such administration provides effective levels of the two compounds in the body of the patient. The latter also applies to cocktail therapy, e.g. the administration of three or more active ingredients.

[0057] "Plasticizers" are compounds used to soften the microencapsulation material or film coatings to make them less brittle. Suitable plasticizers include, e.g., polyethylene glycols such as PEG 300, PEG 400, PEG 600, PEG 1450, PEG 3350, and PEG 800, stearic acid, propylene glycol, oleic acid, triethyl cellulose and triacetin. In some embodiments, plasticizers can also function as dispersing agents or wetting agents.

[0058] The term "polypeptide" or "protein" as used herein can be the full length polypeptide, or a fragment or segment of a polypeptide, and can encompass a stretch of amino acid residues of at least about 8 amino acids, generally at least 10 amino acids, more generally at least 20 amino acids, often at least 30 amino acids, more often at least 50 amino acids or more of the full length polypeptide.

[0059] "Solubilizers" include compounds such as triacetin, triethylcitrate, ethyl oleate, ethyl caprylate, sodium lauryl sulfate, sodium doccusate, vitamin E TPGS, dimethylacetamide, N-methylpyrrolidone, N-hydroxyethylpyrrolidone, polyvinylpyrrolidone, hydroxypropylmethyl cellulose, hydroxypropyl cyclodextrins, ethanol, n-butanol, isopropyl alcohol, cholesterol, bile salts, polyethylene glycol 200-600, glycofurol, transcutol, propylene glycol, and dimethyl isosorbide and the like.

[0060] "Stabilizers" include compounds such as any antioxidation agents, buffers, acids, preservatives and the like.

[0061] "Substantially pure" or "purified" when used in the context of a biological material, amniotic materia and/or a protein context typically means that the material is isolated from other contaminating proteins, nucleic acids, and other biologicals derived from the original source organism. Purity, or "isolation" may be assayed by standard methods, and will ordinarily be at least about 10% pure, more ordinarily at least about 20% pure, generally at least about 30% pure, and more generally at least about 40% pure; in further embodiments at least about 50% pure, or more often at least about 60% pure; in still other embodiments, at least about 95% pure.

[0062] "Suspending agents" include compounds such as polyvinylpyrrolidone, e.g., polyvinylpyrrolidone K12, polyvinylpyrrolidone K17, polyvinylpyrrolidone K25, or polyvinylpyrrolidone K30, vinyl pyrrolidone/vinyl acetate copolymer (S630), polyethylene glycol, e.g., the polyethylene glycol can have a molecular weight of about

[illegible] to about 6900, or about 3350 to about 4000, or about 7000 to about 5400, sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethylcellulose, hydroxymethylcellulose acetate stearate, polysorbate-80, hydroxyethylcellulose, sodium alginate, gums, such as, e.g., gum tragacanth and gum acacia, guar gum, xanthans, including xanthan gum, sugars, cellulosics, such as, e.g., sodium carboxymethylcellulose, methylcellulose, sodium carboxymethylcellulose, hydroxypropylmethylcellulose, hydroxyethylcellulose, polysorbate-80, sodium alginate, polyethoxylated sorbitan monolaurate, polyethoxylated sorbitan monolaurate, povidone and the like.

[0063] "Surfactants" include compounds such as sodium lauryl sulfate, sodium docusate, Tween 60 or 80, triacetin, vitamin E TPGS, sorbitan monooleate, polyoxyethylene sorbitan monooleate, polysorbates, polaxomers, bile salts, glyceryl monostearate, copolymers of ethylene oxide and propylene oxide, e.g., Pluronic® (BASF), and the like. Some other surfactants include polyoxyethylene fatty acid glycerides and vegetable oils, *e.g.*, polyoxyethylene (60) hydrogenated castor oil; and polyoxyethylene alkylethers and alkylphenyl ethers, *e.g.*, octoxynol 10, octoxynol 40. In some embodiments, surfactants may be included to enhance physical stability or for other purposes.

[0064] As used herein, the term "subject" is used to mean an animal, preferably a mammal, including a human or non-human. The terms patient and subject may be used interchangeably.

[0065] The terms "treat," "treating" or "treatment," as used herein, include alleviating, abating or ameliorating a disease or condition symptoms, preventing additional symptoms, ameliorating or preventing the underlying metabolic causes of symptoms, inhibiting the disease or condition, e.g., arresting the development of the disease or condition, relieving the disease or condition, causing regression of the disease or condition, relieving a condition caused by the disease or condition, or stopping the symptoms of the disease or condition either prophylactically and/or therapeutically.

[0066] "Wetting agents" include compounds such as oleic acid, glyceryl monostearate, sorbitan monooleate, sorbitan monolaurate, triethanolamine oleate, polyoxyethylene sorbitan monooleate, polyoxyethylene sorbitan monolaurate, sodium docusate, sodium oleate, sodium lauryl sulfate, sodium doccusate, triacetin, Tween 80, vitamin E TPGS , ammonium salts and the like.

[0067] All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0068] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0069] Fig. 1A is a non-limiting example of a bar graph showing the suppression of TGF- β1 promoter activity by various AM Extracts. PL: plastic control. FRO/P: frozen amniotic membrane, placental portion. FRO/F: frozen amniotic membrane, fetal portion. FRE/P: Fresh amniotic membrane, placental portion. FRE/F: Fresh amniotic membrane, fetal portion. Fig. 1B is a table comparing the P values of the various placental preparations.

[0070] Fig. 2 is a non-limiting example of a bar graph showing the dose response curve of TGF-β1 promoter activity suppression. RLU: Relative luciferase units.

[0071] Fig. 3 is a non-limiting example of a bar graph showing the effect of various AM extract preparations on the suppression of TGF-β1 promoter activity.

[0072] Fig. 4 is a non-limiting example of a bar graph showing the effect of various AM extract preparations on the suppression of TGF-βRII promoter activity.

[0073] Fig. 5 is a non-limiting example of a bar graph demonstrating that soluble AME and jelly extracts derived after centrifugation do not alter the suppressive effect on TGF-β Promoter Activities. HA, AM (Total (T), Low Speed (LS), High speed (HS)) and Jelly (Total (T), Low Speed (LS), High Speed (HS)) showed suppression of TGF- β1 promoter activation compared to the PBS control when normalized with beta-galatosidase activity.

[0074] Fig. 6 is a non-limiting example of a set of microscopic images of human corneal fibroblasts showing cell morphology changes either 18 or 48 hours after treatment with various compounds. PBS: the PBS control; HA: hyaluronic acid; AME: amniotic membrane extract; L/AME: lyophilized amniotic membrane extract; AMJ: amniotic membrane jelly; L/AMJ: lyophilized amniotic membrane jelly.

[0075] Fig. 7 is a non-limiting example of a bar graph demonstrating the effect of AME (at 25 or 125 μg/ml), with or without lyophilized (L), on the suppression of TGFβ1 activity. The activity is measured in relative luciferase units (RLU).

[0076] Fig. 8 is a non-limiting example of a bar graph showing the effect of the addition of collagen gel (Col), AM extract AME, or collagen gel mixed with AM extract (Col + AME) on the suppression of TGF-β promoter activity. BSA was used as a control.

[0077] Fig. 9 is a non-limiting example of a bar graph comparing the effect of treatment with AME, HA, or HA + AME, compared to a control assay with BSA alone, on the suppression of TGFβ1 activity. The promoter activity is displayed as relative luciferase units (RLU).

[0078] Fig. 10 is a non-limiting example of an analysis of hyaluronan MW Ranges in AM Extracts of various AM extracts, separated by agarose gel electrophoresis. Amniotic membrane extracted by buffer A, B, C were treated with or without hyaluronidase and electrophoretically separated by a 0.5 % agarose gel.

[0079] Fig. 11 is a non-limiting example of an analysis of hyaluronan MW Ranges in AM Extracts of various AM extracts, separated by agarose gel electrophoresis. Amniotic membrane extracted by buffer PBS were treated with or without hyaluronidase (10 units/ml in Tris-HCl, pH 7.5, 150 mM NaCl) for 2 hr at 37°C and run through 0.5 % agarose gels. HA: positive hyaluronic acid control; L: AM extract after low speed centrifugation; H: AM extract after high speed centrifugation.

[0080] Fig. 12 is a non-limiting example of a photograph of a western blot demonstrating that the inter-α-trypsin inhibitor (IαI) is present in AM Extracts. IαI was present in AM extract A and C although the signal of bikunin was very weak (~39 kDa). Prior to transfer to the western blot, the extract was separated on a 4-15% denatured acrylamide gel.

[0081] Fig. 13 is a non-limiting example of an immunoblot demonstrating that the inter-α- trypsin inhibitor (IαI) is present in the AM extracts even after low (LS) or high speed (HS) centrifugation.

[0082] Fig. 14 is a non-limiting example of an immunoblot of TSG-6 (Tumor Necrosis Factor-Stimulated Gene 6), either with (+) or without (-) hyaluronidase treatment. The samples included total AM extract without centrifugation (T), AM Extract after extraction in isotonic low salt buffer (buffer A); high salt buffer (B); or 4 M guanidine HCl (C); as detailed in Example 2. TSG-6 was present in the total extract, buffer A extract, and buffer C extract. The addition of hyaluronidase did not appear to alter the TSG-6 level in the extracts.

[0083] Fig. 15 is a non-limiting example of an immunoblot analysis of the deglycosylation of TSG-6 in AM. AM extract A, B, and C were treated with (+) or without 20 units/ml PNGase F at 37 °C for 3 hours. Glycosylation of TSG-6 in AM was then analyzed by western blot. The cell lysate of human corneal fibroblast (HCF) was used as a positive control.

[0084] Fig. 16 is a non-limiting example of an immunoblot analysis of potential TSG-6 complexes in AM by digestion with Chondroitin Sulfate ABC lyase. AM extract A, B, and C were treated without (-) or with (+) 1 unit/ml ABC lyase at 37 °C for 2 hours. The possible disruption of TSG-6 complexes was then analyzed by western blot using an anti-TSG-6 antibody RAH-1: 1:1000.

[0085] Fig. 17 is a non-limiting example of an immunoblot of potential TSG-6 complexes in AM by digestion with Chondroitin Sulfate ABC lyase. This is the same experiment as shown in Fig. 16 except that a different TSG-6 antibody was used. Here, the anti-TSG-6 antibody was obtained from R & D Systems (cat# MAB2104).

[0086] Fig. 18 is a non-limiting example of an immunoblot demonstrating the presence of Pentraxin (PTX3) in AM, using a rat monoclonal anti-PTX3 antibody obtained from Alexis Biochemicals. HCF: human corneal fibroblast, T, A, B, C: AM extract Total, A, B, C, respectively; HAse, Hyaluronidase.

[0087] Fig. 19 is a non-limiting example of an immunoblot demonstrating the presence of TSP-1 in AM. The monomeric TSP-1 (180 kDa) and the putative trimeric TSP-1 (540 kDa) are indicated. The positive control, TSP-1, was purified from human platelets (Calbiochem, Cat# 605225) and loaded as 100 ng/lane.

[0088] Fig. 20 is a non-limiting example of an immunoblot demonstrating the presence of Smad 7 in AM. AM was extracted with PBS or urea (2M urea in 50 mM Tris-HCl, pH 7.5). 20 µg of total protein was loaded for each extract. Smad 7 was detected with goat anti-human Smad 7 (AF2029, 1:1000, R & D Systems). Smad 7 migrated as a band of ~ 51 kDa.

[0089] Fig. 21 a non-limiting example of microscopic images of amniotic membrane stromal cells (AMSC) in AM. A: AMSCs exhibited dendritic morphology and maintained intercellular contacts in situ. B: Staining with Live and Dead assay for cell viability. The dendritic morphology and intercellular contacts were better visualized by this method. C: AMSCs did not express α-SMA. D: AMSCs did not express desmin. In contrast, as a positive control, umbilical cord mesenchymal cells showed strong staining to both α -SMA and desmin (insert of C and D, respectively). E: All AMSCs expressed vimentin. Dotted lines indicate the separation between the AM epithelium and AM stromal layers. Nuclear counterstaining was performed by DAPI (C, D) and PI (E), respectively. Bar represents 50 µm.

[0090] Fig. 22 is a non-limiting example of rapid myofibroblast differentiation of AMSCs in vitro. P0 = primary AMSC cells. P1 =Passage 1; P2 = Passage 2. PO, P1, P2 refer to Passage 0, 1, and 2 respectively. A and E: P0 (4 days); B and F: P0 (7 days); C and G: P1; D and H: P2. Cells in E, F, G, H were immunostained with mouse anti-αSMA monoclonal antibody; A: AMSCs cultured on plastic in DMEM with 10% FBS exhibited a typical fibroblast cell shape. Bar represents 100 µm.

[0091] Fig. 22 I is a non-limiting example of a line graph demonstrating that α-SMA-positive myofibroblasts dramatically increased from 71.9 ± 3.7 % at 1 week primary culture to 93.9 ± 4.1% at passage 1 and 98.5 ± 1.7% at passage 2.

[0092] Fig. 22 J is a non-limiting example of an immunoblot analysis demonstrating the increase of protein expression of α-SMA and ED-A fibronetin (Fn). PO and P2 refer to Passage 0 and Passage 2, respectively. B-actin is used as a control.

[0093] Fig. 23A through 23H are non-limiting examples of microscopic images demonstrating that differentiated myofibroblast from AMSCs reversed to fibroblasts when cultured back on AM stromal matrix. Myofibroblasts derived from AMSCs at P2 were subcultured on type I collagen (A, C, E, G) or AM stromal matrix (B, D, F, H) in DMEM with 10% FBS for 7 days. Bars represent 100 µm. Live and Dead assay showed cells on both collagen (C) and AM stroma matrix (D) remained 100% viability, but exhibited a different cell shape. Phalloidin and α-SMA double staining showed vivid stress fibers (E) and strong α-SMA expression (G) in myofibroblasts on

collagen cultures. In contrast, phalloidin staining became weak and spotty (F), and α-SMA became obscured in cells subcultured on AM stromal matrix (H). I: An immunoblot analysis showed decreased expression of ED-A fibronectin (Fn) and undetectable expression of α-SMA of AMSCs seeded on AM stromal matrix as compared to those seeded on type I collagen.

[0094] Fig. 24A through 24H are non-limiting examples of microscopic images demonstrating that AM Stromal Extracts (ASE) Prevented Myofibroblast Differentiation of AMSCs. Phalloidin staining (upper panel) and α-SMA staining (lower panel) were performed. A and E: cells cultured without ASE for 4 days; B and F: cells cultured for 4 days with ASE; C and G: cells cultured without ASE for 10 days; D and H: Cells cultured with ASE for 10 days. Cells in A, B, C, and D were stained with FITC conjugated phalloidin while cells in E, F, G, and H were stained with mouse anti- α-SMA monoclonal antibody. AMSCs maintained a spindle fibroblastic shape after 4 days cultivation on plastic in DMEM/10%FBS without (A) or with (B) ASE. However, at that time, cells already started to express α-SMA without ASE (E), but did not express α-SMA when ASE was added (F). When cultures were extended for 10 days, cells became enlarged and exhibited prominent stress fibers (C), and strong expression of α-SMA (G) without ASE. In contrast, AMSCs aggregated into spheres of varying sizes with addition of ASE. These spheres did not express stress fibers (D), but expressed weak α-SMA staining (H). Bar represents 100 μm.

[0095] Fig. 25A and 25B are non-limiting examples of microscopic images demonstrating that amiotic stromal extract (ASE) reverses differentiated myofibroblasts. Myofibroblasts differentiated from AMSCs on plastic in DMEM/10% FBS at passage 2 were cultured with addition of ASE for 1 week. A: Cells reverted from a squamous shape to an elongated or spindle shape B: α-SMA staining became notably decreased. C: An immunoblot analysis demonstrating that ED-A fibronectin and α-SMA levels were reduced as compared to the control without ASE. Bar represents 100 μm.

[0096] Fig. 26A through 26L are non-limiting examples of microscopic images demonstrating that the reversal of myofibroblasts by ASE was not associated with cell proliferation. AMSCs (passage 2) were cultured in DMEM/ITS without (A, B, C, D) or with ASE (E, F, G, H) for 0, 2, 4, or 6 days as indicated. α-SMA-expressing stress fibers were gradually decreased from day 0 to day 6 after addition of ASE (I, J, K, L), and correlated with morphological changes (E to H).

[0097] Fig. 27A through 27D are non-limiting examples of microscopic images demonstrating that AM extract suppressed fibroblast migration from human limbal explants and resulted in less fibroblasts in the outgrowth. A, B: The outgrowth from human limbal explants cultured in both SHEM (Ctrl) and SHEM/AME (AME). C, D: After 14 days in culture, human limbal explants were removed from culture wells, embedded, sectioned, and stained with Hematoxylin and Eosin staining.

[0098] Fig. 28A is a non-limiting example of a photograph of a 48 well assay plate demonstrating the suppression of fibroblast outgrowth by AME. A. The outgrowth from human limbal explants after 14 days of growth in either SHEM (Ctrl) and SHEM with 25 μg/ml AME (AME) was separately harvested and seeded in each 96 well at 2000 cells/well. Cells from the Ctrl were seeded in columns 1-3 (1: Ctrl; 2: PBS; 3: AME; and those from the AME were seeded in columns 4-6 (1: Ctrl; 2: PBS; 3: AME. An MTT assay was performed after 10 days of culture.

[0099] Fig. 28B is a non-limiting example of a bar graph showing the quantitation and statistical analysis of the relative suppression of fibroblast outgrowth by AME.

[00100] Fig. 29A and 29B are non-limiting examples of microscopic images of the outgrowth from human limbal explants cultured in both SHEM control (Fig. 29A) and SHEM with AME (Fig. 29B) for 14 days.

[00101] Fig. 30 is a non-limiting example of a bar graph of relative NO production in cells cultured on either plastic or intact amniotic membrane, with or without IFN-γ stimulation. Raw264.7 cells were seeded in each of 24

wells at 2.5×10^5 in DMEM/ITS (n=4 for each group). The cells were stimulated with or without 200 μ/ml of IFN-γ, and the culture medium was collected for the NO assay. Pl: plastic. iAM: intact amniotic membrane.

[00102] Fig. 31 is a non-limiting example of a panel of bar graphs quantitating the levels of prostaglandin D2 (PGD2) and prostaglandin E2 (PGE2) in raw264.7 cells cultured on plastic or iAM, either with or without IFN-γ stimulation. A: PGD2 synthesis. B: PGE2 synthesis. C: The ratio of PGD2/PGE2 in Raw264.6 when cultured on the plastic and iAM.

[00103] Fig. 32 is a non-limiting example of a bar graph demonstrating TGF-β1 promoter activity in Raw264.7 cells cultured on plastic or iAM, either with or without IFN-γ stimulation.

[00104] Fig. 33 is a non-limiting example of a microscopic image showing AME induced macrophage death. Raw264.7 cells were seeded at 2×10^5 in each 24 well in DMEM/10% fetal bovine serum. After 1 hr, 125 μg/ml of PBS (control – Fig. 33A) or 125 μg/ml of AME in PBS (AME – Fig. 33B) was added to the culture medium.

[00105] Fig. 34A through 34C is a non-limiting example of a bar graphs demonstrating that ASE preferentially inhibited HUVEC cells. A: Measurement of viable HUVEC cells. B: Measurement of viable HCF cells. C: Measurement of viable RCE cells.

[00106] Fig. 35 is a non-limiting example of microscopic images of HUVEC cells. The results showed that HUVEC cells were alive in the control without addition of ASE (Fig. 35Aa) but showed pronounced cell death after ASE treatment (Fig. 35Ad). In contrast, both HCF and RCE cells did not reveal any notable cell death in cultures without (Fig. 35Ab and 35Ac, respectively) or with (35Ae and 35Af, respectively) ASE treatment. Hoechst-33342 staining showed that ASE-treated HUVECs had 61.6 ± 7.7% of condensed and fragmented nuclei (Fig. 35Bd), which was significantly higher than 3.1 ± 1.8% of the control without ASE treatment (Fig. 35Ba, also see 2C, $p<0.001$). In contrast, there was no obvious apoptosis in either HCFs or RCEs without (Fig. 35Bb and 35Bc, respectively) or with (Fig. 35Be and 35Bf, respectively) ASE treatment.

[00107] Fig. 36 is a non-limiting example of a bar graph demonstrating that the addition of ASE increases the number of apototic HUVEC cells.

[00108] Fig. 37 is a non-limiting example showing the effect of ASE on the inhibition HUVEC migration stimulated by VEGF. A: Control (no VEGF or ASE). B: Addition of VEGF increased cell migration. C: Addition of VEGF and 200 μg/ml ASE. The addition of the ASE retarded the VEGF-induced migration. Fig. 37D is a bar graph quantitating the cell migration that occurred in Figs. 37A, 37B, and 37C.

[00109] Fig. 38 is a non-limiting example of a panel of microscopic images (A-C) and a bar graph (D) demonstrating that the addition of ASE inhibits tube formation. To perform the *in vitro* tube formation assay, HUVEC cells were seeded on Matrigel. A: Tube-like formation in the control culture. B: Addition of 100 μg/mL ASE added to the culture inhibited tube formation. C: Addition of 200 μg/mL ASE added to the culture inhibited tube formation. D: Bar graph quantitating the number of tubes formed per field from A, B, and C.

DETAILED DESCRIPTION OF THE INVENTION

COMPOSITIONS

[00110] Described herein are purified compositions that exert a number of physiologically significant effects in mammalian cells and intact mammalian tissues. The purified compositions comprise at least four components:

- Cross-linked high molecular weight hyaluronan (HA);
- Tumor necrosis factor-stimulated gene 6 (TSG-6);
- Pentraxin (PTX-3); and
- Thrombospondin (TSP-1).

[00111] Additional components may also be included in purified compositions that have these four components, including: Smad7.

[00112] Any or all of the components of the purified compositions described herein can be prepared from a human amniotic material, including human amniotic jelly preparations and extracts (as described herein), human amniotic membrane preparations and extracts (as described herein), and human amniotic stroma preparations and extracts (as described herein).

[00113] Together, these four components (with or without Smad7) can suppress TGF-β promoter activity; increase apoptosis in macrophages; decrease proliferation, decrease migration, and increase apoptosis of human vascular endothelial cells; decrease viability of human fibroblasts; decrease inflammation; and prevent apoptosis of epithelial cells exposed to storage and injury.

[00114] Hyaluronic acid (HA) is a natural sugar found in the synovial joint fluid, the vitreous humor of the eye, the cartilage, blood vessels, extra-cellular matrix, skin, and umbilical cord. The cross-linking of HA can be through a covalent bound to another molecule, such as a protein. For example, HA can be covalently bound to the heavy chain of inter-α-trypsin inhibitor. The ratio of protein to HA in the AM preparations and purified compositions described herein can be less than about 200:1, less than about 100:1, less than about 50:1, or less than about 10:1.

[00115] TSG-6 is a hyaluronan binding protein that plays a role in extracellular matrix remodeling, cell proliferation, and leucocyte migration. TSG-6 can form a complex with the serine protease inhibitor inter-α-inhibitor. PTX-3 (Pentraxins) are Ca^{2+} dependent ligand binding proteins that have a pentameric discoid structure and are present in plasma. TSP-1 (Thrombospondin I) is a homotrimeric glycoprotein having a potent anti-angiogenic and other biological activities. TSP-1 is secreted into the extracellular matrix by a variety of cell types.

[00116] These components can be obtained from any suitable source. For example, at least one of the components can be obtained from human tissues, such as amniotic membrane, amniotic jelly, amniotic stroma, or a combination thereof. At least one of the components can be obtained from commercial sources. At least one of the components can be isolated from a transgenic organism. The protein sequences can have a similarity of at least 90%, 93%, 95%, 97%, 99% or 99.5% to the human protein sequence. The components can be purified, substantially purified, partially purified, or can also be present in crude extracts. The components can also be prepared from mammalian amniotic membrane tissues, as each of the four components is present in amniotic membrane tissues.

[00117] In additional aspects, the protein Smad7 is also present in the composition. The Smad7 can be obtained from any suitable source, such as from amniotic membrane, from a commercial source, isolated from a transgenic organism. The Smad7 protein can be purified, substantially purified, partially purified, or can be present in a crude extract.

AM Preparations derived from Placental Material

[00118] In some aspects, at least one of the components HA, TSG-6, PTX-3, TSP-1, optionally Smad7 can be obtained from preparations of amniotic membrane. Alternatively, crude amniotic membrane preparations containing the combination of HA, TSG-6, PTX-3, TSP-1 and optionally Smad7 can be prepared. Exemplary methods of preparing various AM preparations are described herein.

[00119] Human placental material can be obtained, for example, from sources such as Bio-Tissue, Inc. (Miami, FL) and Baptist Hospital (Miami, FL) (under IRB approval). The tissue is typically obtained in either a fresh or frozen state. The tissue can be washed to remove excess storage buffer, blood, or contaminants. The excess liquid can be removed, for example, using a brief centrifugation step, or by other means. The tissue can be frozen, using, for example, liquid nitrogen or other cooling means, to facilitate the subsequent homogenization. The source of the AM tissue can be a human. However, other sources of AM tissue, such as bovine or porcine AM tissue, can be used.

[00120] The AM can be used to prepare the composition. AM preparations can include components or portions purified from or extracted from intact AM, AM stromal matrix, HA, AM jelly, and inter-alpha trypsin inhibitor (HA-ITI)). If desired, certain components of the AM preparation can be isolated from the preparation at any time during the process. For example, an extract enriched for a specific protein or set of AM proteins can be isolated from the preparation. After homogenization of the tissue, the larger particles can be separated out, or they can be left in the preparation. The preparation can be dried, if desired. An exemplary preparation method is described in Example 1.

[00121] The compositions can also be obtained from AM jelly. AM jelly can be obtained from the fresh AM tissue, or can be obtained before or after the freezing process. The AM jelly can be frozen, and can also be freeze-ground following the procedure for AM preparations as described herein. The jelly can be centrifuged, and can also be lyophilized.

[00122] In additional embodiments, a composition made substantially from the stromal layer is prepared. To prepare this composition, the stromal layer is separated from the layer of fresh, frozen, thawed, or otherwise treated AM membrane. The stromal removal can occur, for example, by enzymatic methods, mechanical methods, or by other means. The stromal layer material can be fresh or frozen. The stromal material can be ground or freeze-ground following the procedure for AM preparations as described herein. If desired, the stromal matrix material can be centrifuged, and can also be lyophilized.

[00123] The tissue can be frozen prior to the grinding process. The freezing step can occur by any suitable cooling process. For example, the tissue can be flash-frozen using liquid nitrogen. Alternatively, the material can be placed in an isopropanol/dry ice bath or can be flash-frozen in other coolants. Commercially available quick freezing processes can be used. Additionally, the material can be placed in a freezer and allowed to equilibrate to the storage temperature more slowly, rather than being flash-frozen. The tissue can be stored at any desired temperature. For example, -20°C or -80°C or other temperatures can be used for storage.

[00124] Pulverizing the tissue while frozen, rather than grinding the tissue prior to freezing, is one optional method for preparing the tissue. Alternatively, fresh, partially thawed, or thawed tissue can be used in the grinding step. The tissue (fresh, frozen, or thawed) can then be sliced into pieces of a desired size with a suitable device, such as a scalpel, then ground to fine particles using a BioPulverizer (Biospec Products, Inc., Bartlesville, OK) or other suitable devices, and homogenized with a homogenization device such as a Tissue Tearor (Biospec Products, Inc., Dremel, WI, in a suitable solution. Exemplary solutions include but are not limited to phosphate buffered saline (PBS), DMEM, NaCl solution, and water. The pH of the solution can be adjusted as needed. In some embodiments, the pH range is from about 5.5 or 6.0 to about 8.5. In some embodiments, the frozen tissue is ground in a solution having a pH of between about 6.3, about 6.6, or about 7.0 to about 7.4, about 7.6, or about 7.8.

[00125] Any suitable buffer or liquid can be used to prepare the formulations. Example 2 examines the use of various extraction buffers (high salt, low salt, PBS, etc.) on total protein content and HA in the preparation (Table X). Example 2 further examines the levels of the specific proteins TSG-6 (Fig. 14), PTX-3 (Fig. 18), TSP-1 (Fig. 19), and Smad7 (Fig. 20) using several extraction methods.

[00126] The homogenate can then be mixed at any suitable speed, temperature, or other parameters. The mixing can occur, for example, at a temperature range of from about 1°C, or 3°C, to about 6°C, 10°C, 15°C, or 20°C. In some embodiments, the mixing occurs at about 4°C. The homogenate can be mixed, for example, from less than about 1 minute, 10 minutes, or 20 minutes to about 1, 2, 3 or more hours.

[00127] The homogenate can then be centrifuged to remove any remaining large particulates, if desired. The centrifugation can be performed using any suitable range of time, temperature, protein concentration, buffers, and

speed as desired. The centrifugation can occur, for example, at a range of about 1,000, 5,000, or 10,000 x g to about 20,000 x g. In some embodiments, the centrifugation occurs at about 15,000 x g. The centrifugation can occur for a duration of from less than 1 minute, 5 minutes, 10 minutes, 20 minutes, to about 40 minutes, 60 minutes, 1.5 hours, or more. The supernatant can then be collected and stored in aliquots at -80° C. The total protein can be quantitated, if desired, using any suitable commercial protein analysis kit, such as a BCA assay (Pierce, Rockford, IL). Example 2, Table X, and Fig. 13 describe the analysis of AM preparations after low speed or high speed centrifugation.

[00128] For biochemical characterization and purification, the above solutions can be supplemented with protease inhibitors. An exemplary mixture of protease inhibitors is the following: 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 mM PMSF. Typically, however, a protease inhibitor is not added to the preparation if it is to be added to live cells or tissues.

[00129] The formulation can be tested to confirm the presence of specific components or proteins. For example, the formulation can be tested for the presence of molecules including but not limited to HA, TSG-6, PTX-3, TSP-1, Smad7, and the like. The formulation can also be tested to confirm the absence of pathogens at any point during the preparation process.

[00130] AM preparations can be in a liquid, suspension, or lyophilized powder (e.g., ground or pulverized), or other forms. Antimicrobial agents such as antibiotics or anti-fungal agents may be added. Other substances can be added to the compositions to stabilize and/or preserve the compositions. The material can be packaged and stored, for example, at room temperature, or for example, at -20°C or -80°C prior to use.

[00131] In some embodiments, the preparation is present as a dry powder formulation. A dry powder formulation can be stored in a smaller volume, and may not require the same low temperature storage requirements to keep the formulation from degrading over time. A dry powder formulation can be stored and reconstituted prior to use. The dry powder formulation can be prepared, for example, by preparing the freeze-ground AM tissue as described herein, then removing at least a portion of the water in the composition. The excess water can be removed from the preparation by any suitable means. An exemplary method of removing the water is by use of lyophilization using a commercially available lyophilizer or freeze-dryer. Suitable equipment can be found, for example, through Virtis, Gardiner, NY; FTS Systems, Stone Ridge, NY; and SpeedVac (Savant Instruments Inc., Farmingdale, NY). The amount of water that is removed can be from about 5%, 10%, 20%, 30% to about 60, 70, 80, 90, 95 or 99% or more. In some embodiments, substantially all of the excess water is removed. The lyophilized powder can then be stored. The storage temperature can vary from less than about -196°C -80°C, -50°C, or -20°C to more than about 23°C. If desired, the powder can be characterized (weight, protein content, etc) prior to storage.

[00132] The lyophilized powder can be reconstituted in a suitable solution or buffer prior to use. Exemplary solutions include but are not limited to PBS, DMEM, and BSS. The pH of the solution can be adjusted as needed. The concentration of the AM can be varied as needed. In some procedures a more concentrated preparation is useful, whereas in other procedures, a solution with a low concentration of AM is useful. Additional compounds can be added to the composition. Exemplary compounds that can be added to the reconstituted formulation include but are not limited to pH modifiers, buffers, collagen, HA, antibiotics, surfactants, stabilizers, proteins, and the like. The lyophilized AM powder can also be added to a prepared cream, ointment or lotion to result in the desired concentration.

[00133] Additional components can be added to the composition as desired. In some embodiments, water soluble or powdered AM preparations can be mixed with an ECM component such as collagen, fibrin, or HA.

[00134] Collagen is a major structural protein found in the body. It provides support for tissues, connects tissue to bone, and provides the structure of the body. When the body is in the healing process, collagen plays a role in

helping to build a cellular structure. Hyaluronic acid is a natural sugar found in the synovial joint fluid, the vitreous humor of the eye, the cartilage, blood vessels, extra-cellular matrix, skin, and umbilical cord. Fibrin is a protein involved in the clotting of blood.

[00135] Water-soluble AM preparation can be mixed with collagen, fibrin or with HA. Similarly, lyophilized powder AM preparation can be mixed with collagen, fibrin or HA. Collagen, fibrin and HA can be are suitable delivery vehicles, as AM preparations mixed with collagen or HA were shown to exert a suppressive effect upon TGF-β promoter activity. Although AM preparations were mixed with collagen gel and HA gel in the experiments described herein, any soluble forms (e.g., liquid) of collagen and HA or other ECM components (e.g., fibrin) can be used. The collagen, fibrin or HA can be derived from any suitable source organism. When collagen, fibrin or HA are added, the ratio of these compounds to AM can be varied as desired. For example, a ratio of AM to collagen (or fibrin or HA) of less than about 0.001:1, 0.01:1, 0.05:1, or 0.1:1, to about 1:1, 1.5:1, 2:1, 5:1, 10:1, 100:1 or 1000:1 or more can be used.

[00136] Collagen gel can be prepared, for example, by diluting the stock solution (4 mg/ml) with 0.1 N acetic acid and by mixing it with appropriate volume ratios of 20X DMEM or suitable buffer, and 1 N NaOH, as described in Example 1. The collagen in the preparation can be present, for example, at a range of from less than about 2 mg/ml to more than about 4 mg/ml.

[00137] Various dilutions of high MW HA can be prepared, for example, by diluting commercially prepared HA (Healon™ (10 mg HA/ml) (Pharmacia, LaJolla, CA) in DMEM or suitable buffer. Lyophilized powder and water-soluble forms of AM preparations can be diluted in a solution such as PBS, DMEM, or other solutions into the desired collagen concentration. The HA in the preparation can be present, for example, at a range of from less than about 2 μg/ml to more than about 129 μg/ml.

[00138] The following procedures represent illustrative methods for preparing the amniotic preparations and purified compositions described and used herein.

Preparation of Preserved Human AM:

[00139] Human placenta was collected at elective cesarean delivery (Heiligenhaus et al., Invest Ophthalmol Vis Sci. 42:1969-1974, 2001, Lee and Tseng, Am J Ophthalmol. 123:303-312, 1997, Prabhasawat et al., Ophthalmology, 104:974-985, 1997, Tseng et al., Arch Ophthalmol. 116:431-441, 1998). The AM was flattened onto nitrocellulose paper (Hybond N+, Amersham, England), with the epithelium surface up. The AM samples were stored at -80° C in DMEM /glycerol 1:2 (v/v) until use.

Amniotic Membrane Extract Preparations

[00140] Fresh and frozen human placentas were obtained from Bio-Tissue, Bio-tissue, Inc. (Miami, FL). The entire procedure for preparation of total human AM extracts (AME) was carried out aseptically so as to be used for subsequent cell culture-based experiments. The AM was sliced into small pieces to fit into the barrel of a BioPulverizer (Biospec Products, Inc., Bartlesville, OK), frozen in the liquid nitrogen, pulverized into a fine powder, and weighed. Cold 1X PBS buffer, pH 7.4, containing protease inhibitors (protease inhibitor cocktail, P8340, Sigma, and supplemented with 1 mM PMSF) and phosphatase inhibitors (50 mM sodium fluoride and 0.2 mM sodium vanadate) was added to the powder at 1:1 (ml/g). The mixture was kept on ice and homogenized with a Tissue Tearor (Biospec Products, Inc., Dremel, WI) 5 times, 1 minute each, with a 2 minute cooling interval. These water-soluble extracts were designated as "Total" AM extracts (AME).

[00141] Total AM extracts were divided into two 50 ml conical centrifuge tubes. One was centrifuged at high speed (HS, 48,000 x g) and the other one was centrifuged at a low speed (LS, 15,000 X g) at 4°C. Aliquots of the

HS and LS supernatant were transferred to sterile 1.5 ml Eppendorf tubes and were designated as AM/HS, AM/LS, respectively. Desired AM/HS samples were frozen at -20°C for 1 h before lyophilization. The samples then were placed in the chamber of FreeZone (Labconco, Kansas City, MO) with holes on the cap. Samples were lyophilized at -50°C at a vacuum of 0.85 mBar for 5 hours. Before use, the samples were reconstituted with the sterile distilled H_2O to the same volume. The same method was also used to prepare extracts from AM jelly, which was easily scraped from the adherent material on the AM stroma.

Total Soluble Human Amniotic Membrane and Amniotic Membrane Jelly Extract Preparations

[00142] Frozen human placenta material was obtained from Bio-Tissue, Bio-tissue, Inc. (Miami, FL). The entire procedure for preparation of total human AM extracts (AME) was carried out aseptically so as to be used for subsequent cell culture-based experiments. The AM was sliced into small pieces to fit into the barrel of a BioPulverizer (Biospec Products, Inc., Bartlesville, OK), frozen in the liquid nitrogen, pulverized into a fine powder, and weighed. Cold 1X PBS buffer, pH 7.4, containing protease inhibitors (protease inhibitor cocktail, P8340, Sigma, and supplemented with 1 mM PMSF) and phosphatase inhibitors (50 mM sodium fluoride and 0.2 mM sodium vanadate) was added to the powder at 1:1 (ml/g). The mixture was kept on ice and homogenized with a Tissue Tearor (Biospec Products, Inc., Dremel, WI) 5 times, 1 minute each, with a 2 minute cooling interval. These water-soluble extracts were designated as "Total" AM extracts (AME).

[00143] The total water-soluble extract was mixed for 1 hr at 4 °C, subsequently fractionated by two different speeds of centrifugation at 4 °C for 30 min, i.e., 15000 x g and 48000 x g, and the resultant supernatant was designated as L and H, respectively. Each supernatant was divided into aliquots and stored at -80 °C. This method was also used to prepare extracts from AM jelly, which was easily scraped from the adherent material on the AM stroma.

Total Soluble Human Amniotic Membrane and Amniotic Membrane Jelly Extracts by Different Buffers and Fractionation methods

[00144] In examining preparations in different extraction buffers, the powder as prepared from above was weighed and mixed with Buffer A (Isotonic Low salt): 100 mM Tris-HCl, pH 7.6, 150 mM NaCl, 4 mM EDTA, 1 % Triton X-100 at the wet weight (g) of AM to the buffer (ml) at 1:1 ratio by stirring at 4 °C for 1 hr. After centrifugation at 48000 x g, the resultant pellet was subsequently extracted by Buffer B (high salt): 100 mM Tris-HCl, pH 7.6, 1 M NaCl, 4 mM EDTA, 1 % Triton X-100 by stirring at 4 °C for 1 hr. Again after centrifugation at 48000 x g, the pellet was finally extracted by Buffer C (4 M guanidine hydrochloride): 100 mM sodium acetate, pH 5.8, 4 M guanidine hydrochloride, 4 mM EDTA, 1 %Triton X-100 by stirring at 4 °C for 24 hr. All the above three buffers were supplemented with the following protease and phosphatase inhibitors: 1 μg/ml aprotinin, 1 μg/ml leupeptins, 1 μg/ml pepstatin A, 0.5 mM PMSF, 50 μM sodium fluoride and 0.2 μM sodium vanadate. The resultant supernatants, designated as Extract A, B, and C, respectively, were dialyzed against the dislysis buffer (50 mM Tris-HCl, pH7.5, 0.15 M NaCl) supplemented with 0.5 mM PMSF at 4°C for 6 hr and dialysis buffer was changed twice, each with 500 x (the volume ratio- dialysis buffer :samples). After dialysis, the volume of each sample was measured and adjusted to the same volume with the dialysis buffer. The same method was also used to prepare extracts from AM jelly, which was the adherent material on the AM stroma that could be easily scraped off.

Preparation of Total Soluble Human Amniotic Membrane Extracts in PBS

[00145] The entire procedure for preparation of total soluble human AM extracts (T) was carried out aseptically so as to be used for subsequent cell culture-based experiments. Frozen human placenta was obtained from Bio-tissue, Inc. (Miami, FL), from which AM was retrieved. AM was sliced into small pieces to fit into the

barrel of a BioPulverizer (Biospec Products, Inc., Bartlesville, OK), frozen in the liquid nitrogen, and then pulverized into a fine powder. The powder was weighed and mixed with cold PBS buffer (prepared by adding distilled H_2O to 1 x PBS, pH7.4, from10 x PBS, cat# 70011-044, Invitrogen, Carlsbad, CA) with protease inhibitors (protease inhibitor cocktail, P8340, Sigma, and supplemented with 1 mM PMSF) and phosphatase inhibitors (50 mM sodium fluoride and 0.2 mM sodium vanadate) at 1:1 (ml/g). The mixture was kept in the ice and homogenized with a Tissue Tearor (Biospec Products, Inc., Dremel, WI) for 5 times, 1 min each with a 2 min interval cooling.This water-soluble extract was designated as "Total" (T). The total water-soluble extract was mixed for 1 hr at 4 °C, centrifuged at 4 °C for 30 min at 48000 x g. The supernatant was divided into aliquots and stored at -80 °C.

Preparation of Water-soluble AM Stromal Extracts

[00146] Using aseptic techniques, frozen human AM obtained from Bio-Tissue, Inc. (Miami, FL) was briefly washed 2-3 times with HBSS to remove the original storage medium. The AM stroma was scraped by spatula, frozen in the air phase of liquid nitrogen and grounded to fine particles by BioPulverizer (Biospec Products, Inc., Bartlesville, OK) followed by homogenization on ice with Tissue Tearor (Biospec Products, Inc., Dremel, WI) in PBS, pH 7.4, for 1 min. The homogenate was mixed by rotation for 1 h and centrifuged at 14,000 x g for 30 min at 4°C. The supernatant in PBS was then collected, and stored in aliquots at -80°C. The protein concentration was determined by BCA assay. This water-soluble protein extract, designated as amniotic stromal extract (ASE), was used for experiments described herein.

AM Stromal Extract Preparation

[00147] The complete procedure for preparation of protein extracts was carried out aspectically. Frozen human AM obtained from Bio-Tissue (Miami, FL) was briefly washed 2-3 times with HBSS (Invitrogen, Carlsbad, CA) to remove the storage medium. AM stroma was scraped from the stromal side of the AM by spatula for AM stroma extract preparation. Human placenta as well as chorion obtained from Baptist Hospital (Miami, FL) was rinsed 3 times with HBSS to remove blood. To prepare the water-soluble protein extract, total AM, scraped AM stroma, stroma-removed AM, placenta, and chorion were each frozen in the air phase of liquid nitrogen and each ground to fine particles using a BioPulverizer (Biospec Products, Inc., Bartlesville, OK) followed by homogenization on ice with Tissue Tearor (Biospec Products, Inc., Dremel, WI) in PBS (pH 7.4) for 1 min. Each homogenate was mixed for 1 hour and centrifuged at 14,000 g for 30 min at 40° C. Each supernatant (in PBS) was then collected and stored in aliquots (0.5 ml) at -80° C. A BCA assay (Pierce, Rockford, IL) was used to quantitate the total protein in different extracts.

Preparing Water-soluble and Lyophilized Powder Forms of Human AM Extracts

[00148] In a typical procedure for preparing human AM extracts, the entire procedure is carried out aseptically. Unless otherwise noted, the AM extracts can be handled at room temperature during the steps of the procedure. First, fresh or frozen human AM is obtained, preferably from Bio-Tissue, Inc. (Miami, FL). Frozen AM is briefly washed 2-3 times with HBSS (Invitrogen, Carlsbad, CA) to remove the storage medium. Fresh human placenta or chorion is rinsed 3 times with HBSS to remove blood.

[00149] To prepare the water-soluble form of AM extracts, the AM (e.g., AM stroma, stroma-removed AM, placenta, chorion) is transferred to a sterile 50 ml centrifuge tube and centrifuged at 4° C for 5 min at 5000 x g to remove the excess fluid. The AM is weighed, transferred to a 100 mm or 150 mm sterile Petri dish, and frozen in the air phase of a liquid nitrogen container for 20 min to facilitate the subsequent homogenization. The frozen AM is then sliced into small pieces with a disposable scalpel or ground to fine particles using a BioPulverizer (Biospec Products, Inc., Bartlesville, OK) or other suitable device, and homogenized with Tissue Tearor (Biospec Products,

Inc., Dremel, WI) or other suitable device, in phosphate buffered saline (PBS) or DMEM without phenol red (Invitrogen, Carlsbad, CA) at neutral pH. For biochemical characterization and purification, the above solutions are supplemented with the following proteinase inhibitors: 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 mM PMSF. However, if the extract is to be directly added to cell culture, no protease inhibitor is added. The homogenate is mixed at 4° C for 30 min and centrifuged at 15,000 x g for 30 min. The supernatant (i.e., AM extract) is collected and stored in aliquots (0.5 ml) at -80° C. A BCA assay (Pierce, Rockford, IL) is used to quantitate the total protein in each AM extract.

[00150] To prepare the lyophilized powder form of AM extracts, frozen AM is ground to fine particles using a BioPulverizer (Biospec Products, Inc., Bartlesville, OK), or other suitable device, and further homogenized as described herein. Aliquots of approximately 0.5 ml are lyophilized by SpeedVac (Savant Instruments Inc., Farmingdale, NY) at 4° C for 4 h to decrease the weight from 280 mg to 32 mg (~ 89% reduction). The lyophilized powder is weighed and stored at -80° C. Before use, the lyophilized powder can be reconstituted in a suitable buffer.

[00151] To prepare AM stromal extracts, the AM stroma is scraped from the stromal surface of intact total AM leaving the basement membrane and the amniotic epithelium intact, and the frozen AM stroma is ground using a BioPulverizer as described herein. The stroma is extracted with PBS (e.g., 1.4 mg/ml) at a neutral pH at 4° C for 30 min and centrifuged at 15,000 x g for 30 min. The supernatant is collected and stored in aliquots (0.5 ml) at -80° C. A BCA assay (Pierce, Rockford, IL) is used to quantitate the total protein in the AM stromal extract.

[00152] Methods involving conventional molecular biology techniques are described herein. Such techniques are generally known in the art and are described in detail in methodology treatises such as Molecular Cloning: A Laboratory Manual, 3rd ed., vol. 1-3, ed. Sambrook et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2001; and Current Protocols in Molecular Biology, ed. Ausubel et al., Greene Publishing and Wiley-Interscience, New York, 2003 (with periodic updates). Various techniques for culturing animal cells are known in the art and are described in Culture of Animal Cells: A Manual of Basic Technique, 4th ed., R. Ian Freshney, Wiley-Liss, Hoboken, NJ, 2000, and Animal Cell Culture Techniques (Springer Lab Manual), M. Clynos, Springer – Verlag, New York, NY, 1998. Methods involving protein analysis and purification are also known in the art and are described in Protein Analysis and Purification: Benchtop Techniques, 2nd ed., Ian M. Rosenberg, Birkhauser, New York, NY, 2004.

PHARMACEUTICAL COMPOSITIONS

[00153] AM preparations can be formulated for administration purposes as a non-solid dosage form, for example, by combining with a delivery vehicle to create compositions such as solutions, drops, suspensions, pastes, sprays, ointments, oils, emulsions, aerosols, a coated bandage, a patch, creams, lotions, gels, and the like. The formulation used will depend upon the particular application. Gels are useful for administering the compositions because they allow better retention of the active ingredient at the site of introduction, allowing the active ingredient to exert its effect for a longer period of time before clearance of the active ingredient. Alternatively, AM preparations can be formulated as extended-release solid dosage forms (including oral dosage forms). A description of exemplary pharmaceutically acceptable carriers or vehicles and diluents, as well as pharmaceutical formulations, is provided herein and can also be found in Remington's Pharmaceutical Sciences, a standard text in this field, and in USP/NF.

[00154] Pharmaceutical compositions may be formulated in a conventional manner using one or more physiologically acceptable carriers including excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route

of administration chosen. Any of the well-known techniques, carriers, and excipients may be used as suitable and as understood in the art. A summary of pharmaceutical compositions described herein may be found, for example, in *Remington: The Science and Practice of Pharmacy*, Nineteenth Ed (Easton, Pa.: Mack Publishing Company, 1995); Hoover, John E., *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, Pennsylvania 1975; Liberman, H.A. and Lachman, L., Eds., *Pharmaceutical Dosage Forms*, Marcel Decker, New York, N.Y., 1980; and *Pharmaceutical Dosage Forms and Drug Delivery* Systems, Seventh Ed. (Lippincott Williams & Wilkins; 1999), herein incorporated by reference in their entirety.

[00155] In certain embodiments, the compositions include a pharmaceutically acceptable diluent(s), excipient(s), or carrier(s). In addition, the AM preparations and purified compositions described herein can be administered as pharmaceutical compositions in which AM preparations and purified compositions described herein are mixed with other active ingredients, as in combination therapy. In some embodiments, the pharmaceutical compositions may include other medicinal or pharmaceutical agents, carriers, adjuvants, such as preserving, stabilizing, wetting or emulsifying agents, solution promoters, salts for regulating the osmotic pressure, and/or buffers. In addition, the pharmaceutical compositions can also contain other therapeutically valuable substances.

[00156] A pharmaceutical composition, as used herein, refers to a mixture of a AM preparations and purified compositions described herein with other chemical components, such as carriers, stabilizers, diluents, dispersing agents, suspending agents, thickening agents, and/or excipients. The pharmaceutical composition facilitates administration of the compound to an organism. In practicing the methods of treatment or use provided herein, therapeutically effective amounts of AM preparations and purified compositions described herein are administered in a pharmaceutical composition to a mammal having a disease, disorder, or condition to be treated. In some embodiments, the mammal is a human. A therapeutically effective amount can vary widely depending on the severity of the disease, the age and relative health of the subject, the potency of the compound used and other factors. The compounds can be used singly or in combination with one or more therapeutic agents as components of mixtures.

Topical Formulations

[00157] Formulations of the AM preparations and purified compositions described herein include those suitable for topical administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. The amount of active ingredients which can be combined with a carrier material to produce a single dosage form will vary depending upon the host being treated, the particular mode of administration.

[00158] Suspensions may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

[00159] Typical compositions described herein include a wide variety of physical forms. These include, but are not limited to, solutions, lotions, creams, oils, gels, sticks, sprays, ointments, balms, shampoo, and pastes. Generally, such carrier systems can be described as being solutions, emulsions, gels, solids, and aerosols. The compositions may be applied topically to the skin, or may be applied in the form of a transdermal delivery device, such as a microneedle, a patch, bandage, or gauze pad known in the art.

[00160] The ointments, pastes, creams and gels may contain, in addition to the AM preparations and purified compositions described herein, excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.

[00161] Powders and sprays can contain, in addition to the AM preparations and purified compositions described herein, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

[00162] Solvents are generally employed in the preparation of suitable topical compositions. Such solvents can either be aqueous or organic based. The solvent must be capable of having dispersed or dissolved therein the active ingredients while not being irritating to the animal being treated. Water forms the basis for all aqueous solvents, while suitable organic solvents include propylene glycol, butylene glycol, polyethylene glycol, polypropylene glycol, glycerol, 1,2,4-butanetriol, sorbitol esters, 1,2,6-hexanetriol, ethanol, isopropanol, butanediol, and mixtures thereof. Solvents can be included in the overall composition in amounts ranging from 0.1% to 99% and preferably from 2.0% to 75%. In some embodiments, the compositions are produced in the form of an emollient-containing composition. A wide variety of suitable emollients are known and may be used herein.

[00163] In some embodiments, the compositions are formulated as lotions containing from about 0.01% to 10% of the AM preparations and purified compositions described herein. In other embodiments, the compositions are formulated in a solution carrier system as a cream. A cream composition would preferably comprise from about 0.1% to 15% and preferably from 1% to 5% of the the AM preparations and purified compositions described herein. Lotions and creams can be formulated as emulsions as well as solutions. The compositions may also be administered in liquid form, including in the form of liposomes suspended in liquid, as in the different type of sprays available in this industry.

[00164] In other embodiments, the active ingredients are formulated as ointments. Suitable ointments may comprise simple bases of animal or vegetable oils, or semi-solid hydrocarbons (oleaginous). Suitable ointments may also comprise absorption ointment bases which absorb water to form emulsions. Ointment carriers may also be water soluble. An ointment may comprise from 1% to 99% of an emollient plus to about 0.1% to 99% of a thickening agent.

[00165] The proportion of the AM preparations and purified compositions described herein in the compositions can vary from between about 0.01 wt. % to about 100 wt. %, more preferably from about 0.1 wt. % to about 99.9 wt. %, and especially from about 1.0 wt. % to about 99.0 wt. %.

[00166] "Carriers" or "vehicles" preferably refer to carrier materials suitable for topical administration and include any such materials known in the art, such as any liquid, gel solvent, liquid diluent, solubilizer, or the like, which is non-toxic, and which does not interact with other components of the composition in a deleterious manner. Examples of suitable carriers for use herein include water, silicone, liquid sugars, waxes, oils, petroleum jelly, and a variety of other materials.

[00167] In some embodiments, the carrier or vehicle includes one or more solvents, oils, surfactants, humectants, thickening agents, antioxidants, chelating agents, buffers, and preservatives.

[00168] Examples of solvents include C_2-C_{10} alcohols, such as hexanol, cyclohexanol, benzyl alcohol, 1,2-butanediol, glycerol, and amyl alcohol; C_5-C_{10} hydrocarbons such as n-hexane, cyclohexane, and ethylbenzene; C_4-C_{10} aldehydes and ketones, such as heptylaldehyde, cyclohexanone, and benzylaldehyde; C_4-C_{10} esters, such as amyl acetate and benzyl propionate; ethereal oils, such as oil of eucalyptus, oil of rue, cumin oil, limonene, thymol, and 1-pinene; halogenated hydrocarbons having 2-8 carbon atoms, such as 1-chlorohexane, 1-bromohexane, and chlorocyclohexane.

[00169] Examples of oils comprise fats and oils such as olive oil and hydrogenated oils; waxes such as beeswax and lanolin; hydrocarbons such as liquid paraffin, ceresin, and squalane; fatty acids such as stearic acid and

oleic acid; alcohols such as cetyl alcohol, stearyl alcohol, lanolin alcohol, and hexadecanol; and esters such as isopropyl myristate, isopropyl palmitate and butyl stearate.

[00170] Examples of surfactants include anionic surfactants such as sodium stearate, sodium cetyl sulfate, polyoxyethylene laurylether phosphate, sodium N-acyl glutamate; cationic surfactants such as stearyldimethylbenzylammonium chloride and stearyltrimethylammonium chloride; ampholytic surfactants such as alkylaminoethylglycine hydrochloride solutions and lecithin; and nonionic surfactants such as glycerin monostearate, sorbitan monostearate, sucrose fatty acid esters, propylene glycol monostearate, polyoxyethylene oleylether, polyethylene glycol monostearate, polyoxyethylene sorbitan monopalmitate, polyoxyethylene coconut fatty acid monoethanolamide, polyoxypropylene glycol (e.g., the materials sold under the trademark "Pluronic"), polyoxyethylene castor oil, and polyoxyethylene lanolin.

[00171] Examples of humectants include glycerin, 1,3-butylene glycol, and propylene glycol; examples of thickening agents include xanthan gum, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, polyethylene glycol and sodium carboxymethyl cellulose; examples of antioxidants comprise butylated hydroxytoluene, butylated hydroxyanisole, propyl gallate, citric acid and ethoxyquin; examples of chelating agents include disodium edetate and ethanehydroxy diphosphate; examples of buffers comprise citric acid, sodium citrate, boric acid, borax, and disodium hydrogen phosphate; and examples of preservatives are methyl parahydroxybenzoate, ethyl parahydroxybenzoate, dehydroacetic acid, salicylic acid and benzoic acid.

[00172] In certain embodiments, the carrier/vehicle is composed of the foregoing materials to achieve a controlled occlusion of the skin, thereby resulting in optimal enhancement of biologically active moiety penetration across the skin with minimal skin irritation. In certain embodiments, the carrier/vehicle may include a dispersing agent that aids in maintaining a particulate phase of the active ingredients dispersed in the continuous phase. In other embodiments, non-ionic excipients, such as lauric alcohol, propylene glycol monolaurate, myristyl lactate, lauryl lactate, or the like, facilitate dispersion.

The rate of biologically active moiety delivery across a dermal surface can be increased by transdermal delivery enhancers. Suitable transdermal delivery enhancers include proton-accepting solvents such as dimethylsulfoxide and dimethylacetamide. Other suitable transdermal delivery enhancers include 2-pyrrolidine, N,N-diethyl-m-toluamide, 1- dodecylazacycloheptan-2-one, N,N-dimethylformamide, N-methyl-2-pyrrolidine, terpenes, surfactants, and calcium thioglycolate.

[00173] Suitable dermal penetration enhancers include 1-5 carbon fatty acid esters of para-aminobenzoic acid, isopropyl palmitate, isopropyl myristate, ethanol, isobutyl alcohol, isobutyl alcohol, stearyl alcohol, glycerol, 2-pyrrolidone, urea, propylene glycol, oleic acid, palmitic acid, dimethyl sulfoxide, N,N-dimethyl acetamide, N,N-dimethyl formamide, 2-pyrrolidone, 1-methyl-2-pyrrolidone, 5-methyl-2-pyrrolidone, 1,5-dimethyl-2-pyrrolidone, 1-ethyl-2-pyrrolidone, 2-pyrrolidone-5-carboxylic acid, N,N-dimethyl-m-toluamide, urea, ethyl acetate, 1-dodecylazacycloheptan-2-one, oleic acid, imidazoline, butylurea, and pyrrolidone carboxylic acid esters.

[00174] Wetting agents, emulsifiers, surfactants, and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring, and perfuming agents, preservatives and antioxidants can also be present in the compositions.

[00175] Examples of pharmaceutically acceptable antioxidants include: (1) water-soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite, and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal-chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

[00176] As appropriate compositions for topical application there may be cited all compositions usually employed for topically administering therapeutics, e.g., creams, jellies, dressings, shampoos, tinctures, pastes, ointments, salves, powders, liquid or semiliquid formulations, and the like. Application of said compositions may be by aerosol, e.g., with a propellant such as nitrogen carbon dioxide, a freon, or without a propellant such as a pump spray, drops, lotions, or a semisolid such as a thickened composition which can be applied by a swab. In particular compositions, semisolid compositions such as salves, creams, pastes, jellies, ointments, and the like will conveniently be used.

Ophthalmic Formulations

[00177] The AM preparations and purified compositions described herein can be administered in a variety of ways, including all forms of local delivery to the eye. Additionally, the AM preparations and purified compositions described herein can be administered systemically, such as orally or intravenously. The AM preparations and purified compositions described herein can be administered topically to the eye and can be formulated into a variety of topically administrable ophthalmic compositions, such as solutions, suspensions, gels or ointments. Thus, "ophthalmic administration" encompasses, but is not limited to, intraocular injection, subretinal injection, intravitreal injection, periocular administration, subconjuctival injections, retrobulbar injections, intracameral injections (including into the anterior or vitreous chamber), sub-Tenon's injections or implants, ophthalmic solutions, ophthalmic suspensions, ophthalmic ointments, ocular implants and ocular inserts, intraocular solutions, use of iontophoresis, incorporation in surgical irrigating solutions, and packs (by way of example only, a saturated cotton pledget inserted in the fornix).

[00178] A composition comprising the AM preparations and purified compositions described herein can illustratively take the form of a liquid where the agents are present in solution, in suspension or both. Typically when the composition is administered as a solution or suspension a first portion of the agent is present in solution and a second portion of the agent is present in particulate form, in suspension in a liquid matrix. In some embodiments, a liquid composition may include a gel formulation. In other embodiments, the liquid composition is aqueous. Alternatively, the composition can take the form of an ointment.

[00179] Useful compositions can be an aqueous solution, suspension or solution/suspension, which can be presented in the form of eye drops. A desired dosage can be administered via a known number of drops into the eye. For example, for a drop volume of 25 µl, administration of 1-6 drops will deliver 25-150 µl of the composition. Aqueous compositions typically contain from about 0.01% to about 50%, more typically about 0.1% to about 20%, still more typically about 0.2% to about 10%, and most typically about 0.5% to about 5%, weight/volume of the AM preparations and purified compositions described herein.

[00180] Typically, aqueous compositions have ophthalmically acceptable pH and osmolality. "Ophthalmically acceptable" with respect to a formulation, composition or ingredient typically means having no persistent detrimental effect on the treated eye or the functioning thereof, or on the general health of the subject being treated. Transient effects such as minor irritation or a "stinging" sensation are common with topical ophthalmic administration of agents and consistent with the formulation, composition or ingredient in question being "ophthalmically acceptable."

[00181] Useful aqueous suspension can also contain one or more polymers as suspending agents. Useful polymers include water-soluble polymers such as cellulosic polymers, e.g., hydroxypropyl methylcellulose, and water-insoluble polymers such as cross-linked carboxyl-containing polymers. Useful compositions can also comprise an ophthalmically acceptable mucoadhesive polymer, selected for example from carboxymethylcellulose,

carbomer (acrylic acid polymer), poly(methylmethacrylate), polyacrylamide, polycarbophil, acrylic acid/butyl acrylate copolymer, sodium alginate and dextran.

[00182] Useful compositions may also include ophthalmically acceptable solubilizing agents to aid in the solubility of components of the AM preparations and purified compositions described herein. The term "solubilizing agent" generally includes agents that result in formation of a micellar solution or a true solution of the agent. Certain ophthalmically acceptable nonionic surfactants, for example polysorbate 80, can be useful as solubilizing agents, as can ophthalmically acceptable glycols, polyglycols, e.g., polyethylene glycol 400, and glycol ethers.

[00183] Useful compositions may also include one or more ophthalmically acceptable pH adjusting agents or buffering agents, including acids such as acetic, boric, citric, lactic, phosphoric and hydrochloric acids; bases such as sodium hydroxide, sodium phosphate, sodium borate, sodium citrate, sodium acetate, sodium lactate and tris-hydroxymethylaminomethane; and buffers such as citrate/dextrose, sodium bicarbonate and ammonium chloride. Such acids, bases and buffers are included in an amount required to maintain pH of the composition in an ophthalmically acceptable range.

[00184] Useful compositions may also include one or more ophthalmically acceptable salts in an amount required to bring osmolality of the composition into an ophthalmically acceptable range. Such salts include those having sodium, potassium or ammonium cations and chloride, citrate, ascorbate, borate, phosphate, bicarbonate, sulfate, thiosulfate or bisulfite anions; suitable salts include sodium chloride, potassium chloride, sodium thiosulfate, sodium bisulfite and ammonium sulfate.

[00185] Other useful compositions may also include one or more ophthalmically acceptable preservatives to inhibit microbial activity. Suitable preservatives include mercury-containing substances such as merfen and thiomersal; stabilized chlorine dioxide; and quaternary ammonium compounds such as benzalkonium chloride, cetyltrimethylammonium bromide and cetylpyridinium chloride.

[00186] Still other useful compositions may include one or more ophthalmically acceptable surfactants to enhance physical stability or for other purposes. Suitable nonionic surfactants include polyoxyethylene fatty acid glycerides and vegetable oils, e.g., polyoxyethylene (60) hydrogenated castor oil; and polyoxyethylene alkylethers and alkylphenyl ethers, e.g., octoxynol 10, octoxynol 40.

[00187] Still other useful compositions may include one or more antioxidants to enhance chemical stability where required. Suitable antioxidants include, by way of example only, ascorbic acid and sodium metabisulfite.

[00188] Aqueous suspension compositions can be packaged in single-dose non-reclosable containers. Alternatively, multiple-dose reclosable containers can be used, in which case it is typical to include a preservative in the composition.

[00189] The ophthalmic composition may also take the form of a solid article that can be inserted between the eye and eyelid or in the conjunctival sac, where it releases the AM preparations and purified compositions described herein. Release is to the lacrimal fluid that bathes the surface of the cornea, or directly to the cornea itself, with which the solid article is generally in intimate contact. Solid articles suitable for implantation in the eye in such fashion are generally composed primarily of polymers and can be biodegradable or non-biodegradable.

Injectable Formulations

[00190] Formulations suitable for intramuscular, subcutaneous, or intravenous injection may include physiologically acceptable sterile aqueous or non-aqueous solutions, dispersions, suspensions or emulsions, and sterile powders for reconstitution into sterile injectable solutions or dispersions. Examples of suitable aqueous and non-aqueous carriers, diluents, solvents, or vehicles including water, ethanol, polyols (propyleneglycol, polyethylene-glycol, glycerol, cremophor and the like), suitable mixtures thereof, vegetable oils (such as olive oil)

and injectable organic esters such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants. Formulations suitable for subcutaneous injection may also contain additives such as preserving, wetting, emulsifying, and dispensing agents. Prevention of the growth of microorganisms can be ensured by various antibacterial and antifungal agents, such as parabens, chlorobutanol, phenol, sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, such as aluminum monostearate and gelatin.

[00191] For intravenous injections, AM preparations and purified compositions described herein may be formulated in aqueous solutions, in physiologically compatible buffers such as Hank's solution, Ringer's solution, physiological saline buffer, or other suitable solutions. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art. For other parenteral injections, appropriate formulations may include aqueous or nonaqueous solutions, preferably with physiologically compatible buffers or excipients. Such excipients are generally known in the art.

[00192] Parenteral injections may involve bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The pharmaceutical composition described herein may be in a form suitable for parenteral injection as a sterile suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

Transdermal Formulations

[00193] Transdermal formulations described herein may be administered using a variety of devices which have been described in the art. For example, such devices include, but are not limited to, U.S. Pat. Nos. 3,598,122, 3,598,123, 3,710,795, 3,731,683, 3,742,951, 3,814,097, 3,921,636, 3,972,995, 3,993,072, 3,993,073, 3,996,934, 4,031,894, 4,060,084, 4,069,307, 4,077,407, 4,201,211, 4,230,105, 4,292,299, 4,292,303, 5,336,168, 5,665,378, 5,837,280, 5,869,090, 6,923,983, 6,929,801 and 6,946,144, each of which is specifically incorporated by reference in its entirety.

[00194] The transdermal dosage forms described herein may incorporate certain pharmaceutically acceptable excipients which are conventional in the art. In one embodiments, the transdermal formulations described herein include at least three components: (1) a formulation as described herein; (2) a penetration enhancer; and (3) an aqueous adjuvant. In addition, transdermal formulations can include additional components such as, but not limited to, gelling agents, creams and ointment bases, and the like. In some embodiments, the transdermal formulation can further include a woven or non-woven backing material to enhance absorption and prevent the removal of the transdermal formulation from the skin. In other embodiments, the transdermal formulations described herein can maintain a saturated or supersaturated state to promote diffusion into the skin.

[00195] Formulations suitable for transdermal administration of AM preparations and purified compositions described herein may employ transdermal delivery devices and transdermal delivery patches and can be lipophilic emulsions or buffered, aqueous solutions, dissolved and/or dispersed in a polymer or an adhesive. Such patches may be constructed for continuous, pulsatile, or on demand delivery of pharmaceutical agents. Still further, transdermal delivery of the AM preparations and purified compositions described herein can be accomplished by means of iontophoretic patches and the like. Additionally, transdermal patches can provide controlled delivery of the composition. The rate of absorption can be slowed by using rate-controlling membranes or by trapping the compound within a polymer matrix or gel. Conversely, absorption enhancers can be used to increase absorption. An absorption enhancer or carrier can include absorbable pharmaceutically acceptable solvents to assist passage through the skin. For example, transdermal devices are in the form of a bandage comprising a backing member, a reservoir containing the compound optionally with carriers, optionally a rate controlling barrier to deliver the compound to the skin of the host at a controlled and predetermined rate over a prolonged period of time, and means to secure the device to the skin.

Solid Oral Dosage Forms

[00196] The pharmaceutical solid dosage forms described herein can include one or more pharmaceutically acceptable additives such as a compatible carrier, binder, filling agent, suspending agent, flavoring agent, sweetening agent, disintegrating agent, dispersing agent, surfactant, lubricant, colorant, diluent, solubilizer, moistening agent, plasticizer, stabilizer, penetration enhancer, wetting agent, anti-foaming agent, antioxidant, preservative, or one or more combination thereof. In still other aspects, using standard coating procedures, such as those described in *Remington's Pharmaceutical Sciences*, 20th Edition (2000).

[00197] Compressed tablets are solid dosage forms prepared by compacting the bulk blend of the formulations described herein. In various embodiments, compressed tablets which are designed to dissolve in the mouth will include one or more flavoring agents. In other embodiments, the compressed tablets will include a film surrounding the final compressed tablet. In some embodiments, the film coating can provide a delayed release of the composition from the formulation. In other embodiments, the film coating aids in patient compliance (e.g., Opadry® coatings or sugar coating). Film coatings including Opadry® typically range from about 1% to about 3% of the tablet weight. In other embodiments, the compressed tablets include one or more excipients.

[00198] A capsule may be prepared, for example, by placing the bulk blend of the formulation of the composition described herein, inside of a capsule. In some embodiments, the formulations (non-aqueous suspensions and solutions) are placed in a soft gelatin capsule. In other embodiments, the formulations are placed in standard gelatin capsules or non-gelatin capsules such as capsules comprising HPMC. In other embodiments, the formulation is placed in a sprinkle capsule, wherein the capsule may be swallowed whole or the capsule may be opened and the contents sprinkled on food prior to eating. In some embodiments, the therapeutic dose is split into multiple (e.g., two, three, or four) capsules. In some embodiments, the entire dose of the formulation is delivered in a capsule form.

[00199] In various embodiments, the particles of the composition and one or more excipients are dry blended and compressed into a mass, such as a tablet, having a hardness sufficient to provide a pharmaceutical composition that substantially disintegrates within less than about 30 minutes, less than about 35 minutes, less than about 40 minutes, less than about 45 minutes, less than about 50 minutes, less than about 55 minutes, or less than about 60 minutes, after oral administration, thereby releasing the formulation into the gastrointestinal fluid.

[00200] In another aspect, dosage forms may include microencapsulated formulations. In some embodiments, one or more other compatible materials are present in the microencapsulation material. Exemplary materials include,

but are not limited to, pH modifiers, erosion facilitators, anti-foaming agents, antioxidants, flavoring agents, and carrier materials such as binders, suspending agents, disintegration agents, filling agents, surfactants, solubilizers, stabilizers, lubricants, wetting agents, and diluents.

[00201] The pharmaceutical solid dosage forms including AM preparations and purified compositions described herein can be further formulated to provide a controlled release of the composition. Controlled release refers to the release of the composition from a dosage form in which it is incorporated according to a desired profile over an extended period of time. Controlled release profiles include, for example, sustained release, prolonged release, pulsatile release, and delayed release profiles. In contrast to immediate release compositions, controlled release compositions allow delivery of an agent to a subject over an extended period of time according to a predetermined profile. Such release rates can provide therapeutically effective levels of agent for an extended period of time and thereby provide a longer period of pharmacologic response while minimizing side effects as compared to conventional rapid release dosage forms. Such longer periods of response provide for many inherent benefits that are not achieved with the corresponding short acting, immediate release preparations.

[00202] In some embodiments, the solid dosage forms described herein can be formulated as enteric coated delayed release oral dosage forms, i.e., as an oral dosage form of a pharmaceutical composition as described herein which utilizes an enteric coating to affect release in the small intestine of the gastrointestinal tract. The enteric coated dosage form may be a compressed or molded or extruded tablet/mold (coated or uncoated) containing granules, powder, pellets, beads or particles of the active ingredient and/or other composition components, which are themselves coated or uncoated. The enteric coated oral dosage form may also be a capsule (coated or uncoated) containing pellets, beads or granules of the solid carrier or the composition, which are themselves coated or uncoated.

[00203] In other embodiments, the formulations described herein are delivered using a pulsatile dosage form. A pulsatile dosage form is capable of providing one or more immediate release pulses at predetermined time points after a controlled lag time or at specific sites. Pulsatile dosage forms may be administered using a variety of pulsatile formulations known in the art. For example, such formulations include, but are not limited to, those described in U.S. Pat. Nos. 5,011,692, 5,017,381, 5,229,135, and 5,840,329, each of which is specifically incorporated by reference. Other pulsatile release dosage forms suitable for use with the present formulations include, but are not limited to, for example, U.S. Pat. Nos. 4,871,549, 5,260,068, 5,260,069, 5,508,040, 5,567,441 and 5,837,284, all of which are specifically incorporated by reference.

[00204] Many other types of controlled release systems known to those of ordinary skill in the art and are suitable for use with the formulations described herein. Examples of such delivery systems include, e.g., polymer-based systems, such as polylactic and polyglycolic acid, plyanhydrides and polycaprolactone; porous matrices, nonpolymer-based systems that are lipids, including sterols, such as cholesterol, cholesterol esters and fatty acids, or neutral fats, such as mono-, di- and triglycerides; hydrogel release systems; silastic systems; peptide-based systems; wax coatings, bioerodible dosage forms, compressed tablets using conventional binders and the like. See, e.g., Liberman et al., *Pharmaceutical Dosage Forms*, 2 Ed., Vol. 1, pp. 209-214 (1990); Singh et al., *Encyclopedia of Pharmaceutical Technology*, 2nd Ed., pp. 751-753 (2002); U.S. Pat. Nos. 4,327,725, 4,624,848, 4,968,509, 5,461,140, 5,456,923, 5,516,527, 5,622,721, 5,686,105, 5,700,410, 5,977,175, 6,465,014 and 6,932,983, each of which is specifically incorporated by reference.

[00205] In some embodiments, pharmaceutical formulations are provided that include particles of the compositions described herein and at least one dispersing agent or suspending agent for administration to a subject.

The formulations may be a powder and/or granules for suspension, and upon admixture with water, a substantially uniform suspension is obtained.

[00206] The aqueous suspensions and dispersions described herein can remain in a homogenous state, as defined in The USP Pharmacists' Pharmacopeia (2005 edition, chapter 905), for at least 4 hours. The homogeneity should be determined by a sampling method consistent with regard to determining homogeneity of the entire composition. In one embodiment, an aqueous suspension can be re-suspended into a homogenous suspension by physical agitation lasting less than 1 minute. In another embodiment, an aqueous suspension can be re-suspended into a homogenous suspension by physical agitation lasting less than 45 seconds. In yet another embodiment, an aqueous suspension can be re-suspended into a homogenous suspension by physical agitation lasting less than 30 seconds. In still another embodiment, no agitation is necessary to maintain a homogeneous aqueous dispersion.

[00207] Suitable viscosity enhancing agents for the aqueous suspensions or dispersions described herein include, but are not limited to, methyl cellulose, xanthan gum, carboxymethyl cellulose, hydroxypropyl cellulose, hydroxypropylmethyl cellulose, Plasdon® S-630, carbomer, polyvinyl alcohol, alginates, acacia, chitosans and combinations thereof. The concentration of the viscosity enhancing agent will depend upon the agent selected and the viscosity desired.

[00208] In addition to the additives listed above, the liquid formulations can also include inert diluents commonly used in the art, such as water or other solvents, solubilizing agents, and emulsifiers. Exemplary emulsifiers are ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propyleneglycol, 1,3-butyleneglycol, dimethylformamide, sodium lauryl sulfate, sodium doccusate, cholesterol, cholesterol esters, taurocholic acid, phosphotidylcholine, oils, such as cottonseed oil, groundnut oil, corn germ oil, olive oil, castor oil, and sesame oil, glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols, fatty acid esters of sorbitan, or mixtures of these substances, and the like.

[00209] It is to be appreciated that there is overlap between the above-listed additives used in the aqueous dispersions or suspensions described herein, since a given additive is often classified differently by different practitioners in the field, or is commonly used for any of several different functions. Thus, the above-listed additives should be taken as merely exemplary, and not limiting, of the types of additives that can be included in formulations described herein. The amounts of such additives can be readily determined by one skilled in the art, according to the particular properties desired.

Intranasal Formulations

[00210] Intranasal formulations are known in the art and are described in, for example, U.S. Pat. Nos. 4,476,116, 5,116,817 and 6,391,452, each of which is specifically incorporated by reference. Formulations can be prepared according to these and other techniques well-known in the art are prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, fluorocarbons, and/or other solubilizing or dispersing agents known in the art. See, for example, Ansel, H. C. et al., Pharmaceutical Dosage Forms and Drug Delivery Systems, Sixth Ed. (1995). These compositions and formulations can be prepared with suitable nontoxic pharmaceutically acceptable ingredients. These ingredients are known to those skilled in the preparation of nasal dosage forms and some of these can be found in REMINGTON: THE SCIENCE AND PRACTICE OF PHARMACY, 21st edition, 2005, a standard reference in the field. The choice of suitable carriers is highly dependent upon the exact nature of the nasal dosage form desired, e.g., solutions, suspensions, ointments, or gels. Nasal dosage forms generally contain large amounts of water in addition to the active ingredient. Minor amounts of other ingredients such as pH adjusters, emulsifiers or dispersing agents, preservatives, surfactants, gelling agents, or buffering and other stabilizing and solubilizing agents may also be present.

[00211] For administration by inhalation, the compositions described herein may be in a form as an aerosol, a mist or a powder. Pharmaceutical compositions described herein are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, such as, by way of example only, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the AM preparations and purified compositions described herein and a suitable powder base such as lactose or starch.

Other Formulations

[00212] The AM preparations and purified compositions described herein may also be formulated in rectal compositions such as enemas, rectal gels, rectal foams, rectal aerosols, suppositories, jelly suppositories, or retention enemas, containing conventional suppository bases such as cocoa butter or other glycerides, as well as synthetic polymers such as polyvinylpyrrolidone, PEG, and the like. In suppository forms of the compositions, a low-melting wax such as, but not limited to, a mixture of fatty acid glycerides, optionally in combination with cocoa butter is first melted.

METHODS OF DOSING AND TREATMENT REGIMENS

[00213] The compositions can be administered by any suitable technique. Typically, the compositions will be administered directly to a target site (e.g., ocular surface, skin). The administration of formulations to the ocular surface is well known in the art. If delivery of AM preparations to the skin is desired, topical administration can be used. An injectable composition is also envisioned. Administration can also be parenteral (e.g., subcutaneous). Other methods of delivery, e.g., liposomal delivery, diffusion from a device impregnated with the composition, and microemulsion-based transdermal delivery in both cosmetic and pharmaceutical applications, are known in the art.

[00214] The compositions containing the AM preparations and purified compositions described herein can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, the compositions are administered to a patient already suffering from a disease or condition, in an amount sufficient to cure or at least partially arrest the symptoms of the disease or condition. Amounts effective for this use will depend on the severity and course of the disease or condition, previous therapy, the patient's health status, weight, and response to the drugs, and the judgment of the treating physician. It is considered well within the skill of the art for one to determine such therapeutically effective amounts by routine experimentation (including, but not limited to, a dose escalation clinical trial).

[00215] In prophylactic applications, compositions containing the AM preparations and purified compositions described herein are administered to a patient susceptible to or otherwise at risk of a particular disease, disorder or condition. Such an amount is defined to be a "prophylactically effective amount or dose." In this use, the precise amounts also depend on the patient's state of health, weight, and the like. It is considered well within the skill of the art for one to determine such prophylactically effective amounts by routine experimentation (*e.g.*, a dose escalation clinical trial). When used in a patient, effective amounts for this use will depend on the severity and course of the disease, disorder or condition, previous therapy, the patient's health status and response to the drugs, and the judgment of the treating physician.

[00216] In the case wherein the patient's condition does not improve, upon the doctor's discretion the administration of the compounds may be administered chronically, that is, for an extended period of time, including

throughout the duration of the patient's life in order to ameliorate or otherwise control or limit the symptoms of the patient's disease or condition.

[00217] In the case wherein the patient's status does improve, upon the doctor's discretion the administration of the compounds may be given continuously; alternatively, the dose of drug being administered may be temporarily reduced or temporarily suspended for a certain length of time (*i.e.*, a "drug holiday"). The length of the drug holiday can vary between 2 days and 1 year, including by way of example only, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 10 days, 12 days, 15 days, 20 days, 28 days, 35 days, 50 days, 70 days, 100 days, 120 days, 150 days, 180 days, 200 days, 250 days, 280 days, 300 days, 320 days, 350 days, or 365 days. The dose reduction during a drug holiday may be from 10%-100%, including, by way of example only, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%.

[00218] Once improvement of the patient's conditions has occurred, a maintenance dose is administered if necessary. Subsequently, the dosage or the frequency of administration, or both, can be reduced, as a function of the symptoms, to a level at which the improved disease, disorder or condition is retained. Patients can, however, require intermittent treatment on a long-term basis upon any recurrence of symptoms.

[00219] The amount of a given agent that will correspond to such an amount will vary depending upon factors such as the particular compound, disease or condition and its severity, the identity (*e.g.*, weight) of the subject or host in need of treatment, but can nevertheless be routinely determined in a manner known in the art according to the particular circumstances surrounding the case, including, *e.g.*, the specific agent being administered, the route of administration, the condition being treated, and the subject or host being treated. In general, however, doses employed for adult human treatment will typically be in the range of 0.02-5000 mg per day, preferably 1-1500 mg per day. The desired dose may conveniently be presented in a single dose or as divided doses administered simultaneously (or over a short period of time) or at appropriate intervals, for example as two, three, four or more sub-doses per day.

[00220] The pharmaceutical composition described herein may be in unit dosage forms suitable for single administration of precise dosages. In unit dosage form, the formulation is divided into unit doses containing appropriate quantities of one or more compound. The unit dosage may be in the form of a package containing discrete quantities of the formulation. Non-limiting examples are packaged tablets or capsules, and powders in vials or ampoules. Aqueous suspension compositions can be packaged in single-dose non-reclosable containers. Alternatively, multiple-dose reclosable containers can be used, in which case it is typical to include a preservative in the composition. By way of example only, formulations for parenteral injection may be presented in unit dosage form, which include, but are not limited to ampoules, or in multi-dose containers, with an added preservative.

[00221] The daily dosages appropriate for the AM preparations and purified compositions described herein are from about 0.01 to 2.5 mg/kg per body weight. An indicated daily dosage in the larger mammal, including, but not limited to, humans, is in the range from about 0.5 mg to about 100 mg, conveniently administered in divided doses, including, but not limited to, up to four times a day or in extended release form. Suitable unit dosage forms for oral administration include from about 1 to 50 mg active ingredient. The foregoing ranges are merely suggestive, as the number of variables in regard to an individual treatment regime is large, and considerable excursions from these recommended values are not uncommon. Such dosages may be altered depending on a number of variables, not limited to the activity of the compound used, the disease or condition to be treated, the mode of administration, the requirements of the individual subject, the severity of the disease or condition being treated, and the judgment of the practitioner.

[00222] Toxicity and therapeutic efficacy of such therapeutic regimens can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, including, but not limited to, the determination of the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between the toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio between LD_{50} and ED_{50}. Compounds exhibiting high therapeutic indices are preferred. The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with minimal toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized.

COMBINATION TREATMENTS

[00223] The compositions and methods described herein may also be used in conjunction with other well known therapeutic reagents that are selected for their particular usefulness against the condition that is being treated. In general, the compositions described herein and, in embodiments where combinational therapy is employed, other agents do not have to be administered in the same pharmaceutical composition, and may, because of different physical and chemical characteristics, have to be administered by different routes. The determination of the mode of administration and the advisability of administration, where possible, in the same pharmaceutical composition, is well within the knowledge of the skilled clinician. The initial administration can be made according to established protocols known in the art, and then, based upon the observed effects, the dosage, modes of administration and times of administration can be modified by the skilled clinician.

[00224] The particular choice of compounds used will depend upon the diagnosis of the attending physicians and their judgment of the condition of the patient and the appropriate treatment protocol. The compounds may be administered concurrently (e.g., simultaneously, essentially simultaneously or within the same treatment protocol) or sequentially, depending upon the nature of the disease, disorder, or condition, the condition of the patient, and the actual choice of compounds used. The determination of the order of administration, and the number of repetitions of administration of each therapeutic agent during a treatment protocol, is well within the knowledge of the skilled physician after evaluation of the disease being treated and the condition of the patient.

[00225] It is known to those of skill in the art that therapeutically-effective dosages can vary when the drugs are used in treatment combinations. Methods for experimentally determining therapeutically-effective dosages of drugs and other agents for use in combination treatment regimens are described in the literature. For example, the use of metronomic dosing, i.e., providing more frequent, lower doses in order to minimize toxic side effects, has been described extensively in the literature. Combination treatment further includes periodic treatments that start and stop at various times to assist with the clinical management of the patient.

[00226] For combination therapies described herein, dosages of the co-administered compounds will of course vary depending on the type of co-drug employed, on the specific drug employed, on the disease or condition being treated and so forth. In addition, when co-administered with one or more biologically active agents, the compound provided herein may be administered either simultaneously with the biologically active agent(s), or sequentially. If administered sequentially, the attending physician will decide on the appropriate sequence of administering protein in combination with the biologically active agent(s).

[00227] In any case, the multiple therapeutic agents may be administered in any order or even simultaneously. If simultaneously, the multiple therapeutic agents may be provided in a single, unified form, or in multiple forms (by way of example only, either as a single pill or as two separate pills). One of the therapeutic agents may be given in multiple doses, or both may be given as multiple doses. If not simultaneous, the timing between the multiple doses

may vary from more than zero weeks to less than four weeks. In addition, the combination methods, compositions and formulations are not to be limited to the use of only two agents; the use of multiple therapeutic combinations are also envisioned.

[00228] It is understood that the dosage regimen to treat, prevent, or ameliorate the condition(s) for which relief is sought, can be modified in accordance with a variety of factors. These factors include the disorder from which the subject suffers, as well as the age, weight, sex, diet, and medical condition of the subject. Thus, the dosage regimen actually employed can vary widely and therefore can deviate from the dosage regimens set forth herein.

[00229] The pharmaceutical agents which make up the combination therapy disclosed herein may be a combined dosage form or in separate dosage forms intended for substantially simultaneous administration. The pharmaceutical agents that make up the combination therapy may also be administered sequentially, with either therapeutic compound being administered by a regimen calling for two-step administration. The two-step administration regimen may call for sequential administration of the active agents or spaced-apart administration of the separate active agents. The time period between the multiple administration steps may range from, a few minutes to several hours, depending upon the properties of each pharmaceutical agent, such as potency, solubility, bioavailability, plasma half-life and kinetic profile of the pharmaceutical agent. Circadian variation of the target molecule concentration may also determine the optimal dose interval.

[00230] In addition, the AM preparations and purified compositions described herein also may be used in combination with procedures that may provide additional or synergistic benefit to the patient. By way of example only, patients are expected to find therapeutic and/or prophylactic benefit in the methods described herein, wherein pharmaceutical composition of a compound disclosed herein and /or combinations with other therapeutics are combined with genetic testing to determine whether that individual is a carrier of a mutant gene that is known to be correlated with certain diseases or conditions.

[00231] The AM preparations and purified compositions described herein and combination therapies can be administered before, during or after the occurrence of a disease or condition, and the timing of administering the composition containing a compound can vary. Thus, for example, the compounds can be used as a prophylactic and can be administered continuously to subjects with a propensity to develop conditions or diseases in order to prevent the occurrence of the disease or condition. The compounds and compositions can be administered to a subject during or as soon as possible after the onset of the symptoms. The administration of the compounds can be initiated within the first 48 hours of the onset of the symptoms, preferably within the first 48 hours of the onset of the symptoms, more preferably within the first 6 hours of the onset of the symptoms, and most preferably within 3 hours of the onset of the symptoms. The initial administration can be via any route practical, such as, for example, an intravenous injection, a bolus injection, infusion over 5 minutes to about 5 hours, a pill, a capsule, transdermal patch, buccal delivery, and the like, or combination thereof. A compound is preferably administered as soon as is practicable after the onset of a disease or condition is detected or suspected, and for a length of time necessary for the treatment of the disease, such as, for example, from about 1 month to about 3 months. The length of treatment can vary for each subject, and the length can be determined using the known criteria. For example, the compound or a formulation containing the compound can be administered for at least 2 weeks, preferably about 1 month to about 5 years, and more preferably from about 1 month to about 3 years.

KITS/ARTICLES OF MANUFACTURE

[00232] For use in the therapeutic applications described herein, kits and articles of manufacture are also described herein. Such kits can include a carrier, package, or container that is compartmentalized to receive one or more containers such as vials, tubes, and the like, each of the container(s) including one of the separate elements to

be used in a method described herein. Suitable containers include, for example, bottles, vials, syringes, and test tubes. The containers can be formed from a variety of materials such as glass or plastic.

[00233] The articles of manufacture provided herein contain packaging materials. Packaging materials for use in packaging pharmaceutical products are well known to those of skill in the art. See, e.g., U.S. Patent Nos. 5,323,907, 5,052,558 and 5,033,252. Examples of pharmaceutical packaging materials include, but are not limited to, blister packs, bottles, tubes, inhalers, pumps, bags, vials, containers, syringes, bottles, and any packaging material suitable for a selected formulation and intended mode of administration and treatment. A wide array of formulations of the compounds and compositions provided herein are contemplated as are a variety of treatments for any disease, disorder, or condition.

[00234] For example, the container(s) can include one or more AM preparations and purified compositions described herein, optionally in a composition or in combination with another agent as disclosed herein. The container(s) optionally have a sterile access port (for example the container can be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). Such kits optionally comprising a compound with an identifying description or label or instructions relating to its use in the methods described herein.

[00235] A kit will typically may include one or more additional containers, each with one or more of various materials (such as reagents, optionally in concentrated form, and/or devices) desirable from a commercial and user standpoint for use of the AM preparations and purified compositions described herein. Non-limiting examples of such materials include, but not limited to, buffers, diluents, filters, needles, syringes; carrier, package, container, vial and/or tube labels listing contents and/or instructions for use, and package inserts with instructions for use. A set of instructions will also typically be included.

[00236] A label can be on or associated with the container. A label can be on a container when letters, numbers or other characters forming the label are attached, molded or etched into the container itself; a label can be associated with a container when it is present within a receptacle or carrier that also holds the container, e.g., as a package insert. A label can be used to indicate that the contents are to be used for a specific therapeutic application. The label can also indicate directions for use of the contents, such as in the methods described herein.

[00237] In certain embodiments, the pharmaceutical compositions can be presented in a pack or dispenser device which can contain one or more unit dosage forms containing a compound provided herein. The pack can for example contain metal or plastic foil, such as a blister pack. The pack or dispenser device can be accompanied by instructions for administration. The pack or dispenser can also be accompanied with a notice associated with the container in form prescribed by a governmental agency regulating the manufacture, use, or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the drug for human or veterinary administration. Such notice, for example, can be the labeling approved by the U.S. Food and Drug Administration for prescription drugs, or the approved product insert. Compositions containing a compound provided herein formulated in a compatible pharmaceutical carrier can also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition.

METHODS OF TREATMENT

[00238] The AM preparations and purified compositions described herein have many uses including research and clinical applications. Based on the results described herein, the AM preparations and purified compositions described herein can be applied to tissues or cells to achieve a desired modulation of physiology. AM preparations and purified compositions described herein can further be added to cell cultures or tissue cultures to achieve a desired effect (as described herein).

AM Preparations and purified compositions described herein suppress TGF promoter activity

[00239] The anti-scarring, anti-inflammatory, and anti-angiogenic activities of AM preparations and purified compositions described herein is demonstrated by the suppression of TGF-β1 promoter activity as shown herein. The fetal portion of the frozen amniotic membrane has a significantly higher anti-scarring effect than that of fresh amniotic membrane; the placental portion of the frozen amniotic membrane also has a significantly higher anti-scarring effect than the fresh amniotic membrane (Example 1). Therefore, the frozen AM, either the placental or fetal portion, showed more potent suppressive effects in TGF- β than the fresh AM. This suppressive effect mediated by total AM extract obtained from frozen AM was dose-dependent over a range of 0.4 to 125 μg/ml. Furthermore, such a suppressive effect could not be substituted by high MW HA alone (exceeding 100X of equivalent AM extract), and was lost after digestion with hyaluronidase, suggesting that it was mediated by a complex between HA-IαI. Centrifugation at low or high speed did not affect the suppressive effect significantly. However, subsequent lyophilization and reconstitution produced a more potent suppressive effect. Additionally, the overall suppressive effect of AM was more potent than that of AM jelly.

[00240] TGF-β is the prototypic cytokine that is involved in tissue inflammation, in addition to wound healing and scar formation. See Border, et al., J. Clin. Invest., 90:1-7 (1992); Grande, Proc. Soc. Exp. Biol. Med., 214:27-40 (1997); Jester, et al., Prog. Retin. Eye Res., 18(3):311-356 (1999); and Marek, et al., Med. Sci. Monit., 8(7):RA145-151 (2002). Mammalian cells express three different TGF-βs: TGF-β1, TGF-β2, and TGF-β3. TGF-β is the most potent cytokine promoting myofibroblast differentiation by up-regulating expression of α-SMA, integrin α5β1, and EDA domain-containing fibronectin (Fn) in a number of cell types, including fibroblasts. See Tseng, et al., Ocular Surface J., 2(3):177-187 (2004); Ronnov-Jessen, et al., Lab. Invest., 68:696-707 (1993); Verbeek, et al., Am. J. Pathol., 144:372-82 (1994); Hales, et al., Curr. Eye Res., 13:885-90 (1994); Jester, et al., Cornea, 15:505-16 (1996); Serini, et al., J. Cell. Biol., 142:873-81 (1998); Grande, Proc. Soc. Exp. Biol. Med., 214(1):27-40 (1997); and Jester, et al., Prog. Retin. Eye. Res., 18:311-56 (1999). TGF-β also up-regulates the expression of such matrix components as collagens and proteoglycans, down-regulates proteinase and matrix metalloproteinases, and up-regulates their inhibitors. Collectively, these actions result in increased cell-matrix interactions and adhesiveness, as well as deposition and formation of scar tissue. See Tseng, et al., Ocular Surface J., 2(3):177-187 (2004); Grande, Proc. Soc. Exp. Biol. Med., 214(1):27-40 (1997); Jester, et al., Prog. Retin. Eye. Res., 18:311-56 (1999); and Lawrence, Eur. Cytokine Netw., 7:363-74 (1996).

[00241] TGF-βs exert their actions via binding with TGF-beta receptors (TGF-βRs) on the cell membrane. In human cells, there are three TGF-βRs, namely TGF-βR type I (TGF-βRI), type II (TGF-βRII), and type III (TGF-βRIII). TGF-βs, serving as ligands, bind with a serine, threonine kinase receptor complex made of TGF-βRI and TGF-βRII; such a binding is facilitated by TGF-βRIII, which is not a serine, threonine kinase receptor. See Tseng, et al., Ocular Surface J., 2(3):177-187 (2004); and Massague, et al., Genes and Development., 14:627-44 (2000). Binding with TGF-βRII activates TGF-βRI, which is responsible for direct phosphorylation of a family of effector proteins known as Smads, which modulate transcription of a number of target genes, including those described herein, participating in scar formation. See Tseng, et al., Ocular Surface J., 2(3):177-187 (2004); Massague, et al., Genes and Development., 14:627-44 (2000); and Derynck, et al., Biochem. Biophys. Acta., 1333:F105-F150 (1997).

[00242] Suppression of TGF-β can be achieved by neutralizing antibodies to TGF-β and agents that intercede the signaling mediated by TGF-β such as decorin. See Shahi, et al., Lancet, 339:213-214 (1992); Petroll, et al., Curr. Eye Res., 1739:736-747 (1998); Yamaguchi, et al., Nature, 346(6281):281-284 (1990); and Logan, et al., Exp. Neurol., 159:504-510 (1999). Most of the literature has shown suppression of TGF-β being achieved at the level of modulating the TGF-β activation, binding with its receptor, or its signal transduction. It has been shown that

amniotic membrane can achieve such an inhibition at the level of transcription, i.e., to turn off transcription of TGF-β genes. In particular, amniotic membrane has been shown to suppress TGF- β signaling in human corneal and limbal fibroblasts, and human conjunctival and pterygium body fibroblasts. See Tseng, et al., J Cell Physiol., 179:325-335 (1999); and Lee, et al., Curr. Eye Res., 20(4):325-334 (2000).

[00243] Application of the AM preparations and purified compositions described herein can be used to lower the production or activity of TGF-β. Several types of AM compositions, such as AME (total human AM extract), the AME supernatant after centrifugation, AM jelly, and AM stroma were prepared as detailed in Example 1. The effect of various buffers, such as PBS, low salt buffer, high salt buffer, and guanidine HCl on TGF-β activity was examined. Additionally, the effect of various freeze-grinding procedures on TGF-β activity was examined.

[00244] The suppression of TGF-β activity was lost after hyaluronidase digestion, demonstrating that the suppressive effect may be mediated by a HA-related complex (Fig. 3). The suppressive effect was not recovered by addition of HA. The centrifugation step did not alter the suppression of TGF-β activity, in either AME or AM jelly extracts (Fig. 5). Lyophilization enhanced the suppression of TGF-β activity of both AME and jelly extract (Fig. 6). Fig. 8 and Fig. 9 demonstrate that collagen and HA, when added to AME, can enhance the suppression of TGF-β activity. Accordingly, addition of collagen and HA to the AM-based compositions may be useful to treat various diseases involving TGF-β.

[00245] As shown herein, TGF-β is downregulated by the disclosed compositions. Accordingly, the compositions described herein can be used to treat diseases related to TGF-β is downregulation, such as angiogenesis, wound healing, and tissue inflammation.

AM preparations and purified compositions described herein can prevent apoptosis

[00246] The AM preparations and purified compositions described herein can be used to prevent, lessen, or treat apoptosis in tissues. In some embodiments, the AM preparations and purified compositions described herein can decrease or prevent apoptosis in tissues that have been injured. This anti-apoptotic effect demonstrates that the compositions can be used to prolong the life of organs being stored prior to transplant. The compositions can also be used to treat or prevent damage during and after surgical procedures. Example 3 demonstrates the anti-apoptotic effect of AM extract using a murine model of eye damage. Mouse eyes were collected and damaged either by enzymatic treatment or by mechanical injury, AM extract was administered, and the effect on cellular damage was determined, using an assay that measures apoptotic damage to the nucleus. Incubation with AM extract was found to decrease the levels of apoptosis.

[00247] Because of the anti-apoptotic effects exerted by AM preparation, AM preparations and compositions are expected to be useful for preserving tissues (e.g., cornea) before transplantation. The addition of AM preparations to tissues that are being stored can be helpful in lessening cellular damage due to the storage process. For example, the AM preparations and purified compositions described herein can be used to decrease the amount of degradation that occurs in a tissue that is being stored prior to transplantation or surgical procedures. The AM preparations and purified compositions described herein can be added to the storage medium, with or without collagen and/or HA. Stored tissues such as eyes, organs, skin, and the like can benefit from the decreased cellular apoptosis that occurs when an AM composition is added.

[00248] Once a donor tissue is harvested, it is typically stored in a storage medium until transplantation. The compositions can be added to the storage medium to prevent cellular apoptosis. For example, the compositions can be added to storage media for preserving limbal epithelial stem cells. Similarly, AM preparation-containing compositions can be added to cell culture medium or digestion medium to prevent cellular (e.g., keratocyte) apoptosis. Because studies described herein show that incubation of AM preparation during dispase digestion (a

treatment which mimics surgical and pathological insults such as excimer ablation in PRK and recurrent corneal erosion, respectively) significantly reduced apoptosis of both epithelial cells and keratocytes, the compositions can also be administered to an eye receiving mechanical scraping or excimer laser photoablation to attempt to reduce keratocyte apoptosis, and hence reduce corneal haze. As another example, AM preparation-containing formulations can also be used in surgical conditions or diseases such as recurrent corneal erosion or keratoconus where the basement membrane is dissolved to reduce the keratocyte apoptosis.

The AM preparations and purified compositions described herein can prevent or reverse scar formation and can be used to assist in wound healing

[00249] In adult humans and other mammalian vertebrates, wound healing in tissue such as skin is generally a reparative process, in contrast to a regenerative process which appears to take place in healing of fetal and embryonic tissue. The outcome of a wound repair process appears to be influenced by a number of different factors, including both intrinsic parameters, e.g. tissue oxygenation; and extrinsic parameters, e.g. wound dressings. There is, however, considerable evidence indicating that the overall process of healing and repair of wound damaged tissue, including the necessary cellular communication, is regulated in a coordinate manner in adult humans and other mammals by a number of specific soluble growth factors which are released within the wound environment and which, among other things, appear to induce neovascularization, leukocyte chemotaxis, fibroblast proliferation, migration, and deposition of collagen and other extra-cellular matrix molecules within the wounds. Such growth factors that have been identified and isolated are generally specialized soluble proteins or polypeptides and include transforming growth factor alpha (TGF-α), transforming growth factor beta (TGF-β1, TGF-β2, TGF-β3, etc.), platelet derived growth factor (PDGF), epidermal growth factor (EGF), insulin-like growth factors I and II (IGFI and IGFII) and acidic and basic fibroblast growth factors (acidic FGF and basic FGF).

[00250] Myofibroblasts are phenotypically intermediate between smooth muscle cells and fibroblasts. Myofibroblasts play an important role in morphogenesis and oncogenesis, inflammation, wound healing, and fibrosis in most organs and tissues. During normal wound healing, fibroblasts migrate to the wound area and differentiate into myofibroblasts under the influence of growth factors such as TGF-β1 and the mechanical stress developed within a given tissue. Normally, myofibroblasts gradually disappear by apoptosis in the resolution phase. However, under certain pathological situations, myofibroblasts persist and continue to remodel the extracellular matrix, resulting in scar formation. Thus, the ability to control myofibroblast differentiation can be useful to prevent scar formation in various tissues during wound healing. The AM preparations and purified compositions described herein are able to both prevent and reverse scar formation, and can thus be useful for treatment of any diseases where scar formation can occur.

[00251] In some embodiments, the AM preparations and purified compositions described herein can decrease or prevent the formation of scar tissue. This effect is demonstrated in Example 4. AMSCs were cultured on either plastic, collagen, or AM tissue surface material. The AMSCs cultured on plastic rapidly differentiated to myofibroblasts *in vitro*. However, these differentiated myofibroblasts could be reversed to AMSCs when subcultured on AM stromal matrix (Fig. 23). Reversal of myofibroblast differentiation could also be obtained when amniotic stromal extract was added to differentiated myofibroblasts (Fig. 25). Further, AM stromal extract was also found to prevent myofibroblast differentiation of AMSCs (Fig. 26).

[00252] Accordingly, the AM preparations and purified compositions described herein can be used to prevent or reverse scar formation that is caused by any means. The compositions can be administered to treat wrinkles, stretch marks, surgical scars, wound scars, scars from burns or mechanical injuries, and the like.

[00253] The AM preparations and purified compositions described herein can be used to treat or prevent scar formation due to wounds. Wounds are internal and external bodily injuries or lesions caused by physical means, such mechanical, chemical, viral, bacterial, fungal and other pathogenic organisms, or thermal means, which disrupt the normal continuity of tissue structures. Wounds may be caused by accident, surgery, pathological organisms, or by surgical procedures.

[00254] Additionally, the AM preparations and purified compositions described herein can suppress fibroblast migration. As shown in Example 5 and Figs. 27 through 29, AME was found to suppress migration of fibroblasts. Human limbal explants (HLE) were cultured in SHEM or SHEM supplemented with AME to study the biological activity of AME. AME (at 25 μg/ml) delayed the onset of epithelial outgrowth from the limbal explant. AME suppressed migration of explant fibroblasts from the stroma, resulting in an outgrowing epithelial sheet with much less fibroblasts. Furthermore, the epithelial sheet expanded in SHEM containing AME had a smooth edge, a phenomenon resembling when HLE was cultured in SHEM with 10 μM SB203580 - a MAPK p38 inhibitor. Histological sections of the remaining explant after outgrowth revealed that without AME, an increase in dissolution of the stromal matrix was evident. Thus, AME may be able to inhibit fibroblast migration by preventing stromal matrix lysis. These discoveries indicate that AME can be used for developing new products to modify wound healing in the direction promoting expansion of human corneal stem cells and against inflammation, scarring, and angiogenesis.

[00255] The AM preparations and purified compositions described herein can be used during or after surgery, to improve healing and to decrease the amount of tissue damage from mechanical insults to the tissue. The applicable use of the compositions and methods described herein is widespread and includes, but is not limited to all types of surgery, such as plastic, spinal cord, or caesarian section; disease, such as cancer, congestive heart failure, and kidney disease; and conditions as a result of burns, acne, or other injuries. The methods described herein can be used by physicians in reconstructive or plastic surgeries. The AM preparations and purified compositions described herein can also be applied topically on the body surface or tissues to achieve short-term and long-term therapeutic effects.

[00256] The AM preparations and purified compositions described herein can be used to treat or prevent damage due to eye disease. Types of eye diseases that can be treated by administering the AM preparations and purified compositions described herein include but are not limited to dry eye, corneal injury, corneal ulcer, Sjogren's syndrome, damage from contact lenses, fungal infection, viral infection, or bacterial infection, mechanical injury, surgical damage, burn damage, conjunctival inflammation, ocular pemphigoid, Stevens-Johnson syndrome,chemical injury, and the like.

[00257] The AM preparations and purified compositions described herein can also be used to treat epidermal diseases. Types of epidermal layer diseases that can be treated by administering the AM preparations and purified compositions described herein include but are not limited to fungal diseases, viral diseases, bacterial diseases, rash, eczema, psoriasis, ichthyosis, epidermalytic hyperkeratosis, and the like.

Treatment and Prevention of Angiogenesis-related Diseases

[00258] As described herein, the anti-angiogenic activity of the AM preparations and purified compositions described herein was demonstrated using soluble protein extracts prepared from the avascular stroma of cryopreserved AM. The AM stromal extract (ASE) was found to have a potent anti-angiogenic action on cultured human umbilical vein endothelial cells (HUVECs) by inhibiting proliferation, inducing apoptosis, attenuating migration, and inhibiting tube formation. Example 8 demonstrates that an amniotic membrane stromal extract (ASE) has anti-angiogenic properties. ASE was found to inhibit HUVEC cell proliferation (Fig. 34). ASE was also found to

induce apoptosis in HUVEC cells (Fig. 35). ASE The inhibitory effect of ASE on VEGF-induced cell migration was demonstrated using a transwell assay (Fig. 37). ASE was further found to inhibit tube formation of HUVEC cells (Fig. 38).

[00259] Taken together, these results demonstrate that the AM preparations and purified compositions described herein can be useful for treatment of angiogenesis-related diseases. Exemplary angiogenesis-related diseases that can be treated include but are not limited to tumor growth, cancer, and of ophthalmological conditions such as corneal graft rejection, ocular neovascularization, retinal neovascularization including neovascularization following injury or infection, diabetic retinopathy, macular degeneration, retrolental fibroplasia, neovascular glaucoma, retinal ischemia, vitreous hemorrhage, and the like.

[00260] Exemplary cancer types that can be treated using the AM preparations and purified compositions described herein include but are not limited to Acute Lymphoblastic Leukemia, Acute Myeloid Leukemia, Adrenocortical Carcinoma, AIDS-Related Cancers, AIDS-Related Lymphoma, Anal Cancer, Astrocytoma, Basal Cell Carcinoma, Bile Duct Cancer, Bladder Cancer, Bladder Cancer, Bone Cancer, Brain Stem Glioma, Brain Tumor, Breast Cancer , Bronchial Adenomas, Burkitt's Lymphoma, Carcinoid Tumor, Carcinoma, Central Nervous System Lymphoma, Cerebellar Astrocytoma, Cervical Cancer, Chronic Lymphocytic Leukemia, Chronic Myelogenous Leukemia, Chronic Myeloproliferative Disorders, Colon Cancer, Colorectal Cancer, Cutaneous T-Cell Lymphoma, Endometrial Cancer, Ependymoma, Esophageal Cancer, Extragonadal Germ Cell Tumor, Eye Cancer, Intraocular Melanoma, Eye Cancer, Retinoblastoma, Gallbladder Cancer, Gastrointestinal Carcinoid Tumor, Gastrointestinal Stromal Tumor (GIST), Germ Cell Tumor (Extracranial), Germ Cell Tumor (Extragonadal), Germ Cell Tumor (Ovarian), Gestational Trophoblastic Tumor, Glioma, Hairy Cell Leukemia, Head and Neck Cancer, Hepatocellular (Liver) Cancer, Hodgkin's Lymphoma, Hypopharyngeal Cancer, Hypothalamic and Visual Pathway Glioma, Intraocular Melanoma, Islet Cell Carcinoma (Endocrine Pancreas), Kaposi's Sarcoma, Kidney (Renal Cell) Cancer, Laryngeal Cancer, Leukemia (Acute Lymphoblastic), Leukemia (Acute Myeloid), Leukemia (Chronic Lymphocytic), Leukemia (Chronic Myelogenous), Lip and Oral Cavity Cancer, Liver Cancer, Lung Cancer (Non-Small Cell), Lung Cancer (Small Cell), Lymphoma, (Cutaneous T-Cell), Lymphoma (Non-Hodgkin's), Malignant Fibrous Histiocytoma of Bone/Osteosarcoma, Medulloblastoma, Melanoma, Merkel Cell Carcinoma, Mesothelioma, Metastatic Squamous Neck Cancer with Occult Primary, Multiple Endocrine Neoplasia Syndrome, Multiple Myeloma/Plasma Cell Neoplasm, Mycosis Fungoides, Myelodysplastic Syndromes, Myelodysplastic/Myeloproliferative Diseases, Myelogenous Leukemia, Myeloid Leukemia, Myeloproliferative Disorders, Nasal Cavity and Paranasal Sinus Cancer, Nasopharyngeal Cancer, Neuroblastoma, Oral Cancer, Oropharyngeal Cancer, Osteosarcoma/Malignant Fibrous Histiocytoma of Bone, Ovarian Cancer, Ovarian Epithelial Cancer, Ovarian Germ Cell Tumor, Ovarian Low Malignant Potential Tumor, Pancreatic Cancer, Parathyroid Cancer, Penile Cancer, Pheochromocytoma, Pineoblastoma and Supratentorial Primitive Neuroectodermal Tumors, Pituitary Tumor, Plasma Cell Neoplasm/Multiple Myeloma, Pleuropulmonary Blastoma, Prostate Cancer, Rectal Cancer, Retinoblastoma, Rhabdomyosarcoma, Salivary Gland Cancer, Sarcoma (Kaposi's), Sarcoma (uterine), Sezary Syndrome, Skin Cancer (non-Melanoma), Skin Cancer (Melanoma), Skin Carcinoma (Merkel Cell), Small Intestine Cancer, Soft Tissue Sarcoma, Squamous Cell Carcinoma, Stomach (Gastric) Cancer, T-Cell Lymphoma, Testicular Cancer, Thymoma, Thyroid Cancer, Trophoblastic Tumor, Gestational, Urethral Cancer, Uterine Cancer, Endometrial, Uterine Sarcoma, Vaginal Cancer, Visual Pathway and Hypothalamic Glioma, Vulvar Cancer, Waldenström's Macroglobulinemia, Wilms' Tumor, and the like.

Treatment of Inflammation

[00261] The AM preparations and purified compositions described herein can be used to decrease inflammation. The AM contains many factors that may contribute/mediate its anti-inflammatory ability, such as interleukin-10 (IL-10), members of the transforming growth factor-beta (TGF-β) superfamily, protease inhibitors, and IL-1 receptor antagonist (IL-1RA). IL-10 is known to suppress and counteract pro-inflammatory cytokines, such as IL-6, TNFα, and IL-8. See Foutunato, et al., Am. J. Obstet. Gynecol.,175:1057-65 (1996); Foutunato, et al., Am. J. Obstet. Gynecol.,177:803-9 (1997); and Foutunato, et al., Am. J. Obstet. Gynecol.,179:794-9 (1998). Activin and inhibin, which are members of the TGF-β superfamily, are produced by the AM. Varying doses of activin give rise to different results. At low doses of activin, production of IL-6, IL-8, and prostaglandin E2 (PGE2) is stimulated, but at high doses, it inhibits production. See Petraglia, et al., J. Clin. Endocrinol. Metab., 77:542-8 (1993); Riley, et al., Hum. Reprod. 15:578-83 (2000); and Keelan, et al., Placenta, 31:38-43 (2000). The AM also contains protease inhibitors such as α1 anti-trypsin, which can exert an anti-inflammatory effect. See Na, et al., Trophoblast Res., 13:459-66 (1999). IL-1RA is an inhibitor of IL-1 and therefore, suppresses the inflammation mediated by IL-1. See Romero, et al., Am. J. Obstet. Gynecol., 171:912-21 (1994). The inventors have demonstrated that AM has down-regulates the expression and production of IL-1 and up-regulates IL-1RA. See Tseng, et al., Ocular Surface J., 2(3):177-187 (2004).

[00262] Activated macrophages play an important role in initiating, maintaining and resolving host inflammatory responses. In addition to killing viruses, bacteria and parasites, and to act as scavenger cells, macrophages also exert deleterious effects on the host by producing excessive free radicals, lytic enzymes and inflammatory cytokines, collectively aggravating tissue damage and being responsible for many of the systemic symptoms associated with acute and chronic inflammation.

[00263] The AM preparations and purified compositions described herein can suppress macrophages leading to inflammatory effects, as shown in Example 6 and Figures 30-33. The mouse macrophage cell line, Raw 264.7, was used as a model to test the anti-inflammatory action with or without activation of IFNγ. Cryopreserved amniotic membrane (AM) suppressed the production of NO by Raw264.7 cells, especially under the stimulation of IFNγ. Furthermore, production of PGD2 was favored more so than PGE2 when cells were cultured on AM as compared to cells cultured on plastic. The ratio between PGD2 and PGE2, an index of anti-inflammatory action, was significantly higher in cells cultured on AM compared to those that were cultured on plastic.

[00264] The above anti-inflammatory action was correlated with the suppression of TGF-β signaling as the suppression of TGF-β1 promoter activity in Raw264.7 was significantly higher when cultured on AM than on cultured on plastic. Further, addition of 125 μg/ml of AME induced cell death of Raw264.7 cells. Together, these results demonstrate that the AM preparations and purified compositions described herein can suppress macrophages leading to anti-inflammatory actions.

[00265] Accordingly, the AM preparations and purified compositions described herein can be used to treat diseases related to tissue inflammation. Inflammatory disorders are typically characterized by one or more of the signs of pain, (dolor, from the generation of noxious substances and the stimulation of nerves), heat (calor, from vasodilatation), redness (rubor, from vasodilatation and increased blood flow), swelling (tumor, from excessive inflow or restricted outflow of fluid), and loss of function (functio laesa, which may be partial or complete, temporary or permanent). Inflammation takes many forms and includes, but is not limited to, inflammation that is one or more of the following: acute, adhesive, atrophic, catarrhal, chronic, cirrhotic, diffuse, disseminated, exudative, fibrinous, fibrosing, focal, granulomatous, hyperplastic, hypertrophic, interstitial, metastatic, necrotic,

obliterative, parenchymatous, plastic, productive, proliferous, pseudomembranous, purulent, sclerosing, seroplastic, serous, simple, specific, subacute, suppurative, toxic, traumatic, ulcerative, and the like.

[00266] Exemplary inflammatory disorders that can be treated by the AM preparations and purified compositions described herein include but are not limited to arthritis (including rheumatoid arthritis, spondyloarthopathies, gouty arthritis, degenerative joint diseases (i.e. osteoarthritis), systemic lupus erythematosus, Sjogren's syndrome, ankylosing spondylitis, undifferentiated spondylitis, Behcet's disease, haemolytic autoimmune anaemias, multiple sclerosis, amyotrophic lateral sclerosis, amylosis, acute painful shoulder, psoriatic, and juvenile arthritis), asthma, atherosclerosis, osteoporosis, bronchitis, tendonitis, bursitis, skin inflammation disorders (i.e. psoriasis, eczema, burns, dermatitis), enuresis, eosinophilic disease, gastrointestinal disorders (including inflammatory bowel disease, peptic ulcers, regional enteritis, diverticulitis, gastrointestinal bleeding, Crohn's disease, gastritis, diarrhoea, irritable bowel syndrome and ulcerative colitis), gastroesophageal reflux disease, eosinophilic esophagitis, gastroparesis such as diabetic gastroparesis; food intolerances and food allergies and other functional bowel disorders, such as non-ulcerative dyspepsia, non-cardiac chest pain, and the like.

[00267] The AM preparations and purified compositions described herein can also be used to treat, for example, inflammation associated with: vascular diseases, migraine headaches, tension headaches, periarteritis nodosa, thyroiditis, aplastic anemia, Hodgkin's disease, scierodoma, rheumatic fever, type I diabetes, myasthenia gravis, sarcoidosis, nephrotic syndrome, Behcet's syndrome, polymyositis, gingivitis, hypersensitivity, conjunctivitis, multiple sclerosis, and ischemia (e.g., myocardial ischemia), and the like. The compounds may be useful for treating neuroinflammation associated with brain disorders (e.g., Parkinson's disease and Alzheimer's disease) and chronic inflammation associated with cranial radiation injury. The compounds may be useful for treating acute inflammatory conditions (such as those resulting from infection) and chronic inflammatory conditions (such as those resulting from asthma, arthritis and inflammatory bowel disease). The compounds may also be useful in treating inflammation associated with trauma and non-inflammatory myalgia.

[00268] The compositions and methods described herein are provided further detail in the following examples. These examples are provided by way of illustration and are not intended to limit the invention in any way.

EXAMPLES

Example 1: Suppressive Activities of Various Amniotic Membrane Preparations

Hyaluronidase Digestion

[00269] AM total water-soluble extracts (AME) prepared from frozen AM were mixed with or without 10 units/ml hyaluronidase (Sigma #H1136) in the reaction buffer (50 mM HEPES, pH7.5, 0.1 M NaCl, 1% Triton X-100, 0.1 % BSA supplemented with the above protease and phosphatase inhibitors for 2 hours at 37 °C using a positive control of high MW HA (cat# H1876, Sigma) purified from human umbilical cords.

Cell Culture and TGF-β1 Promoter Suppression

[00270] When human corneal fibroblasts cultured on 100 mm plastic dish in DMEM/10% FBS reached 80 % confluency (~1.0 x 10^6 cells), cells were washed twice with DMEM/10% FBS. Adenoviruses-TGF- β1 promoter-luciferase (MOI= 37.5) and Adeno-CMV-beta-gal (MOI=30) were added to the culture plates with 10 ml of the fresh DMEM/10% FBS and cells were incubated at 37 °C for 4 hours, and trypsinized for 5 minutes using 4 ml prewarmed trypsin/EDTA. After trypsin/EDTA activity was neutralized with 8 ml of DMEM/10% FBS, cells were collected into a 15 ml tube and centrifuged at 1,500 rpm (~600 x g) for 5 min. After decanting the medium, cells were resuspended in 15 ml DMEM/10% FBS, and cell viability was measured by trypan blue stain. Viable $3x10^4$

cells were seeded on a plastic 24 well or on the stromal surface of AM inserts. A total of 4 wells or inserts were prepared. Cells were then incubated at 37°C in a CO_2 incubator for 48 hours.

[00271] After carefully removing the growth medium from each well, cells were rinsed with 0.5 ml PBS at lease twice, taking care not to dislodge attached cells. After removing as much as PBS in the well, 100 µl 1x lysis buffer was added to cover the cells, and cells were mechanically scraped and transferred to a microcentrifuge tube placed on ice. Cell lysates were collected by vortexing for 10-15 sec and centrifuging at 12,000 x g for 15 sec at room temperature. The supernatant designated as cell lysate was stored at -80°C prior to assaying for luciferase activities.

Suppression of TGF-β1 Promoter Activity by Different AM Extracts

[00272] In Fig. 1, compared to the plastic control (PL), both the placental portion and the fetal portion of frozen amniotic membrane (FRO/P and FRO/F, respectively) showed significant suppression of TGF-β1 promoter activity (each $P<0.01$). For the fresh placenta, the placental portion of amniotic membrane (FRE/P) also exhibited a significant suppression of TGF-β1 promoter activity ($P<0.05$). Nevertheless, the fetal portion of the fresh amniotic membrane (FRE/F) did not show any suppressive effect ($P=0.5$). These results indicated that the fetal portion of the fresh amniotic membrane does not have the same anti-scarring effect as the frozen counterpart. For the frozen amniotic membrane, the suppressive effect by the placental portion (FRO/P) was not significantly different from that by the fetal portion ($P=0.3$). For the fresh amniotic membrane, the suppressive effect by the fetal portion (FRE/F) was not significantly from the placental portion (FRE/P) ($P=0.1$). For the placental portion, the suppressive effect by the frozen amniotic membrane (FRO/P) was significantly better than the fresh amniotic membrane (FRE/P) ($P<0.05$). In the fetal portion, however, the suppressive effect by the frozen amniotic membrane (FRO/F) was not significantly different than the suppressive effect of the fresh amniotic membrane (FRE/F) ($P=0.1$).

Suppression of TGF-β1 Promoter Activity is Dose-dependent and Lost after Digestion with Hyaluronidase

[00273] The suppression of TGF-β1 promoter activity by total water-soluble AM extracts prepared from frozen AM obeyed a dose-responsive curve from 0.04 to 125 µg/ml (Fig. 2). As shown by the promoter activity of TGF-β1 and TGF-βRII, the suppressive effect of 25 µg/ml of total water-soluble AM extracts prepared from frozen AM was lost when pre-treated with hyaluronidase, indicating that such a suppressive effect was mediated by an HA-related complex (Fig. 3). It should be noted that 25 µg/ml AM extracts contained less than 0.78 µg/ml HA.

Lost suppressive effect from hyaluronidase cannot be recovered by addition of HA

[00274] Although 100 µg/ml high MW HA alone showed a mild suppressive activity, its magnitude was still significantly less than 25 µg/ml AM extracts. Taken together, these data suggest that the suppressive effect of AM extracts was mediated by HA-linked complex, i.e., HA-IαI complex.

Soluble AME and Jelly Extracts Derived after Centrifugation Do Not Change the Suppressive effect on TGF-β1 Promoter Activities

[00275] Compared to the PBS control, HA, AM (Total, Low Speed, High speed) and Jelly (Total, Low Speed, High Speed) showed suppression of TGF-β1 promoter activation when normalized with beta-galatosidase activity. P value indicated there was not statistically significant due to the variation among the control group (data not shown). By comparing total AME and two conditions of centrifuged soluble AME, results suggested that there was no significant difference. However, without centrifugation of AME showed less suppression compare with low or high soluble AME. Likewise, Jelly/T indicated less TGF-β suppression activities in comparison with Jelly/HS (Fig. 5).

Lyophilization Enhanced the Suppressive Effect of AME and Jelly Extract

[00276] Human corneal fibroblasts showed no change in cell morphology in the control, HA alone, and low concentrations of AME or jelly extracts (Data not shown). However, cells showed a marked change to slender and

small cells after the treatment with high concentration of AME and L/AME, as early as 18 hrs after seeding (Fig. 6). Furthermore, the cell density also decreased. The above changes were even more dramatic in lyophilized AME or L/AME than their non-lyophilized counterparts in AME or Jelly extracts, respectively (Fig. 6).

[00277] To examine whether the TGF-beta promoter was suppressed during AME treatment, luciferase assays were performed. Beta-galatosidase assay was used as the transfection control. The result indicated that AME-125, L-AME-25, L-AME-125, L-Jelly-125 showed a significant differences in inhibiting TGF-beta 1 promoter activities, the percentage of inhibitions were 86%($P<0.01$), 55% ($P<0.1$), 95% ($P<0.01$), and 46% ($P<0.1$), respectively (Fig. 7). The data suggests that lyophilized form of AME or Jelly at a high concentration of 125 μg/ml was more effective than the non-lyophilized AME form. Although the lowest concentration of commercial HA (4 μg/ml) was close to the concentration HA (3.8 μg/ml) in AME/125, the effectiveness suppression in AME/125 is far more potent than HA. (Data not shown) Furthermore, the AME form overall illustrated a better TGF-beta suppression than the Jelly form.

Suppression of TGF-β1 Promoter Activity by AM Extracts Mixed With Collagen Gel or HA

[00278] A mixture of native type 1 collagen gel and water-soluble AM extract was then prepared. To prepare this mixture, collagen gel was first prepared by diluting a 4mg/ml stock collagen solution prepared from rat tail tendon (BD Biosciences, San Jose, CA) with 0.1 N acetic acid and mixing it with a 1/20 volume ratio of 20X DMEM and 1 N NaOH. A collagen gel formed after incubation at 37° C. Next, water-soluble AM extract (prepared as described herein) was diluted in DMEM to a concentration of 25 μg/ml and then mixed with the collagen gel. The suppressive effect of AM extract mixed in type 1 collagen gel was similar to that of AM extracts (AME) used alone, when compared to the control which was added with BSA alone (FIG. 8, $p<0.01$). Although collagen gel alone (Col) also showed a similar suppressive activity when compared to the plastic control (FIG. 8, $p<0.01$), addition of AME in collagen gel (Col+AME) resulted in further suppression (Fig. 8, $p<0.01$). When water-soluble AM extracts (AME) were mixed in HA gel, the suppressive effect on TGF-β1 promoter activity was better preserved as compared to HA alone (mixed with BSA as a control) (FIG. 5, $p<0.01$) similar to that exerted by AME alone (FIG. 9). Accordingly, an AM extract composition, or its combination with collagen can be useful to suppress TGF-β activity in eye tissue.

Example 2: Characterization of Amniotic Membrane Components

Material and Methods

[00279] The concentration of proteins in each extract was quantitated by the BCA Protein Assay Kit (Pierce, Rockford, IL). The concentration of hyaluronic acid (HA) in each extracts was assayed with Hyaluronic Acid (HA) Quantitative Test Kit (Corgenix, Westminster, CO) based on ELISA using a standard curve provided by the manufacturer prepared by serial dilution of HA.

HA Molecular Weight Range Analysis by Hyaluronidase Digestion

[00280] The HA molecular weight ranges of the extracts were analysed by agarose gel electrophoresis according to the method described by Lee and Cowman (Lee H.G. and Cowman, M. K. An Agarose Gel Electrophoretic Method for Analysis of Hyaluronan Molecular Weight Distribution. Analytical Biochemistry, 1994, 219, 278-287). The samples were subjected to 0.5% agarose gel electrophoresis followed by staining using 0.005 % Stains-All (Sigma, cat# 23096-0) in 50% ethanol. The gel was stained overnight under a light-protective cover at room temperature (Shorter staining periods of 3-4 hr can also give acceptable results). HA was visualized as blue bands after destaining by transferring the gel to H_2O and exposed to the room light for approximately 6 hr. The molecular weight standards included lamda DNA-BstE II digested restriction fragments (cat# D9793, Sigma) ranging in MW from 0.9 to 5.7 x 10^6. The authenticity of HA was further verified by incubation of the extract with

for without 10 units/ml hyaluronidase (Sigma #H1136) in the reaction buffer (50 mM Tris-HCl, pH7.5, 0.1 M NaCl, 1% Triton X-100, 0.1 % BSA supplemented with the above protease and phosphotase inhibitors) for 2 h at 37 °C using a positive control of high MW HA (cat# H1876, Sigma) purified from human umbilical cords.

Western Blot Analyses

[00281] The above extracts were electrophoresized on 4-15 % denatured acrylamide gels and transferred to the nitrocellulose membrane, and then immunoblotted with a rabbit anti-human inter-α-trypsin inhibitor (rabbit polyclonal antibody (cat# A0301, DAKO at 1:1000), a rabbit anti-human TSG-6 polyclonal antibody (provided by Dr. Tony Day at 1:1000 dilution), a rat monoclonal anti-PTX3 antibody (Alexis Biochemicals, ALX-804-464, 1 μg/ml), an anti-thrombospondin-1 antibody obtained from Calbiochem (Cat# BA24), and a goat anti-human Smad 7 antibody (AF2029, 1:1000, R & D Systems). Imunoreactive protein bands were detected by Western LightingTMChemiluminesence Reagent (PerkinElmer).

Results

[00282] Experiments showed that the observed suppressive effect on the TGF-β1 promoter activity was abolished when water-soluble AM extracts were pre-heated at 90° C for 10 minutes, suggesting that the responsible component(s) most likely contained protein(s), of which the conformation is important.

Quantitation of HA and Proteins in AM Extracts

[00283] The results summarized in the Table below showed that all AM and jelly extracts contained both HA and proteins. In general, the weight ratio between proteins and HA was high in the Total Extract than the supernatant (e.g., L and H for PBS, and A for Buffer A) after centrifugation for AM, suggesting that most protein-containing materials were eliminated by centrifugation. However, this trend was not noted in AM Jelly, suggesting that AM extracts contained more proteins than Jelly (see T under PBS and T under A/B/C). The ratio between proteins and HA was also increased from Extract A to Extracts B and C for both AM and AM jelly, further supporting that HA was mostly present in the soluble form, and vice versa proteins were found more in the water-insoluble components. Furthermore, HA was largely removed from AM Jelly after centrifugation in A/B/C.

Tissue	**AM**							**Jelly**						
Buffer	**PBS**			**A/B/C**				**PBS**			**A/B/C**			
Fraction	T	L	H	T	A	B	C	T	L	H	T	A	B	C
Protein (μg/ml)	8645	1370	1467	8645	2731	930	2698	3836	3645	3589	3836	3893	527	1364
HA (μgml)	75	62	44	60	74	7	35	80	90	96	129	94	2	7
Protein/ HA	115	22	33	144	37	133	77	48	41	37	30	41	264	195

[Note]: T: Total, L: the supernatant following the low speed centrifugation of the total extract, H: the supernatant following the low speed centrifugation of the total extract, A, B, C: Extracts, see text.

HA in Different AM Extracts Have Molecular Weights Greater than One Million Daltons

[00284] High molecular weight (> 10^6 daltons) of HA was present in the total extracts and Extract A (Fig. 10). However, even higher MW of HA was present in Extract B, while HA was found in a narrow band with even higher MW in Extract C (Fig. 10). All of the HA-containing components disappeared after hyaluoridase digestion, confirming that they indeed contained HA. Compared to the positive control of HA obtained from Sigma (cat# H1136), a similar high molecular weight (> 10^6 daltons) of HA was also found in both supernatants obtained after low and high speeds of centrifugation (Fig. 11). Again these HA-containing bands disappeared after hyaluonidase digestion. A similar result was obtained for AM jelly (not shown).

Inter-α-Trypsin Inhibitor (IαI) is Present in Different AM Extracts and Its Heavy Chains (HCs) Are Covalently Linked with HA

[00285] Fig. 12 showed that before digestion with hyaluronidase, free heavy chains were present in different complexes, and a small amount of light chain was also present (UTI or bikunin). However, in all extracts, i.e., total and Extracts A, B, and C, there was also a covalently linked complex between HA and heavy chains of IαI as the latter was released only after hyaluronidase digestion. The same result was obtained in Extracts H and L obtained by two different speeds of centrifugation (Fig. 13).

Tumor Necrosis Factor-Stimulated Gene 6 (TSG-6) is also Present in AM Extracts

[00286] Fig. 14 showed that TSG-6 (~38 kDa) was present in Total, Extract A and Extract C. In Extract A, there was a band of ~38 kDa migrated close to that of the purified TSG-6 (35kD). The identity of other bands of ~45 and 55kDa was unknown. Total AM extract (without centrifugation) "T" showed two bands (both above 35 kD), and the higher one (55kD) that were found in Extract A (after centrifugation), while the lower one (45kD) was found in Extract C. All of these bands were not significantly altered when samples were treated with hyaluronidase (Fig. 14) or with F-glycosidase (Fig. 15). However, digestion with chondroitin sulfate ABC lyase resulted in more noticeable 38 kD band using antibody RAH-1 (Fig 16) but not using antibody MAB2104 (Fig. 17).

Pentraxin (PTX-3) Is Exclusively Present in Water-soluble AM Extracts and Forms a Complex with HA

[00287] Fig. 18 showed that PTX3 could also be present in AM extracts and is complexed with HA in the water soluble extract A only.

Thrombospondin (TSP-1) Is Present in Different AM Extracts

[00288] Fig. 19 showed that all AM extracts had a high molecular weight band of TSP-1 while the total extract (T) and Extract C also had some bands between 35-120 kDa. Hyaluronidase digestion did not change the reactive pattern except some bands became a little stronger or weaker.

Smad7 Is Present in Mostly in Water-insoluble AM Extracts

[00289] Smad 7 was found in both PBS extracts and urea extracts of AM (Fig. 20).

Example 3: Water-soluble AM Extracts Prevent Cell Death of Corneal Epithelial Cells (Basal Cells and Keratocytes) Induced by Storage and by Injuries Caused by Mechanical and Enzymatic Means

Results

[00290] To demonstrate that AM extracts can prevent apoptosis in injured tissues, the following experiment was performed using a murine model. A total of 22 mouse eye balls were enucleated, two of which were immediately embedded in OCT for frozen sections as a pretreatment control. The remaining of 20 eye balls were subdivided into three subgroups, namely, 1) mechanical scraping (n=8), 2) dispose digestion (enzymatic) (n=6), and 3) without treatment control (n=6). For each group, equal numbers of eye balls were preincubated at 4° C for 24 h in the presence (+) or absence (-) of 125 μg/ml AM extracts in keratinocyte serum-free medium (KSFM) with defined supplement (Gibco, Carlsbad, CA) prior to the treatments. At the end of the first 24 h of incubation in KSFM +/- AM extract (prepared as described herein), 8 eyeballs in Subgroup 1 were then subjected to mechanical scraping with a surgical blade, and were further divided into two groups (n=4 each) and incubated at 37° C in KSFM +/- AM extract. Six eye balls in Subgroup 2 were subjected to enzymatic digestion with 10 mg/ml Dispase II in KSFM +/- AM extract (n=3 each) for 18 h at 4° C. One eye ball from each group was embedded in OCT for frozen sections. The remaining two eye balls from each group were incubated in KSFM +/-AM extract for another 24 h before analysis. For the non-treatment control (n=6), 3 eye balls each were incubated in KSFM +/- AM extract at 37° C continuously for two days; one eye ball was removed at the end of the first day while two eye balls were removed at the end of 2 days.

[00291] The frozen sections from these eyeballs were subjected to TUNEL staining. In short, terminal deoxyribonucleotidyl transferase-mediated FITC-linked dUTP nick-end DNA labeling (TUNEL) assay was performed using DeadEndTM fluorometric TUNEL system obtained from Promega (Madison, WI) according to the manufacturer's instructions. Sections were fixed in 4% formaldehyde for 20 min at room temperature and permeabilized with 1% Triton X-100. Samples were then incubated for 60 min at 37° C with exogenous TdT and fluorescein-conjugated dUTP for repair of nicked 3'-hydroxyl DNA ends. Cells were treated with DNase I as the positive control, while the negative control was incubated with buffer lacking rTdT enzyme. The apoptotic nuclei were labeled with green fluorescence, and the nuclei were counterstained with DAPI as red fluorescence.

[00292] The water-soluble form of AM extract was prepared by the method for preparing water-soluble AM extract. A BCA assay (Pierce, Rockford, IL) was used to quantitate the total protein in the AM extract.

[00293] The normal mouse eye ball showed a minimal amount of apoptosis only in the superficial layer of the corneal epithelium of the uninjured control before incubation in KSFM; no apoptosis was noted in the stromal keratocytes. However, after 24 h incubation at 4° C in KSFM, there was a mild increase of apoptosis in keratocytes of the superficial stroma. Such an increase of keratocyte apoptosis was suppressed by AM extract.

[00294] The AM extract was also shown to reduce apoptosis after mechanical damage to the cells. After mechanical scraping, the mouse eye ball showed a significant increase of keratocyte apoptosis. However, incubation with AM extract following mechanical scraping resulted in a decrease in keratocyte apoptosis.

[00295] The mouse eyes were also treated enzymatically to damage the cells. Dispase digestion at 4° C for 18 h in KSFM resulted in a significant amount of apoptosis in not only keratocytes but also in epithelial cells; for the latter apoptosis was found to be present not only in the superficial epithelial cells, but also in the basal epithelial cells. The extent of epithelial and keratocyte apoptosis was far greater than that noted after mechanical scraping. Incubation of AM extract during dispase digestion significantly reduced apoptosis of both epithelial cells and keratocytes. This is significant because dispase treatment mimics the surgical (e.g., excimer ablation in PRK) and pathological insults (e.g., recurrent corneal erosion) that can be directed to the basement membrane. The results of this experiment demonstrate that the application of AM extract to tissues with damaged cells can be used to reduce or prevent cellular damage.

Example 4: Amniotic Membrane Stromal Extract De-differentiates Myofibroblasts

Materials

[00296] Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS), amphotericin B, gentamicin, fetal bovine serum (FBS), 0.25% trypsin/0.53 mM EDTA, Live and Dead cell viability assay reagent, and FITC conjugated phalloidin were purchased from Invitrogen (Carlsbad, CA). Bovine serum albumin (BSA), insulin-transferrin-sodium selenite media supplement, formaldehyde, protease inhibitor cocktail, mouse anti-desmin antibody, FITC conjugated anti-mouse, goat, and rat IgG, propidium iodide, and Hoechst-33342 dye were from Sigma (St. Louis, MO). Transwell inserts were from Corning Incorporated (Corning, NY). Type I collagen was from BD Biosciences (Bedford, MA). BCA™ protein assay kit was from Pierce (Rockford, IL). Dispase II and collagenase were from Roche (Penzberg, Germany). Mouse anti-αSMA and Ki67 antibodies were from DakoCytomation (Carpinteria, CA). Rabbit anti-vimentin antibody was from Abcam (Cambridge, MA). Mouse anti-EDA fibronectin antibody was from Chemicon (Temecula, CA). HRP conjugated anti-mouse IgG was from BioRad (Hercules, CA). Anti-fade mounting solution was from Vector Laboratories (Burlingame, CA). Cryopreserved human AM was obtained from Bio-Tissue (Miami, FL).

Cell cultures

[00297] Human tissue was handled according to the Declaration of Helsinki. The fresh human placenta was obtained from Baptist Hospital (Miami, FL) after cesarean section after an informed consent was obtained under an IRB-approved protocol. After two times rinse with PBS including gentamicin and amphotericin B, AM was mechanically peeled from the chorion, cut into pieces (~30 mm in diameter), and digested with 10 mg/mL Dispase II in DMEM with 10% FBS at 37 °C for 20 min. After that, the amniotic epithelium was removed by surgical peeling under dissecting microscope, and the remaining stroma was further digested by 2 mg/mL collagenase in DMEM with 10% FBS at 37 °C for 14 h. Cells were collected by centrifuge at 800 x g for 5 min, and resuspended and cultured in DMEM with 10% FBS under a humidified atmosphere of 5% CO_2 in air at 37 °C, the culture medium was changed every two days. AM before enzyme digestion was also embedded in O.C.T for cryosectioning. Human corneoscleral tissues were obtained from the Florida Lions Eye Bank (Miami, FL), from which corneal fibroblasts (HCFs) were harvested, and cultured in DMEM containing 10% FBS, secondary passage (P1) cells were used in all experiments.

Preparation of water-soluble AM stromal extract and AM inserts

[00298] Using aseptic techniques, cryopreserved human AM was briefly washed 2-3 times with HBSS to remove the storage medium. The AM stroma was scraped off by a spatula, frozen in the air phase of liquid nitrogen, and grounded to fine particles by BioPulverizer (Biospec Products, Inc., Bartlesville, OK) followed by homogenization on ice with Tissue Tearor (Biospec Products, Inc., Dremel, WI) in PBS, pH 7.4, for 1 min. The homogenate was mixed by rotation for 1 h and centrifuged at 14,000 × g for 30 min at 4 °C. The supernatant in PBS was then collected, and stored in aliquots at -80 °C. BCA assay was used to quantitate the protein concentration. This water-soluble protein extract was designated as amniotic stromal extract (ASE). For preparation of AM inserts, AM was thawed immediately before use, washed three times with HBSS, cut into pieces approximately 2.5 × 2.5 cm in size, and fastened onto a culture insert with the stromal matrix side facing up.

Immunostaining

[00299] Cryostat sections (4-μm) of AM were fixed in acetone for 10 min at -20 °C; cultured AMSCs and AM whole mount with AMSCs were fixed in 4% paraformaldehyde for 30 min at 4 °C. Sections or cultured cells were rinsed three times for 5 min each with PBS, and then incubated in 0.2% Triton X-100 for 10 min. After three rinses with PBS for 5 min each and preincubation with 2% BSA to block nonspecific staining, sections or cells were incubated with anti-αSMA (1:200), anti-desmin (1:200), and anti-vimentin (1:200) antibodies for 1 h. After three washes with PBS for 15 min, they were incubated with an FITC or Texas Red conjugated secondary antibodies for 45 min. For labeling of F-actin, cells were further stained with FITC conjugated phalloidin at the concentration of 200 units/mL for 15 min. After three additional PBS washes for 15 min each, nuclei were stained with PI (1:2000) for 1 min or Hoechst-33342 for 15 min, then analyzed with a fluorescence microscope. For immunohistochemical staining of Ki67, endogenous peroxidase activity was blocked by 0.6% hydrogen peroxide for 10 min. Nonspecific staining was blocked by 1% normal goat serum for 30 min. Cells were then incubated with anti-Ki67 antibody (1:50) for 1 h. After three washes with PBS for 15 min each, cells were incubated with biotinylated rabbit anti-mouse IgG (1:100) for 30 min, followed by incubation with ABC reagent for 30 min. The reaction product was developed with DAB for 5 min, and examined under a light microscope.

Western Blot Analysis

[00300] Cultured AMSCs or myofibroblasts from plastic, collagen, or AM surface were collected and extracted in cold RIPA buffer [50 mM Tris·Cl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail]. Equal amounts of proteins extracted from lysates were separated on 4%-15%

sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE), and then electrophorectically transferred to nitrocellulose membranes. After 1 h of blocking in 5% nonfat milk, the blots were incubated with primary antibodies to αSMA and ED-A fibronectin using α-actin as a loading control. The specific binding was detected by anti-mouse or anti-rabbit horseradish peroxidase (HRP)-conjugated antibodies, and visualized by enhanced chemiluminescence method.

Statistical Analysis

[00301] All experiments described above were repeated three times, each in triplicate or more. Group means were compared using the appropriate version of Student's unpaired t-test. Test results were reported as two-tailed p values, where $p < 0.05$ was considered statistically significant. Summary data are reported as means ± S.D.

Results

The In Vivo Phenotype of AMSCs

[00302] After removal of amniotic epithelial cells by Dispase II, AMSCs in AM could be observed in situ. Through a phase contrast microscope, they exhibited a dendritic morphology and maintained intercellular contacts via thin processes (Fig. 21A). Cell viability, the dendritic morphology and intercellular contacts were better visualized by staining with Live and Dead assay (Fig. 21B). Immunostaining of AM cross-sections showed that AMSCs did not express α-SMA (Fig. 21C) and desmin (Fig. 21D). In contrast, as a positive control, umbilical cord mesenchymal cells showed strong staining to both α -SMA and desmin (see the inset of Fig.21C and 21D, respectively). However, all AMSCs expressed vimentin (Fig. 21E). These data collectively indicated that AMSCs are of a fibroblast phenotype in vivo.

Rapid Myofibroblast Differentiation of AMSCs In Vitro

[00303] To investigate the differentiation of AMSCs in vitro, collagenase-isolated AMSCs were plated on plastic dishes and cultured in DMEM with 10% FBS at a density of 200 cells/mm^2. Within 4 to 5 days, cells adopted a typical fibroblast cell shape (Fig. 22A) and remained α-SMA negative (Fig. 22E). However, some cells started to increase the cell size, change the shape (Fig. 22B), and express α-SMA (Fig. 22F) at the end of 1 week of culturing. After subcultured to another plastic dishes in the same medium, the majority of cells exhibited a typical myofibroblastic cell shape at the end of one week of the passage one (Fig. 22C), and eventually almost all cells turned into a myofibroblastic shape and had prominent microfilaments at the end of one week of the second passage (Fig. 22D). Accordingly, α-SMA-positive myofibroblasts dramatically increased from 71.9 ± 3.7 % at 1 week primary culture to 93.9 ± 4.1% at passage one culture and 98.5±1.7% at passage two culture (Fig. 22I). Nevertheless, expression of desmin was still negative at the passage two culture (data not shown). Western blot analysis revealed that AMSCs in vivo weakly expressed ED-A fibronectin but did not express α-SMA. However, α-SMA and ED-A fibronectin expression dramatically increased at the end of the primary culture and maintained at the passage 2 (Fig. 22J). These results indicated that AMSCs rapidly differentiated into myofibroblasts on plastic in this serum-containing medium.

Differentiated Myofibroblasts from AMSCs Could Be Reversed If Subcultured on AM Stromal Matrix

[00304] In our previous studies, we have shown that AM can inhibit myofibroblast differentiation of human or mouse keratocytes when cultured on the stromal matrix of AM from the primary culture. To further investigate whether AM stromal matrix was also potent in modulating the phenotype of differentiated myofibroblasts or not, myofibroblasts differentiated from AMSCs at passage 2 were subcultured onto the stromal matrix of AM, and compared to those subcultured on collagen I-coated dish as a control. After 7 days of cultivation in DMEM with 10% FBS, AMSCs on collagen I still maintained a myofibroblastic shape (Fig. 23A). In contrast, cells seeded on AM stromal matrix exhibited a mixture of round, spindle, elongated, and dendritic shapes (Fig. 23B). Live and Dead

assay confirmed that cells on both collagen I (Fig. 23C) and AM matrix (Fig. 23D) remained 100% viability, but revealed a significant difference in the cell shape. Immunostaining to phalloidin showed vivid stress fibers (Fig. 23E), which also contained strong α-SMA expression (Fig. 23G) in myofibroblasts seeded on collagen I. In contrast, the phalloidin staining became weak and spotty (Fig. 23F), and α-SMA staining became obscured while cells were seeded on AM stromal matrix (Fig. 23H). Western blot analysis confirmed that myofibroblasts derived from AMSCs continuously expressed abundant amounts of ED-A fibronectin and α-SMA when seeded on type I collagen (Fig. 23I). In contrast, expression of ED-A fibronectin was decreased and that of α-SMA became undetectable when seeded on AM stromal matrix (Fig. 23I). These results collectively indicated that myofibroblasts differentiated from AMSCs could still be reversed to a fibroblast phenotype when subcultured on the AM stromal matrix.

Amniotic Stromal Extract Prevented Myofibroblast Differentiation of AMSCs and Reverse Differentiated Myofibroblasts

[00305] To further investigate whether the aforementioned reversal activity by the AM stromal matrix was retained in the water-soluble AM stromal extracts, primary AMSCs (P0) were cultured on plastic in DMEM containing 10% FBS with or without 100 μg/ml ASE. The results showed that AMSCs maintained a spindle fibroblastic shape after 4 days of cultivation with or without ASE (Fig. 24A and 24B, respectively). However, at that time, cells already expressed α-SMA without ASE (Fig. 24E), but remained devoid of α-SMA expression when ASE was added (Fig. 24F). When cultures were extended to 10 days, as shown in Fig. 2, AMSCs showed an enlarged cell shape, and vividly expressed phalloidin-positive stress fibers (Fig. 24C) containing positive expression of α-SMA (Fig. 24G). Strikingly, AMSCs aggregated into spheres of varying sizes with a smaller nucleus in the presence of ASE (Fig. 24D). These cells in the sphere remained viable based on the Live and Dead assay (data not shown). Some spheres were detached from the plastic dish, but they could reattach to a new plastic dish to generate new growth of myofibroblasts when switched back to DMEM/10%FBS (data not shown). Phalloidin staining did not show any stress fiber (Fig. 24D), while α-SMA expression in the sphere was weak (Fig. 24H). These results indicated that ASE indeed could prevent myofibroblast differentiation of AMSCs.

[00306] To examine whether the aforementioned reversal activity of AM stromal matrix was retained in ASE, we added 100μg/ml ASE for 1 week to passage 2 AMSCs cultures on plastic containing DMEM with 10% FBS. As described earlier (Fig. 22), by this time nearly all cells turned into myofibroblasts with a squamous morphology, prominent stress fibers and strong expression of α-SMA. Addition of ASE allowed cells to be reverted to an elongated or spindle shape (Fig. 25A) with a significant decrease of α-SMA expression (Fig. 25B). Western blot analysis further confirmed decrease of EDA fibronectin and α-SMA expression after the addition of ASE (Fig. 25C). These results indicated that AM stroma contained soluble factor(s) that could suppress myofibroblast differentiation of AMSCs if added before, but could reverse differentiated myofibroblasts to fibroblasts if added later.

Reversal of Myofibroblasts Was Not Associated with Cell Proliferation

[00307] To further determine whether the aforementioned phenotypic reversal of AMSCs from myofibroblasts to fibroblasts by ASE was accompanied by cellular proliferation or not, we switched the medium from DMEM plus 10% FBS to serum-free DMEM/ITS in passage 3 cultures, in which as described in Fig. 2 nearly all cells turned into myofibroblasts. During a course of 6 days of observation, cells in the control culture maintained the same myofibroblast morphology with prominent stress fibers in the cytoplasm (Fig. 26A to 26D). However, cells in the experimental cultures with addition of ASE gradually changed the shape from being large and flattened on day 0 (Fig. 24E) to spindle and elongated on day 2 and day 4 (Fig. 26F and 26G, respectively), and finally some cells shrank to a small size on day 6 (Fig. 26H). Such a dramatic morphological change caused by addition of ASE was accompanied by the loss of α-SMA-expressing stress fibers (Fig. 26I to 26L). Ki67 staining confirmed that

myofibroblasts in DMEM/FTS of PB cultures did not exhibit any cellular proliferation no matter if ASE was added or not (Fig. 26M and 26N, respectively). As a control, AMSCs in P1 cultures showed occasional Ki67-positive nuclei when cultured on plastic in DMEM/10%FBS (Fig. 26O), while many human corneal fibroblasts cultured on plastic in DMEM/10%FBS showed Ki67-positive nuclei (Fig. 26P). These results strongly supported the notion that ASE not only prevented myofibroblast differentiation of AMSCs, but also reversed differentiated myofibroblasts of AMSCs to fibroblast without affecting their cellular proliferation.

Differences of Morphology and Smad Signaling and Suppression of TGF-β Promoter Activity by AM Stromal Matrix

[00308] Mouse stromal cells freshly isolated by collagenase exhibited a fibroblastic morphology when cultured on plastic in DMEM with 10% FBS, but a dendritic morphology when cultured on AM stromal matrix in the same medium. Immunostaining showed nuclear exclusion of Smad4 for dendritic keratocytes cultured on AM in DMEM with ITS even after being challenged with 10 ng/ml TGF-β1. In contrast, an increasing percentage of cells from 13% when cultured on plastic to 67% and 85% after 10 ng/ml TGF-β1 was added for 3 hours and 5 days, respectively. These results suggest that AM stromal matrix suppresses Smad-mediated T- TGF-β signaling, which helps maintain the keratocyte phenotype.

[00309] Mouse corneal fibroblasts cultured on plastic and intact cryopreserved AM (prepared as described herein for preparing cryopreserved intact AM) were cotransfected with TGF-β2 promoter-luciferase plus CMV-β-galactosidase or TGF-βRII promoter-luciferase plus CMV-β-galactosidase for 48 hours. Cell extracts were assayed for both activities of luciferase and β-galactosidase. The relative luciferase unit of the promoter activity of TGF-β2 and TGF-βRII was suppressed in cells cultured on AM.

Example 5: AM Extracts Suppress Fibroblast Migration from Human Limbal Explants

Materials and Methods

Preparation of Total Soluble Human Amniotic Membrane Extracts in PBS

[00310] The entire procedure for preparation of total soluble human AM extracts (T) was carried out aseptically so as to be used for subsequent cell culture-based experiments. Frozen human placenta was obtained from Bio-tissue, Inc. (Miami, FL), from which AM was retrieved. AM was sliced into small pieces to fit into the barrel of a BioPulverizer (Biospec Products, Inc., Bartlesville, OK), frozen in the liquid nitrogen, and then pulverized into a fine powder. The powder was weighed and mixed with cold PBS buffer (prepared by adding distilled H_2O to 1 x PBS, pH7.4, from10 x PBS, cat# 70011-044, Invitrogen, Carlsbad, CA) with protease inhibitors (protease inhibitor cocktail, P8340, Sigma, and supplemented with 1 mM PMSF) and phosphatase inhibitors (50 mM sodium fluoride and 0.2 mM sodium vanadate) at 1:1 (ml/g). The mixture was kept in the ice and homogenized with a Tissue Tearor (Biospec Products, Inc., Dremel, WI) for 5 times, 1 min each with a 2 min interval cooling.This water-soluble extract was designated as "Total" (T). The total water-soluble extract was mixed for 1 hr at 4 °C, centrifuged at 4 °C for 30 min at 48000 x g. The supernatant was divided into aliquots and stored at -80 °C

Human Corneolimbal Explant Cultures

[00311] Human tissue was handled according to the Tenets of the Declaration of Helsinki. Limbal rims were obtained either from the donor corneas (Medical Eye Bank of Florida, Orlando, FL) or after transplantation of donor corneas (Florida Lions Eye Bank ,Miami, FL). The excessive sclera, iris, corneal endothelium, conjunctiva, and Tenon's capsule were removed, and the remaining rims were briefly rinsed 3 times with SHEM media made of an equal volume mixture of HEPES-buffered DMEM and Ham's F12 supplemented with 5 % FBS, 0.5 % DMSO, 2 ng/ml mouse EGF, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 0.5 μg /ml hydrocortisone, 10 nM cholera toxin, 50 μg/ml gentamicin, 1.25 μg /ml amphotericin B. Each limbal rim was equally divided into two halves and

each half was further equally subdivided into 6 explants, i.e., to make 12 explants per limbal rim. To eliminate variations of age, sex, and race, explants from the corresponding position of the same donor cornea were selected for the control and the experimental group, respectively. An explant was placed on the center of each 6 well with the epithelial side up and cultured in SHEM or SHEM with 25 μg/ml the above total AM extract. The culture was maintained at 37 °C under 95% humidity and 5% CO_2, the medium was changed every other day, and their outgrowth was monitored daily for 14 days using an inverted phase microscope (Nikon, Japan). The outgrowth area was digitized every other day by Adobe Photoshop 5.5 and analyzed by NIH ImageJ 1.30v (NIH, Bethesda, MD)

MTT Assay

[00312] The MTT assay (Cell Proliferation Kit I, cat# 11465007001, Roche Applied Science, Indianapolis, IN) is a colorimetric assay based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolic active cells. The outgrowing cells from human limbal explants cultured in SHEM (the Ctrl group) or SHEM with 25 μg/ml AME (the AME group) for 14 days were harvested separately by trypsin/EDTA digestion and resuspended into SHEM medium. Cells were counted with hemacytometer and seeded at 2000 cells/per 96 well with 100 μl medium. Each group was further divided into 3 subgroups (Ctrl, PBS, AME) in which no supplement, 2 μl of PBS, or 2 μl of 1250 μg/ml AME (to make a final concentration of 25 μg/ml AME) was added immediately after the cell seeding. Each subgroup had a total of 16 wells (from the duplicate). Cells were incubated at 37 °C under 95% humidity and 5% CO_2 for 10 days with the medium being changed every other day. When MTT assay was performed, 10 μl of the MTT labeling reagent (final concentration 0.5 mg/ml) was added to each 96 well. The 96 well plates were incubated for 4 hr under the same culture conditions. Then 100 μl of the Solubilization solution was added to each well and the plate was further incubated at the same condition for 20 hr. The spectrophotometrical absorbance of samples was measured using a microplate reader (Fusion™, Meriden, CT) with a wavelength of 550 nm minus the absorbance of a reference wavelength at 650 nm.

Hematoxylin and Eosin (H & E) Staining

[00313] After cultured for 14 days, explants were removed from wells and embedded with OCT (Tissue-Tek), briefly frozen in liquid nitrogen, and stored at – 80 °C. Tissues were sectioned with Microtome Plus (Triangle Biomedical Sciences, Durham, NC) at 6 μm on snowcoat X-Tra™ microslides (Surgipath, Richmond, IL), fixed in 10 % formalin for 10 min, and sequentially stained with Harris hematoxylin for 5 min, 1 % Eosin yellowish for 1 min at 25 °C. Tissues were dehydralated with a series of 70%, 95%, and 100% alcohol, each for at least 5 min, and finally treated in xylene (SUB-X™ Xylene Substitute, Surgipath) and mounted with a cover slip. The slides were observed under an inverted microscopy (ECLIPSE, TE 2000-U, Nikon).

Results

AM Extract Slowed Down Epithelial Migration from Human Limbal Explants and Resulted in Less Epithelial Cells in the Outgrowth but with More Progenitor Cells

[00314] The Table shown below indicates that the onset of epithelial outgrowth from the limbal explant was delayed and the resultant epithelial outgrowth contained less cells in cultures added with AM extracts.

Table: Explant Outgrowth

Outgrowth	**Explant** (6) SHEM/PL	**Explant** (6) SHEM/AME/PL 25 μg/ml
Day 3	2	0
Day 4	6	1
Day 5	6	6
Day 14 (cells from 4 explants)	7.35×10^6 (3.6)	2.05×10^6 (1.0)

AM Extract Suppressed Fibroblast Migration from Human Limbal Explants and Resulted in Less Fibroblasts in the Outgrowth

[00315] In Fig. 27A, the outgrowth from human limbal explants cultured in both SHEM (Ctrl) and SHEM/AME (AME) formed a similar epithelial sheet. However, some fibroblast-like cells only appeared in Ctrl but not in AME culture, indicating AME may suppress the migration of fibroblasts. In Fig. 27B, after 14 days culture, human limbal explants were removed from culture wells, embedded, sectioned, and stained with H & E. There were much more stromal cells in the AME than those in the Ctrl. This is possibly caused by AME suppressing the migration of fibroblasts.

Suppression of Fibroblast Outgrowth by AME

[00316] The outgrowth from human limbal explants cultured in both SHEM (Ctrl) and SHEM/AME (AME) for 14 days were separately harvested and seeded in each 96 well at 2000 cells/well as described in MTT Assay. Cells from the Ctrl were seeded in columns 1-3 (1: Ctrl; 2: PBS; 3: AME; n=8 for one plate shown here but n=16 for the duplicate with the similar result) and those from the AME were in columns 4-6 (1: Ctrl; 2: PBS; 3: AME). After cultured 10 days, cells were used for MTT assay. There was no significant difference of fibroblast growth among subgroups (Ctrl, PBS, and AME) but there was statistically significant difference of fibroblast growth between cells derived from outgrowth in SHEM and in SHEM/AME (Fig. 28A). Because fibroblasts grew much quickly than epithelial cells did so wells became pink after adding MTT reagents contained fibroblasts. Statistical analysis of the fibroblast growth in Ctrl and AME showed AME significantly suppressed the fibroblast growth ($p=0.01$) (Fig. 28B).

More Clonal Growth from Outgrowth Epithelial Cells Cultured in SHEM/AME than Those from Cultured with SHEM only

[00317] Human limbal explants were cultured in either SHEM only (Ctrl) or SHEM/AME (100 μg/ml). After cultured for 10 days, outgrowing epithelia cells were harvested with trypsin/EDTA and counted again, the growth of epithelial cells were significantly suppressed by AME. That is, total cells from 3 explants cultured in SHEM is 9×10^5 while total cells from 3 explants cultured in SHEM/AME is 2.3×10^5, ratio is Ctrl/AME= 3.9. When these cells were seeded at 500 or 1000 cells in each 60 mm dish (~18 or 36 cell/cm^2) on a swiss 3T3 feeder layer with MMC pre-treatment (4 μg/ml for 2 hr at 37 °C) in SHEM medium. After seeding for 4-5 days, epithelial clones started to form. Clones from cells cultured previously with SHEM/AME were more and larger than those cultured previously with SHEM only ($P < 0.05$). Clones were allowed to grow until day 10 and then clones were stained with the crystal violet dye to show the number and size of clones.

AME Inhibited MAPKp38 of the Outgrowth

[00318] The outgrowth from human limbal explants cultured in both SHEM (Ctrl) and SHEM/AME (AME) for 14 days formed a similar epithelial sheet. However, the edges of epithelial sheets were different: the edge of the Ctrl epithelial sheet appeared rough while that of the AME was smooth (Fig 29, Ctrl –left panel; AME –right panel). This phenomenon resembled the effect of MAPK p38 inhibitor (10 μM SB203580 in SHEM) on the outgrowth of human limbal explants (data not shown). It provided another evidences that AME contained component(s) that can inhibit MAPK p38.

Example 6: Amniotic Membrane Preparations Suppress Macrophage Function

Materials and Methods

Cell Culture and IFN-γ Stimulation

[00319] Raw 264.7 cells, a mouse macrophage cell line, were obtained from the ATCC and cultured in phenol red-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 μg/ml

Gentamicin and 1.25 µg/ml fungizone in 100 mm culture dishes. When cells became 70%-80% confluent, the medium was removed and cells were treated with trypsin/EDTA for 10 min at 25 °C. After digestion, cells were still firmly attached to the dish, so the trypsin/EDTA solution was removed. 5 ml of DMEM with insulin-transferrin-sodium selenite (ITS; including 5 mg insulin, 5 mg human transferrin, and 5 µg sodium selenite in 1000 ml. Sigma, St. Louis, MO) was then added to the dish and cells were harvested by pipetting. After centrifugation at 500 x g for 5 min, the medium was removed, the cell pellet resuspended in the DMEM/ITS medium, cells counted, and cell density adjusted to $0.5x10^6$ /ml.

[00320] To seed cells, 0.5 ml ($0.25x10^6$) of the cell suspension was added to each 24 well. After one hour to let cells attach, 5ul of IFN-γ ($2x10^4$U) was added to each well to reach a final concentration of IFN-γ: 200U/ml. 48 hr later, the cell culture medium was collected for NO and PGD_2/PGE_2 assays. Cells were used for Live & Dead Assay or harvested for Western Blots.

Live & Dead Assay

[00321] The medium was removed and 200 µl of the combined LIVE/DEAD assay reagents (Molecular Probes, Eugene, OR) was added to each well. After incubation with cells for 15 min at room temperature, cells were observed under a fluorescent-light microscope. Live cells produce an intense uniform green fluorescence in the cytoplasm and dead cells produce a bright red fluorescence in the nucleus.

NO Assay

[00322] At the end of the cultivation, the cell culture medium in each well was harvested individually and centrifuged at 12,000 x g for 10 min at 4 °C. The cleared medium was transferred to a new tube and was subjected to assays directly or stored at -80°C before assays later. NO production was assessed by measuring the nitrite, its stable degradation product, using Griess reagent according to the manufacturer's protocol. Briefly, 50 µl of the cleared medium was mixed with 100 µl of the Griess reagents (ICN Biomedicals, Aurora, Ohio), after 15 min the absorbance was measured at 550 nm using Fusion™ universal microplate analyzer (Packard, Meriden, CT). The amount of nitrite was calculated from a standard curve of sodium nitrite ($NaNO_2$; Sigma, St. Louis, MO).

Prostaglandin D_2 (PGD_2) and Prostaglandin E_2 (PGE_2) Assay

[00323] Cell culture supernatants were collected 48 hr later with or without IFN-γ stimulation. PGD2 and PGE2 concentrations were measured using an EIA kit (cat# 512021 and 514010, respectively, Cayman Chemical, Ann Arbor, MI), with the assays conducted according to the instructions of the supplier.

TGF-β1 Promoter Assay

[00324] The TGF-β promoter activity assay involves plasmids containing human TGF-β1 promoter (-1362 to +11)-luciferase, TGF-β2 promoter (-1729 to +63, Noma et al., Growth Factors, 4:247-255, 1991) and TGF-β3 promoter (-1387 to +110). Both the TGF-β2 promoter and the TGF-β3 promoter were inserted into Kpn I and Hind III of pGL3-basic. Human TGF-βRII promoter (-1883 to +50, Bae et al., J Biol Chem., 270:29460-29468, 1995) was amplified by PCR using genomic DNA of human corneal fibroblasts as the template, the forward primer of 5'-GTACGGTACCCATCAAAGAAGTTATGGTTC-3' and the reverse primer of 5'-GTACAAGCTTACTCAACTTCAACTCAGCGC-3'. The amplified TGF-β RII promoter fragment was then digested with Kpn I and Hind III, gel-purified (Qiagen, Valencia, CA), and inserted into the same sites of pGL3-basic. Replication-defective adenoviral viruses were generated for each promoter construct by Core Lab of University of Michigan according to a previously published method (Chen et al., J Immunol. 167:1297-1305, 2001; He et al., Proc Natl Acad Sci USA 95:2509-2514, 1998).

[00325] In 2 x 100 mm plastic dishes, human corneal fibroblasts (passage 1-4) were cultured in DMEM/10% FBS. When cells reached ~80 % confluence (~8 x 10^5 cells/dish), the medium was removed, and cells were washed

twice with the respective medium. The fresh medium containing replication-defective adenoviruses containing TGF-β1, TGF-β2, TGF-β3 or TGF-βRII promoters, each linked to luciferase (each at a multiplicity of infection (MOI) of 100) and a control adenovirus containing CMV-β-galactosidase (at a MOI of 30), was incubated with cells for 4 h. The medium containing adenoviruses was then removed and cells were trypsinized for 5 min at room temperature with pre-warmed trypsin/EDTA. Cells were collected into 15 ml sterile tubes and centrifuged at 1000 rpm (~600 x g) for 5 min. The medium was carefully removed and cells were washed once with the respective medium. The cell pellet was resuspended into the medium at the desired density. A total volume of 100 µl of cell suspension (5000 cells) with or without different amounts (e.g., 25 µg/ml) of water-soluble AM extract (prepared by the method described herein for preparing water-soluble AM extract) or other reagents were transferred to each well of a 96-well plate.

[00326] Cells were further cultured for 44 h (a total time of 48 h for adenovirus transduction). After that, the medium was removed and cells were rinsed with PBS once. Cells from each 96 well were lysed in 50 µl of Cell Culture Lysis Reagent (Promega. Madison, WI). Cell lysate was vortexed for 15 sec and cleared by centrifuging at 4° C for 2 min at 12,000 x g. The supernatant was transferred to a clean tube. 20 µl of the supernatant was either used for a luciferase assay or a β-galactosidase assay. For a luciferase assay, 100 µl of the luciferase assay reagent (Promega, Madison, WI) was dispensed into each well and 20 µl of cell lysate was added and mixed by pipeting 3 times. The luciferase analyzer (FusionTM, Packard Instrument Company, Boston, MA) was programmed to perform a 10-sec measurement. For a high sensitivity β-galactosidase assay (Stratagene, LaJolla, CA), 20 µl of cell lysate was mixed with 130 µl of chlorophenol red-β-D-galactopyranoside (CPRG) substrate in each well and incubated for 30 min to 2 h at 37° C until the sample turned red. The reaction was stopped and the absorbance of each sample was read at 570 nm. The β-galactosidase activity of each sample was calculated and used to normalize the luciferase activity as relative luciferase activity.

Results

AM Decreased NO Production by Macrophage Raw264.7 Cells

[00327] Raw264.7 cells were seeded in each 24 well at 2.5 x 10^5 in DMEM/ITS (n=3 for each group). After cells were stimulated with or without 200 u/ml of IFN-γ, the culture medium was collected for NO assay as described in Methods. Without IFN-γ stimulation, macrophages barely produced detectable NO when cultured on either plastic or the stromal surface of intact amniotic membrane (iAM) (Fig. 30). After IFN-γ stimulation, macrophages produced much more NO in both cultures, but significantly less when macrophages were cultured on the stromal surface of iAM as compared to those cultured on plastic (p=0.048) (Fig. 30).

AM Favored PGD_2 Synthesis while Down-regulated PGE_2 Production

[00328] Without IFN-γ stimulation, Raw264.7 cells produced very little PGD_2 when cultured on the plastic but much more when cultured on iAM, presumably due to PGD_2 released from AM epithelial cells (Fig. 31A). With IFN-γ stimulation, PGD2 production in Raw264.7 cells cultured on both the plastic and iAM increased (Fig. 31A). Without IFN-γ stimulation, Raw264.7 cells produced very little PGE_2 when cultured on the plastic but much more when cultured on iAM, presumably due to PGE_2 released from AM epithelial cells (Fig. 31B). With IFN-γ stimulation, PGE_2 production in Raw264.7 cells cultured on the plastic increased dramatically but PGE_2 production on iAM barely changed (Fig. 31B). The ratio of PGD_2/PGE_2, as an index of anti-inflammatory action, was much higher in Rraw264.7 when cultured on iAM with or without IFN-γ stimulation (Fig. 31C).

AM Suppressed TGF-β1 Promoter Activation in Macrophages

[00329] Without IFN-γ stimulation, TGF-β1 promoter activity in Raw264.7 cells was suppressed on iAM compared with that on the plastic, but not statistically significant (p=0.4) (Fig. 32). With IFN-γ stimulation, TGF-β1 promoter activity in Raw264.7 decreased on both the plastic and iAM. However, the suppression of TGF-β1 promoter activity was much more and was statistically significant when compared with that cultured on plastic ($p<0.01$) (Fig. 32).

AME Induced Macrophage Cell Death

[00330] Raw264.7 cells were seeded at 2 x 10^5 in each 24 well in DMEM/10 % fetal bovine serum. After I hr, 125 μg/ml of AME (in PBS) was added to the culture medium while the same volume of PBS was added to the Ctrl. After 24 hr culture, Raw264.7 cells were attached well in the Ctrl but a lot of cells were floated and appeared to be dead when AME was added (Fig. 33).

In vitro Assay Measuring Macrophage Apoptosis

[00331] An *in vitro* macrophage apoptosis assay was used to measure how AM exerts an anti-inflammatory action based on facilitating apoptosis of an IFN-γ-activated mouse macrophage cell line (Raw 264.7 cells). The Cell Death Detection ELISAPLUS kit was obtained from Roche Diagnostics (Mannheim, Germany). Cell lysates equivalent to 10^4 cells and conditioned media after cultivation were subjected to Cell Death Detection ELISAPLUS assay according to the manufacturer's instructions. This ELISA is a photometric enzyme-immunoassay for the qualitative and quantitative *in vitro* determination of cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleosomes) generated by apoptotic cell death using mouse monoclonal anti-histone and anti-DNA antibody. Positive and negative controls were included as provided by the manufacturer, and the absorbance was measured at 405 nm using FusionTM universal microplate analyzer. Macrophages were cultured on the stromal side of the cryopreserved intact AM that was prepared by collecting human placenta, harvesting AM from the placenta, flattening the AM onto nitrocellulose paper (Hybond N+, Amersham, England) with the epithelium side up, and storing the AM at -80° C in DMEM/glycerol (1:2 v/v) until use. To culture macrophages on AM stromal matrix, the macrophages were seeded on the stromal side of the intact cryopreserved AM.

[00332] The results showed that a small amount of apoptosis was detected in plastic cultures after 48 h in DMEM+ITS, and the apoptosis decreased when macrophages were activated by IFN-γ. Macrophages cultured on cryopreserved intact AM also showed some apoptosis, but there was no significant difference between AM and plastic. In contrast, macrophages activated by IFN-γ showed a significant increase of apoptosis when cultured on AM. Results of this assay were correlated with those obtained by LIVE/DEAD assay staining (Molecular Probes, Carlsbad, CA), Hoechst-33342 nuclear staining, and DeadEnd™ fluorometric terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL) method (Promega, Madison, WI) as described herein.

[00333] Macrophages cultured on plastic (PL) distributed evenly, and most were oval, but following activation with 200 U/ml IFN-γ (PL/IFN-γ) cells became larger and spindle. In contrast, macrophages cultured on AM stromal matrix aggregated in clusters and remained round, and upon IFN-γ activation (AM/IFN-γ) cells shrank and were degenerated into debris. These dramatic morphological changes caused by AM were correlated with marked promotion of macrophage apoptosis measured by Cell Death Detection ELISA with or without IFN-γ activation for 48 h. (FIG. 1, $p > 0.05$ for PL vs. AM, $p < 0.05$ for PL vs. PL/IFN-γ and AM vs. AM/IFN-γ, $p < 0.01$ for PL/IFN-γ vs. AM/IFN-γ). This result indicated that macrophages cultured on AM stroma altered their morphology suggestive of cell death after being activated by IFN-γ.

[00334] To determine if the aforementioned morphological changes on AM cultures indeed represented cell death, cells in parallel experiments were subjected to LIVE/DEAD assay. Cells cultured on plastic with or without activation by IFN-γ and cells on AM without IFN-γ for 48 h were predominantly alive (i.e., yielding green fluorescence). In contrast, a large number of cells on AM after IFN-γ activation were dead (i.e., yielding red fluorescence).

[00335] To determine if the aforementioned cell death was mediated by apoptosis, cells in parallel experiments were subjected to Hoechst-33342 staining. Fragmented nuclei with strong Hoechst staining were found more in AM/ IFN-γ as compared to AM without IFN-γ and to their plastic controls. Percentages of Hoechst-33342-positive apoptotic nuclei were 12.4 ± 2.7% (PL), 16.5 ± 3.1% (PL/ IFN-γ), 14.7 ± 1.1% (AM), and 63.8 ± 7.9% (AM/ IFN-γ). There was no statistical difference between PL and PL/ IFN-γ and between PL and AM (all $p > 0.05$), while there was a statistical difference between AM and AM/ IFN-γ and between PL/ IFN-γ and AM/ IFN-γ (all $p < 0.001$). Furthermore, Hoechst-33342 staining also revealed multinucleated giant cells in plastic cultures when activated by IFN-γ. In contrast, there was no such giant cell in AM/ IFN-γ cultures.

[00336] To verify that such fragmented nuclei were indeed caused by DNA fragmentation, cells in parallel experiments were subjected to TUNEL assay staining. TUNEL-positive fragmented nuclei were sporadic in PL, PL/IFN-γ, and AM without IFN-γ cultures, but markedly increased in AM/IFN-γ cultures. This result was consistent with that shown by Hoechst-33342 staining.

AM Induces Apoptosis of IFN-γ Activated Macrophages in vitro by Interrupting Survival Pathways Mediated by NF-κB and Akt

[00337] It has been reported that endogenously generated or exogenously applied NO can induce apoptotic cell death in macrophages. To determine whether NO was responsible for the aforementioned apoptosis of macrophages cultured on AM stromal matrix, nitrite concentrations were measured in the conditioned media collected at the end of different culture durations from 24 h to 72 h, while TNF-α production was also detected. The results showed that macrophages cultured on PL or AM without IFN-γ activation produced low levels of NO and TNF-α, and there was no difference of NO production between plastic and AM through out the culture duration (all $p>0.05$), while TNF-α concentration was higher on AM than on plastic at all time points (all $p<0.05$). However, when activated with IFNγ, macrophages cultured on plastic continuously produced increasing levels of NO and TNF-α from 24 h to 72 h. In contrast, although macrophages cultured on AM also increased production of NO and TNF-α from 24 h to 48 h, there was no increase in their levels between 48 h and 72 h ($p>0.05$), indicating that the production of NO and TNF-α had ceased after 48 h. NO concentration was higher on AM than that on plastic at 24 h and 48 h ($p<0.01$), while TNF-α concentration was higher on AM at 24 h only ($p<0.01$). The control of incubating AM alone in the culture medium with or without IFN-γ for 48 h did not reveal any detectable level of NO or TNF-α in the supernatant, indicating AM itself did not produce NO and TNF-α. Taken together, these data also indicated that AM acted synergistically with IFN-γ to produce more NO, and AM itself weakly induced TNF-α production by macrophages. The nitrite level promoted by IFN-γ was more pronounced when cells were cultured on AM as compared to plastic at 48 h ($p<0.01$), a finding correlating well with that of apoptosis.

[00338] To determine whether a higher level of NO and apoptosis were causatively related, NO production was blocked by adding NG-monomethyl-L-arginine acetate (L-NMMA) or L-N6-(l-iminoethyl) lysine hydrochloride (L-NIL), which are inhibitors of NO synthase. The results showed that 500 μM L-NMMA or L-NIL significantly attenuated IFN-γ induced nitrite levels more than 50% for cells cultured on AM, i.e., from the baseline of 46.2 ± 4.3 μM to 19.8 ± 4.9 μM and 14.0 ± 9.8 μM for L-NMMA and L-NIL, respectively (both $p<0.01$). Nevertheless, the extent of macrophage apoptosis as determined by Hoechst 33342 staining was not significantly attenuated

Accordingly, these results collectively indicated that macrophage apoptosis on AM under the activation of IFN-γ was not mediated by overproduction of NO.

[00339] To further investigate the mechanism of macrophage apoptosis on AM, two major cell survival signaling pathways were studied, NF-κB and Akt-FKHR signaling pathways. These pathways were studied by determining the expression of total IKK-α, IKK-β, p65 (RelA) subunit of NF-κB, Akt, phospho-Akt (Ser473), and phospho-FKHR (Thr24)/FKHRL1 (Thr32) from macrophages cultured on plastic or AM with or without IFN-γ for 24 h, when quantitative ELISA for apoptosis was not apparent. Western blot analyses showed that the p65 (RelA) subunits of NF-κB and total Akt were slightly up-regulated in macrophages cultured on plastic with IFN-γ activation. The levels of IKK-α, IKK-β total Akt, and phosphor-Akt (Ser473) were only down-regulated in macrophages cultured on AM with IFN-γ activation, while the p65 (RelA) subunits of NF-κB and phospho-FKHR (Thr24)/FKHRL1 (Thr32) were down-regulated in macrophages cultured on AM without IFN-γ activation, and further down-regulated when activated with IFN-γ. These results indicated that these two signaling pathways were both involved in the apoptosis of macrophages cultured on AM.

Example 7: Comparing the Relative Potency of Using Collagen or HA as a Vehicle to Deliver Two Different AM Extracts

[00340] To determine the optimal concentration of the water-soluble and lyophilized forms of AM extracts (prepared by the methods described herein for preparing water-soluble and lyophilized forms of AM extracts, respectively) and compare the relative potency between the two different vehicles containing an appropriate concentration of each form of AM extracts in suppressing TGF-β promoter activity and in promoting macrophage apoptosis, respectively, these two forms of AM extracts are compared by serial dilution in either type I collagen gel or HA and their protein concentrations are monitored accordingly. For type I collagen gel, the protein concentration varies from 0.05 to 2 mg/ml; for HA gel, the concentration varies from 0.05 to 10 mg/ml. These serially diluted solutions or gels of these two forms of AM extracts are pre-coated on plastic dishes before human corneal fibroblasts are seeded or added directly in DMEM with 10% FBS while cells are seeded on the plastic. The anti-scarring effect is measured by assaying the promoter activity of TGF-β1, β2, β3 and RII and comparing the promoter activity to the positive or negative controls where cells are seeded on plastic with or without a given form of AM extracts (without the vehicle), respectively. The positive control, in which cells were seeded on plastic with DMEM plus 10% FBS, showed a high promoter activity. In contrast, the negative control, in which cells were seeded on plastic with DMEM plus 10% FBS but added with 25 μg/ml AM extracts, showed at least 50% reduction of the promoter activity. Based on these control values, the experimental groups using different concentrations of AM extracts mixed in either collagen gel or HA can be measured.

[00341] Once the most effective concentration of these two forms of AM extracts in either collagen or HA is determined, the results are verified by repeating the experiment in serum-free DMEM with ITS added with 10 pg/ml to 5 ng/ml TGF-β1. The anti-scarring effect is further correlated with suppression of Smad-mediated signaling by immunocytolocalization of Smads 2, 3 and 4 and α-smooth muscle actin (α-SMA), a marker for myofibroblasts (Gabbiani G., J Pathol. 200:500-503, 2003; Jester and Petroll, Prog Retin Eye Res. 18:311-356, 1999). Another positive control is performed by adding 10 μg/ml neutralizing antibody to all three isoforms of TGF-β. The anti-inflammatory effect is similarly tested in murine macrophages with or without activation by 200 U/ml IFN-γ in DMEM with ITS by measuring the extent of apoptosis using Cell Death Detection ELISAPLUS kit (Roche, Mannheim, Germany), and correlating the data with those obtained by cell morphology, LIVE/DEAD assay (Molecular Probes, Carlsbad, CA), Hoechst-33342 nuclear staining, and TUNEL assay (Promega, Madison, WI) as recently reported (Li et al., Exp Eye Res. 2005, In Press).

Example 8: Amniotic Membrane Stromal Extract has Anti-Angiogenic Properties

Materials and Methods

Materials

[00342] HUVECs and endothelial cell growth medium were from PromoCell GmbH (Heidelberg, Germany). Cell proliferation kit I (MTT) was from Roche (Penzberg, Germany). BCA™ protein assay kit was from Pierce (Rockford, IL). Dulbecco's modified Eagle's medium (DMEM), Ham's/F12 medium, HEPES buffer, Hank's balanced salt solution (HBSS), phosphate-buffered saline (PBS), amphotericin B, gentamicin, fetal bovine serum (FBS), vascular endothelial growth factor(VEGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), 0.25% trypsin/0.53 mM EDTA, LIVE/DEAD assay reagent were purchased from Invitrogen (Carlsbad, CA). Propidium iodide, Hoechst-33342 dye, triton X-100, bovine serum albumin (BSA), insulin, hydrocortisone, formaldehyde, crystal violet, FITC conjugated anti-mouse, goat, and rat IgG were from Sigma (St. Louis, MO). Transwell inserts were from Corning Incorporated (Corning, NY). Matrigel was from BD Biosciences (Bedford, MA).

Cell Culture

[00343] HUVECs were cultured in the endothelial cell growth medium supplemented by 2% fetal calf serum, 0.1 ng/mL EGF, 1 μg/mL hydrocortisone, and 1 ng/mL basic fibroblast growth factor (bFGF). Human corneoscleral tissues were obtained from the Florida Lions Eye Bank (Miami, FL), from which corneal fibroblasts (HCFs) were harvested and cultured in DMEM containing 10% FBS. Passage 2 cells were used for the experiments. SV40-immortalized rabbit corneal epithelial cells (RCEs), kindly provided by Dr. Peter Reinach (Department of Biological Science, College of Optometry, State University of New York, New York), were grown in DMEM/F12 containing 10% FBS, 5 ng/mL insulin, and 5 ng/mL EGF.

Preparation of Water-soluble AM Stromal Extracts

[00344] Using aseptic techniques, frozen human AM obtained from Bio-Tissue, Inc. (Miami, FL) was briefly washed 2-3 times with HBSS to remove the original storage medium. The AM stroma was scraped by spatula, frozen in the air phase of liquid nitrogen and grounded to fine particles by BioPulverizer (Biospec Products, Inc., Bartlesville, OK) followed by homogenization on ice with Tissue Tearor (Biospec Products, Inc., Dremel, WI) in PBS, pH 7.4, for 1 min. The homogenate was mixed by rotation for 1 h and centrifuged at 14,000 x g for 30 min at 4°C. The supernatant in PBS was then collected, and stored in aliquots at -80°C. The protein concentration was determined by BCA assay. This water-soluble protein extract, designated as amniotic stromal extract (ASE), was used for experiments described herein.

Cell Proliferation Assay

[00345] Cells were seeded in 96-well plates (n = 6) in the respective growth medium mentioned above at the density of 2,000 cells per well with increasing concentrations of ASE and cultured for 48 h. The cell growth was determined by the MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium bromide)-based cell proliferation assay according to a protocol recommended by the manufacturer.

Apoptosis Assay

[00346] LIVE/DEAD assay and Hoechst-33342 staining were used for detecting apoptotic cells. Briefly, after 48 h of culturing, each respective growth medium was removed from the culture, added with 200 μl of the combined LIVE/DEAD assay reagents, and incubated for 15 min at room temperature. Live cells are distinguished by green fluorescence staining of the cell cytoplasm, while dead cells are stained with red fluorescence in the nuclei. To characterize the nuclear morphology as a means to assess apoptosis, Hoechst-33342 dye was added to each respective growth medium at a final concentration of 10 μg/mL, and incubated at 37°C for 15 min. Hoechst-33342

stains the nuclei of live cells and identifies apoptotic cells by the increased fluorescence and nuclear fragmentation or condensation.

Migration Assay

[00347] The inhibitory effect of ASE on VEGF-induced chemotaxis was tested on HUVECs using the transwell assay. HUVECs were grown in the complete growth medium for 36 h. After trypsinization, washing, and resuspension of cells in the incomplete growth medium supplemented with 0.5% FBS, 60,000 cells in 200 μL were seeded on the inside of the transwell inserts (8 mm in diameter), which is separated by a polycarbonate membrane of 8-μm pore size, with or without 200 μg/mL of ASE. The growth medium in the volume of 800 μL supplemented with 1% FBS and 10 ng/mL VEGF was added as a chemotactant directly in 24-well plate outside of the transwell insert. The growth medium supplemented with 0.5% FBS was added directly in 24-well plate as the negative control. After incubation at 37°C with 5% CO_2 and 95% humidity for 4.5 h, non-migrated cells were aspirated, while the membrane was washed with PBS, fixed in 4% formaldehyde in PBS, stained with crystal violet, and observed under the microscope. Cells migrating to the other side of the membrane were counted.

Endothelial Tube Formation Assay

[00348] Matrigel was added in 320 μL to each well of a 24-well plate and allowed to polymerize at 37°C for 30 min. HUVECs (50,000 cells/well) in the endothelial cell culture medium with 10% FBS were seeded on Matrigel in the presence of different concentrations of ASE. Control cells were incubated with BSA at the same concentration. Cells were incubated for 24 h at 37°C and photographed under a microscope (Nikon, Japan). Ten random 40× fields were photographed for each well, and tubes were counted, averaged, and compared.

Statistical Analysis

[00349] All experiments described above were repeated three times, each in triplicate or more. Group means were compared using the appropriate version of Student's unpaired t-test. Test results were reported as two-tailed p values, where $p < 0.05$ was considered statistically significant. Summary data are reported as means ± S.D.

Results

ASE preferentially inhibited proliferation of HUVEC cells

[00350] We first tested whether ASE added to the culture medium would inhibit cellular proliferation. As compared to the control without addition of ASE, proliferation of HUVECs was significantly inhibited by 50 to 200 μg/mL of ASE (Fig. 34A, $p<0.001$). However, proliferation of RCEs and HCFs was significantly inhibited by ASE only at the concentration of 200 μg/mL (Fig. 34B and 34C, respectively, each $p<0.01$). This result indicated that ASE preferentially inhibited proliferation of HUVECs

ASE induced apoptosis in HUVEC cells

[00351] Phase contrast images showed that HUVEC cells shrank from a spindle shape to a small and round shape and the cell density was notably reduced when treated with 200 μg/mL of ASE for 48 h. In contrast, cell morphology and density of HCF and RCE cells were not changed when treated with 200 μg/mL of ASE for 48 h (Data not shown). To determine whether such a change in HUVEC cells caused by 200 μg/mL of ASE was accompanied by cell death, the LIVE/DEAD assay and Hoechst-33342 staining were performed after incubation with 200 μg/mL of ASE for 48 h. The results showed that HUVEC cells were alive in the control without addition of ASE (Fig. 35Aa) but showed pronounced cell death after ASE treatment (Fig. 35Ad). In contrast, both HCF and RCE cells did not reveal any notable cell death in cultures without (Fig. 35Ab and 35Ac, respectively) or with (35Ae and 35Af, respectively) ASE treatment. Hoechst-33342 staining showed that ASE-treated HUVECs had 61.6 ± 7.7% of condensed and fragmented nuclei (Fig. 35Bd), which was significantly higher than 3.1 ± 1.8% of the control without ASE treatment (Fig. 35Ba, also see 2C, $p<0.001$). In contrast, there was no obvious apoptosis in

either HGFs or RCEs without (Fig. 35Bb and 35Bc, respectively) or with (Fig. 35Be and 35Bf, respectively) ASE treatment (also see Fig. 36, p=0.84 and 0.30, respectively). These results indicated that ASE inhibited proliferation of HUVECs by inducing apoptosis.

ASE inhibited migration of HUVEC cells with or without VEGF stimulation

[00352] We then tested whether ASE could inhibit HUVEC migration stimulated by VEGF. Some cells migrated through pores of the polycarbonate membrane in the control without VEGF as a chemoattractant during the 4.5 h testing period (Fig. 37A). Cell migration increased dramatically when treated with VEGF as a chemoattractant (Fig. 37B, $p<0.001$). However, when HUVECs were treated with 200 μg/mL of ASE, the magnitude of cell migration under the influence of VEGF was significantly retarded (Fig. 37C) when compared to either the positive control with VEGF ($p<0.001$) or the negative control without VEGF ($p<0.01$). These results indicated that ASE not only abrogated the chemokine function of VEGF, but also inhibited migration intrinsically present in HUVECs.

ASE inhibited tube formation of HUVEC cells

[00353] To further investigate the anti-angiogenic action of ASE, we performed the in vitro tube formation assay. When HUVECs were seeded on Matrigel, a solid gel of basement membrane proteins, they rapidly aligned and formed hollow tube-like structures after 24 h (Fig. 38A). In contrast, when ASE was added to the culture, tube formation of HUVECs was significantly inhibited at the concentration of either 100 μg/mL (Fig. 38B) or 200 μg/mL (Fig. 38C) (both $p<0.001$). The difference between these two doses of ASE was not statistically significant (Fig. 38D). Collectively, these results indicated that ASE also exerted its anti-angiogenic action by inhibiting the tube formation during the angiogenic process.

Example 9: Skin lotion composition containing AM extract

[00354] A skin lotion is prepared by the following method. 0.25 g methyl hydroxybenzoate and 7.5 g gycerin are dissolved in 75 ml of water at 150°F. 0.7 g sorbitan monolaurate, 0.7 g polysorbate 20, and 1.0 g cetostearyl alcohol are melted at 150°F and are then compounded into the solution. The mixture is allowed to cool while mixing. When the mixture reaches a temperature below approximately 90°F, 4 ml of AM preparations and purified compositions described herein is added while mixing. A trace amount of fragrance is also added while mixing. The lotion is packaged into 10 ml aliquots and stored at room temperature.

Example 10: Topical AM Stromal Matrix Gel Composition and treatment of skin inflammation

[00355] To prepare a pharmaceutical topical gel composition, 100 mg of a freeze-ground, lyophilized AM stromal matrix material is mixed with 1.75 g of hydroxypropyl celluose, 10 mL of propylene glycol, 10 mL of isopropyl myristate and 100 mL of purified alcohol USP. The resulting gel mixture is then incorporated into containers, such as tubes, which are suitable for topical administration. The gel is applied to inflammed skin 2 times per day. By use of this method, the inflammation of the skin decreases.

Example 11: Ophthalmic Solution Composition and Treatment of an Eye Disease

[00356] An opthalmic eye drop solution is prepared by mixing 100 mg of ground, lyophilized AM extract with 0.9 g of NaCl in 100 mL of purified water and filtered using a 0.2 micron filter. The resulting isotonic solution is then incorporated into ophthalmic delivery units, such as eye drop containers, which are suitable for ophthalmic administration. Two drops of the composition is applied to a burn-damaged eye 4 times per day. By use of this method, the eye returns to normal health.

Example 12: Eye ointment composition and treatment of eye disease using same

[00357] A sterile eye ointment composition is prepared by compounding 90 grams white petrolatum, 10 grams liquid petrolatum, and 0.5 grams lyophilized AM powder. The mixture is pasteurized and packaged into individual tube containers of 2.0g each.

[00358] To treat an eye disease using the composition, an aliquot of approximately 0.1 g is gently applied directly from the tube to the inner edge of the bottom eye lid. The ointment is applied 4 times per day. The patient progress is monitored every other week by an opthamologist. By use of this method, the eye disease improves.

Example 13: Treatment of human eye disease using AM preparation

[00359] An individual with burn damage to the eye is identified. A preparation of 1% AM, 0.5% collagen in DMEM is prepared. The individual is treated 4X per day with 2 drops of the composition. By use of this method, the eye damage improves as compared to a non-treated burn damaged eye.

Example 14: Treatment of human skin disease using AM preparation

[00360] An individual with psoriasis is identified. The individual is treated with a 5% preparation of reconstituted AM, supplemented with 1 mg/ml purified Smad7 derived from a commercial source. The formulation is dissolved in a lotion composition. The treatment is administered 2 times per day. By use of this method, the psoriasis is alleviated or disappears.

Example 15: Rectal Gel Composition containing AM extract

[00361] A rectal gel composition is prepared by combining 100 mg of commercially available HA and TSG-6 (purified) with 5 ml sterile AM extract prepared from frozen AM membrane material as described in Example 1. To this mixture is added 2.5 g of methylcelluose (1500 mPa), 100 mg of methylparapen, 5 g of glycerin and 95 mL of purified water. The resulting gel mixture is then incorporated into rectal delivery units, such as syringes, which are suitable for rectal administration.

Example 16: A Kit for treating skin inflammation

[00362] An over-the-counter kit for treatment of skin inflammation is prepared. The kit is made of a package containing a 1 ml container of AM extract in a paste formulation, an applicator, and an instruction sheet.

Example 17: Extended release solid dosage form and use to lessen arthritis inflammation

[00363] An extended release dosage form is prepared by mixing 100 mg of lyophilized, freeze-ground AM stromal matrix with 800 mg methylcellulose and is then microencapsulated in a carboxymethylcelluose coating. Approximately 15 mg of the microencapsulated material is implanted as a bolus under the skin in an inflammed knee joint of an arthritic patient. The implantation process is repeated once a week. By use of this method, the knee inflammation decreases.

Example 18: Parenteral Composition

[00364] A parenteral composition for intramuscular administration is prepared by mixing 10 mg each of: HA, TSG-6, PTX-3, and TSP-1, each of which is obtained from a commercial source, with 100 mg of a water-soluble salt of a compound described herein is dissolved in DMSO and then mixed with 10 mL of 0.9% sterile saline. The mixture is incorporated into a dosage unit form suitable for administration by injection.

Example 19: Treatment of a human tumor by direct injection of an AM parenteral preparation

[00365] A patient having a subcutaneous tumor of approximately 2 cm in width is identified. 10 grams of liquid AM extract is mixed with 10% PEG 300 in water for 2 hours at 4°C. The mixture is filtered through a 0.20 μm filter,

and aliquoted to sterile glass vials which were closed with rubber stoppers and sealed with aluminum caps. The vials are stored at 4°C for up to 2 weeks prior to use. The patient is treated by directly injecting the 0.50 ml solution through the skin into the tumor site, dividing the administration volume into 4 separate regions of the tumor mass (approximately 125 µl to each of the four sites). The composition is administered every 48 hours. The tumor size is monitored weekly. By use of this method the tumor size decreases.

[00366] While preferred embodiments have been shown and described herein, numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein can be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

WHAT IS CLAIMED IS:

1. A purified composition comprising:
   - Cross-linked high molecular weight hyaluronan (HA);
   - Tumor necrosis factor-stimulated gene 6 (TSG-6);
   - Pentraxin (PTX-3); and
   - Thrombospondin (TSP-1).

2. The purified composition of Claim 1, wherein at least portion of the components are prepared from a human amniotic material selected from a human amniotic membrane, a human amniotic jelly, a human amniotic stroma, or a combination thereof.

3. The purified composition of Claim 1, further comprising Smad7.

4. The purified composition of Claim 1, wherein the cross-linking of the HA comprises a covalent bond to a heavy chain of inter-α-trypsin inhibitor.

5. The purified composition of Claim 1, wherein the ratio of protein to HA is less than about 100.

6. The purified composition of Claim 2, wherein the preparation procedure includes
   - Obtaining a frozen or previously-frozen human placenta;
   - Thawing the placenta and isolating the human amniotic material from the thawed placenta; and
   - homogenizing the human amniotic material in a suitable buffer.

7. The purified composition of Claim 6, wherein the preparation procedure further includes:
   - Freezing the human amniotic material; and
   - Grinding the frozen amniotic material.

8. The purified composition of Claim 6, wherein the preparation procedure further comprises:
   - Lyophilizing the homogenate; or
   - Centrifuging the homogenate and isolating the supernatant from the centrifuged homogenate.

9. The purified composition of Claim 8, wherein the preparation procedure further comprises:
   - Lyophilizing the supernatant into powder.

10. The purified composition of Claim 1 further comprising a pharmaceutically acceptable carrier for a non-solid dosage form or an extended release solid dosage form.

11. A method for inhibiting scar formation in a subject comprising the step of providing an effective amount of a scar formation inhibition composition to a subject in need of scar formation inhibition; wherein the scar formation inhibition composition comprises at least one component prepared from a human amniotic material selected from a human amniotic membrane, a human amniotic jelly, a human amniotic stroma, or a combination thereof extracted from an amniotic membrane.

12. The method of Claim 11, wherein at least one component was extracted from the human amniotic material.

13. The method of Claim 12, wherein the human amniotic material is the human amniotic stroma.

14. The method of Claim 12, wherein the extraction procedure comprises:
   - Obtaining a frozen or previously-frozen human placenta;
   - Thawing the placenta and isolating the human amniotic material from the thawed placenta;
   - Homogenizing the human amniotic material in a suitable buffer;
   - Optionally lyophilizing the homogenate to a powder; and
   - Admixing the homogenate or the powder with a pharmaceutically acceptable carrier for a non-solid dosage form or an extended release solid dosage form.

15. The method of Claim 14, wherein the preparation procedure substitutes the step of lyophilizing the homogenate with the step of:

- Centrifuging the homogenate, isolating the supernatant from the centrifuged homogenate, and optionally lyophilizing the supernatant to a powder.

16. A method for reversing scar formation in a subject comprising the step of providing an effective amount of a scar reversal composition to a scarred subject; wherein the scar reversal composition comprises at least one component prepared from a human amniotic material selected from a human amniotic membrane, a human amniotic jelly, a human amniotic stroma, or a combination thereof extracted from an amniotic membrane.

17. The method of Claim 16, wherein at least one component was extracted from the human amniotic material.

18. The method of Claim 17, wherein the human amniotic material is the human amniotic stroma.

19. The method of Claim 17, wherein the extraction procedure comprises:

- Obtaining a frozen or previously-frozen human placenta;
- Thawing the placenta and isolating the human amniotic material from the thawed placenta;
- Homogenizing the human amniotic material in a suitable buffer;
- Optionally lyophilizing the homogenate to a powder; and
- Admixing the homogenate or the powder with a pharmaceutically acceptable carrier for a non-solid dosage form or an extended release solid dosage form.

20. The method of Claim 19, wherein the preparation procedure substitutes the step of lyophilizing the homogenate with the step of:

- Centrifuging the homogenate, isolating the supernatant from the centrifuged homogenate, and optionally lyophilizing the supernatant to a powder.

21. A method for inhibiting angiogenesis in a subject comprising the step of providing an effective amount of an angiogenesis inhibition composition to a subject in need of angiogenesis inhibition; wherein the angiogenesis inhibition composition comprises at least one component prepared from a human amniotic material selected from a human amniotic membrane, a human amniotic jelly, a human amniotic stroma, or a combination thereof extracted from an amniotic membrane.

22. The method of Claim 21, wherein at least one component was extracted from the human amniotic material.

23. The method of Claim 22, wherein the human amniotic material is the human amniotic stroma.

24. The method of Claim 21, wherein the composition comprises:

- Cross-linked high molecular weight hyaluronan (HA);
- Tumor necrosis factor-stimulated gene 6 (TSG-6);
- Pentraxin (PTX-3); and
- Thrombospondin (TSP-1).

25. The method of Claim 22, wherein the extraction procedure comprises:

- Obtaining a frozen or previously-frozen human placenta;
- Thawing the placenta and isolating the human amniotic material from the thawed placenta;
- Homogenizing the human amniotic material in a suitable buffer;
- Optionally lyophilizing the homogenate to a powder; and
- Admixing the homogenate or the powder with a pharmaceutically acceptable carrier for a non-solid dosage form or an extended release solid dosage form.

26. The method of Claim 25, wherein the preparation procedure substitutes the step of lyophilizing the homogenate with the step of:

- Centrifuging the homogenate, isolating the supernatant from the centrifuged homogenate, and optionally lyophilizing the supernatant to a powder.

27. The method of Claim 21, wherein the subject in need is a human with cancer.

28. The method of Claim 21, wherein the subject in need is a human with age-related macular degeneration.

29. The method of Claim 21, wherein the angiogenesis inhibition composition is provided in the form of a non-solid dosage form or an extended release solid dosage form.

30. The method of Claim 21, wherein the angiogenesis inhibition composition has the following properties:

- induces apoptosis of endothelial cells involved in vascular formation;
- prevents migration of endothelial cells involved in vascular formation; and
- prevents tube formation of endothelial cells involved in vascular formation.

31. A method for reducing or preventing inflammation in a subject comprising the step of providing an effective amount of an inflammation inhibition composition to a subject in need of inflammation inhibition or prevention; wherein the inflammation inhibition composition comprises at least one component prepared from a human amniotic material selected from a human amniotic membrane, a human amniotic jelly, a human amniotic stroma, or a combination thereof extracted from an amniotic membrane..

32. The method of Claim 31, wherein at least one component was extracted from the human amniotic material.

33. The method of Claim 32, wherein the human amniotic material is the human amniotic membrane.

34. The method of Claim 31, wherein the composition comprises:

- Cross-linked high molecular weight hyaluronan (HA);
- Tumor necrosis factor-stimulated gene 6 (TSG-6);
- Pentraxin (PTX-3); and
- Thrombospondin (TSP-1).

35. The method of Claim 32, wherein the extraction procedure comprises:

- Obtaining a frozen or previously-frozen human placenta;
- Thawing the placenta and isolating the human amniotic material from the thawed placenta;
- Homogenizing the human amniotic material in a suitable buffer;
- Optionally lyophilizing the homogenate to a powder; and
- Admixing the homogenate or the powder with a pharmaceutically acceptable carrier for a non-solid dosage form or an extended release solid dosage form.

36. The method of Claim 35, wherein the preparation procedure substitutes the step of lyophilizing the homogenate with the step of:

- Centrifuging the homogenate, isolating the supernatant from the centrifuged homogenate, and optionally lyophilizing the supernatant to a powder.

37. The method of Claim 31, wherein the human has arthritis.

38. The method of Claim 31, wherein the human has inflammation in at least one eye.

39. The method of Claim 31, wherein the inflammation inhibition composition is provided as a non-solid dosage form or an extended release solid dosage form.

40. The method of Claim 31, wherein the inflammation inhibition composition has at least two of the following properties:

- induces apoptosis of macrophages at the site of inflammation;

- increases the ratio of prostaglandin D2 to prostaglandin E1 at the site of inflammation;
- suppresses TGF-β1 activity at the site of inflammation; or
- inhibits interferon-gamma signal transduction at the site of inflammation.

5

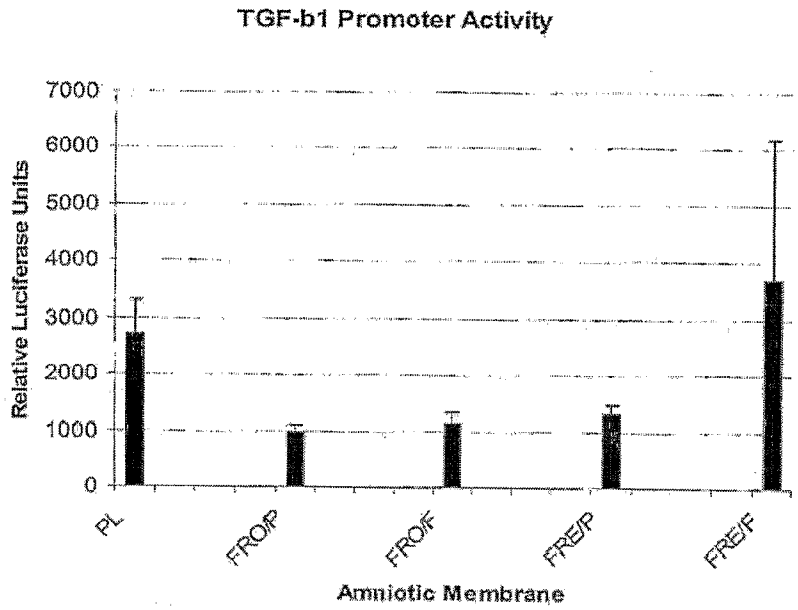


Fig. 1A

P Value Comparison on Two Sides

	PL	FRO/P	FRO/F	FRE/P	FRE/F
PL	X	x	x	x	x
FRO/P	0.007692	x	x	x	x
FRO/F	0.007268	0.30195	x	x	x
FRE/P	0.012165	0.024442	0.23750452	x	x
FRE/F	0.500177	0.115462	0.12833157	0.14812627	x

Fig. 1B

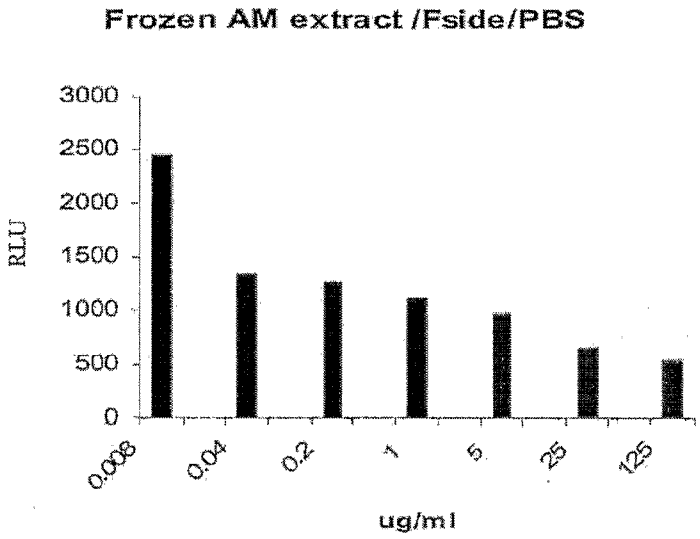

Frozen AM extract /Fside/PBS
RLU
3000
2500
2000
1500
1000
500
0
0.008
0.04
0.2
1
5
25
125
ug/ml

Fig. 2

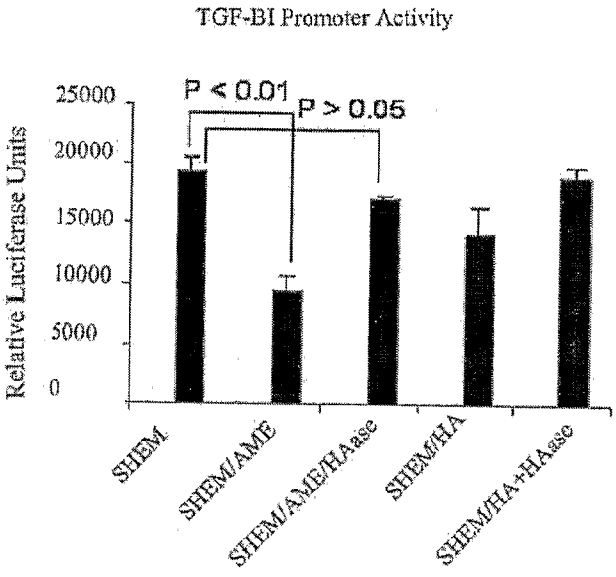

TGF-BI Promoter Activity
Relative Luciferase Units
25000
20000
15000
10000
5000
0
P < 0.01
P > 0.05
SHEM
SHEM/AME
SHEM/AME/HAase
SHEM/HA
SHEM/HA+HAase

Fig. 3

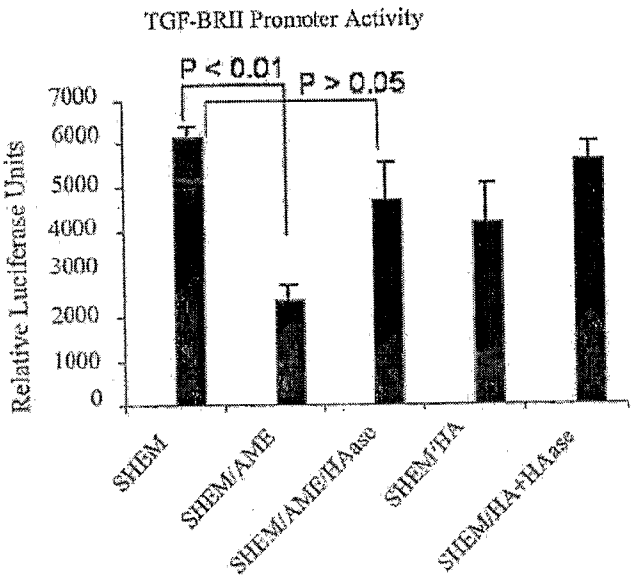

TGF-BRII Promoter Activity
P < 0.01
P > 0.05
Relative Luciferase Units
7000
6000
5000
4000
3000
2000
1000
0
SHEM
SHEM/AME
SHEM/AME/HAase
SHEM/HA
SHEM/HA+HAase

Fig. 4

Comparison of AM vs Jelly and Low vs High Speed

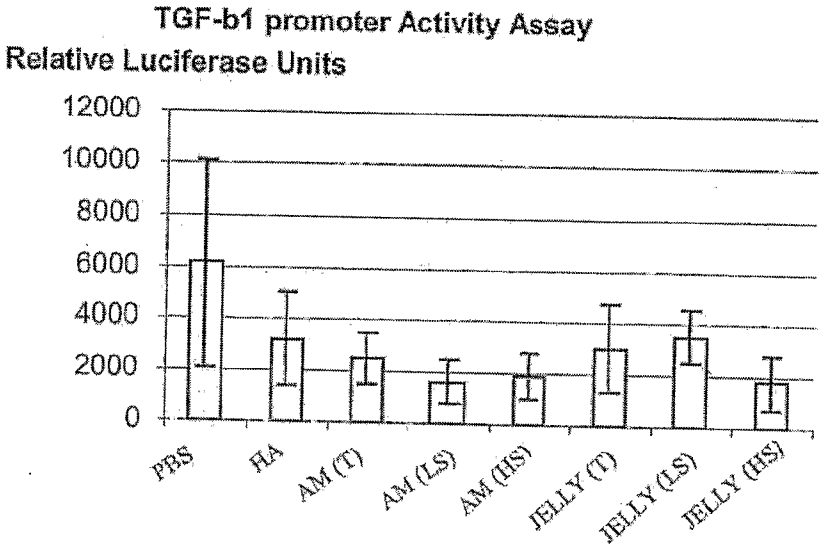


Fig. 5

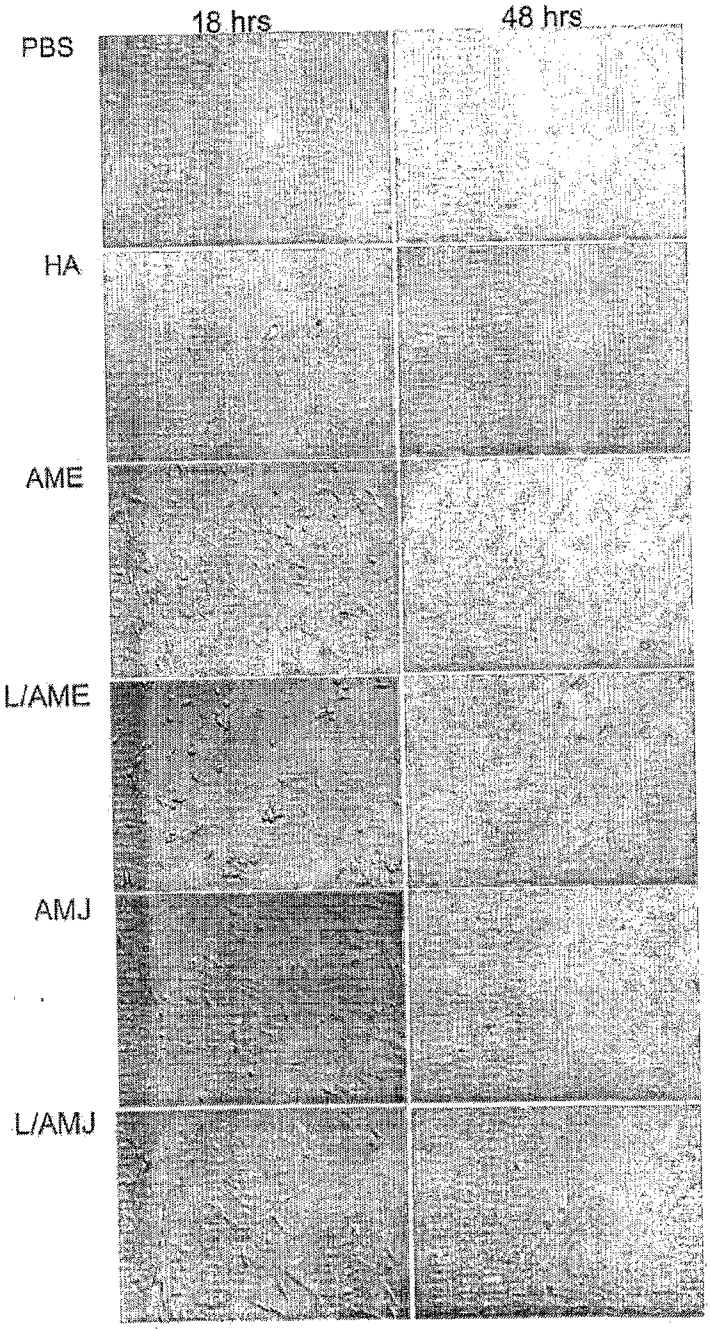


Fig. 6

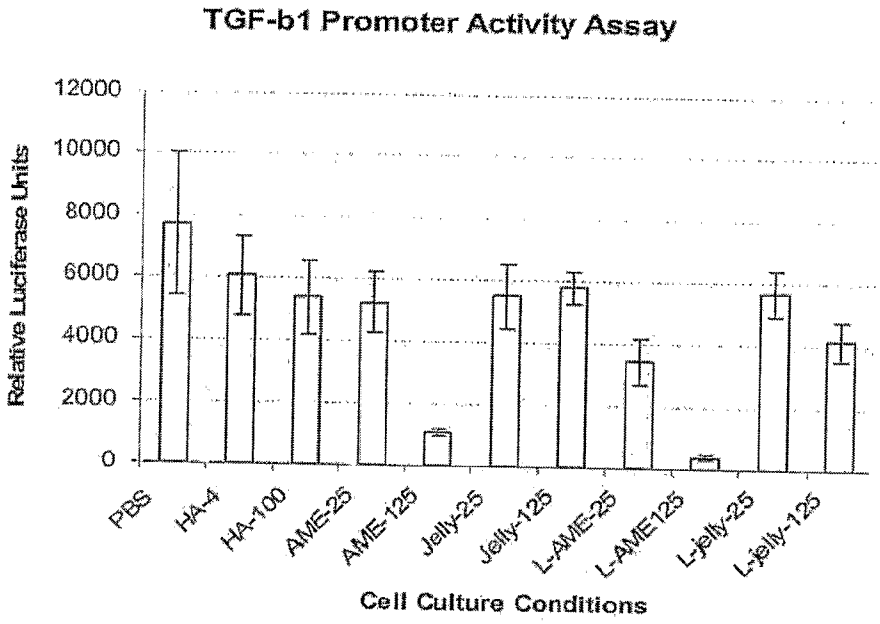

TGF-b1 Promoter Activity Assay
Relative Luciferase Units
12000
10000
8000
6000
4000
2000
0
PBS
HA-4
HA-100
AME-25
AME-125
Jelly-25
Jelly-125
L-AME-25
L-AME125
L-jelly-25
L-jelly-125
Cell Culture Conditions

Fig. 7

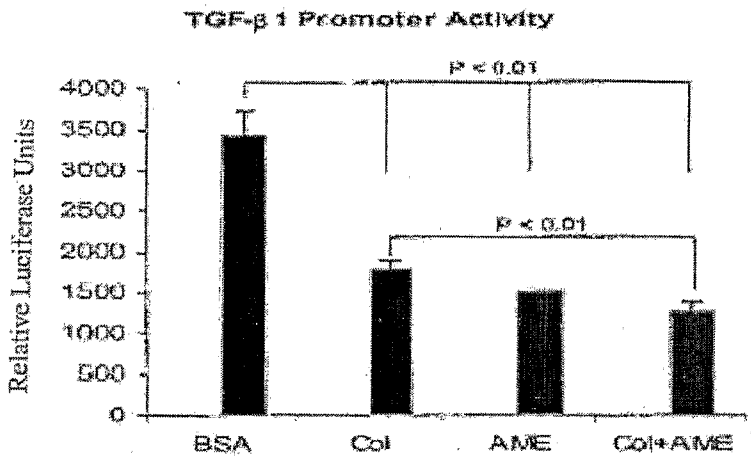

TGF-β 1 Promoter Activity
P < 0.01
P < 0.01
Relative Luciferase Units
4000
3500
3000
2500
2000
1500
1000
500
0
BSA
Col
AME
Col+AME

Fig. 8

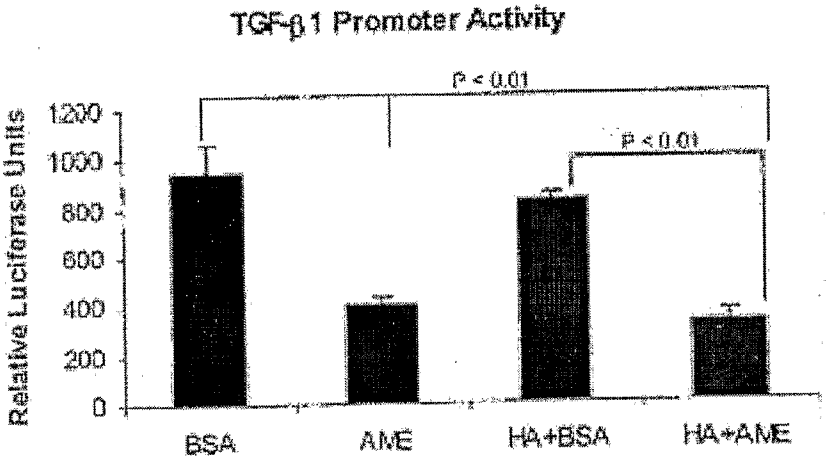

TGF-β1 Promoter Activity
P < 0.01
P < 0.01
Relative Luciferase Units
1200
1000
800
600
400
200
0
BSA
AME
HA+BSA
HA+AME

Fig. 9

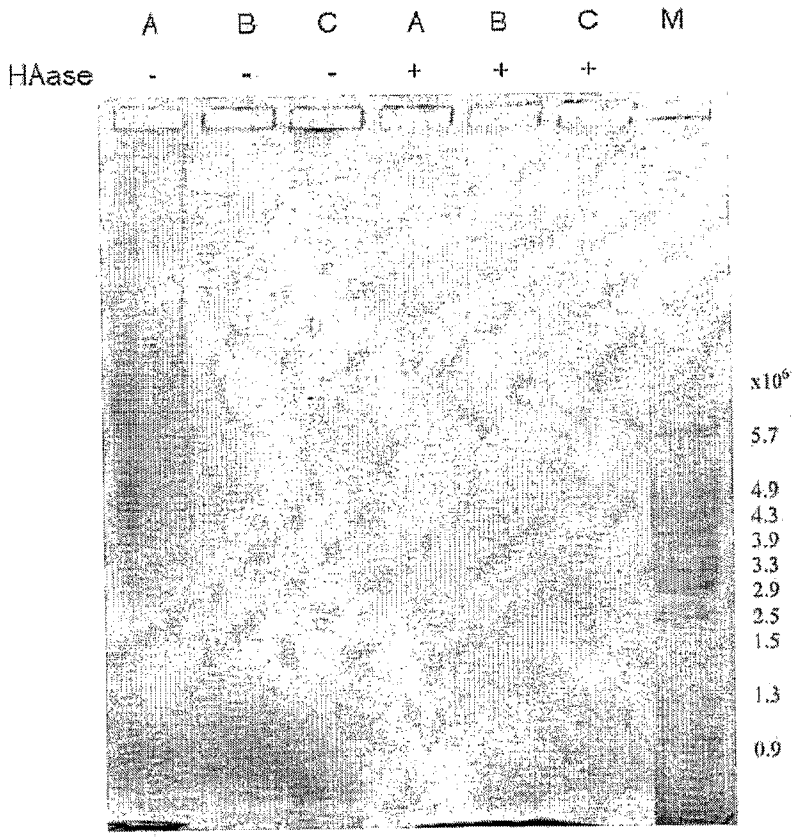


Fig. 10

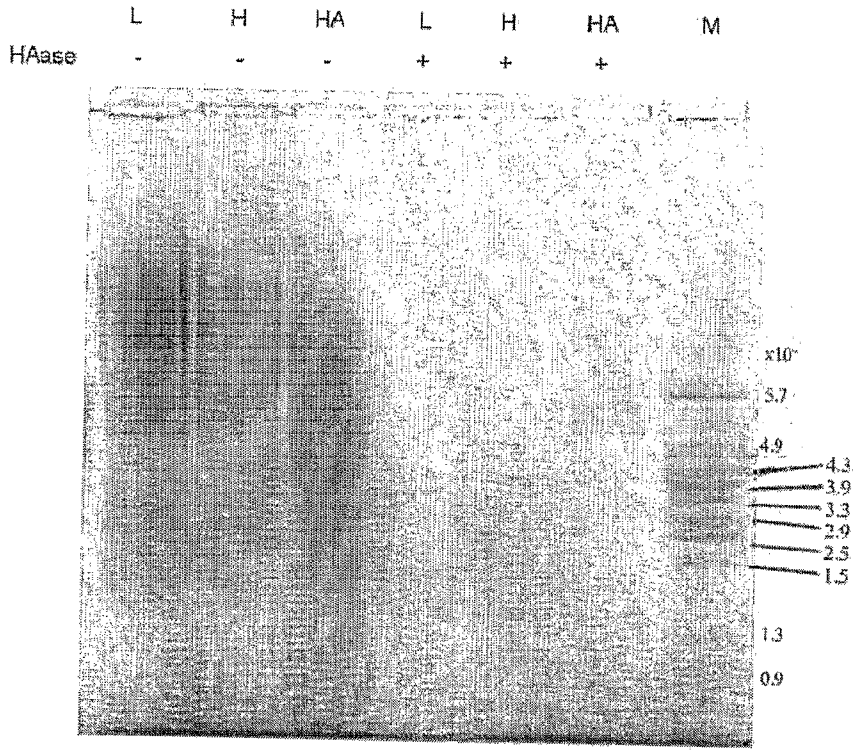


Fig. 11

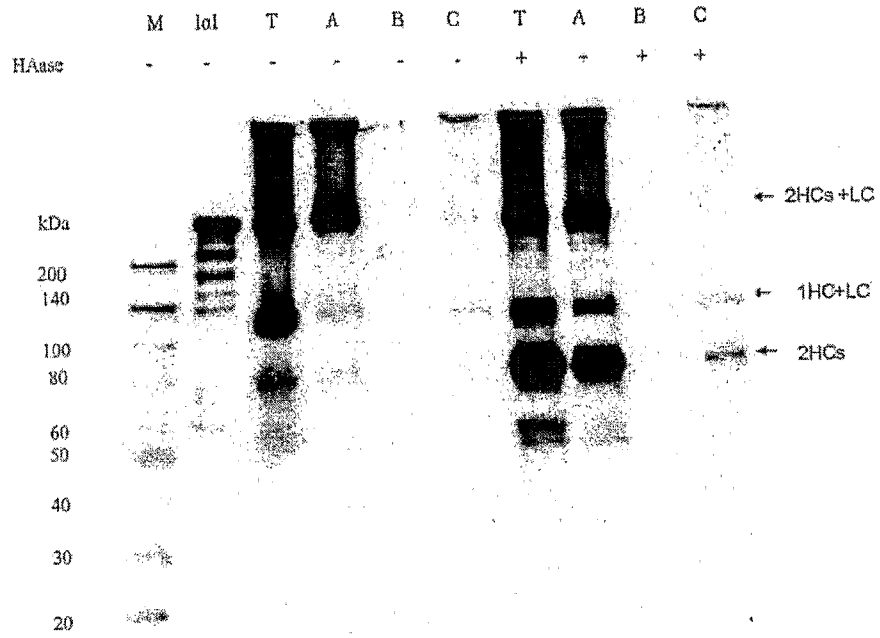

M
lol
T
A
B
C
T
A
B
C
HAase
- - - - - - + + + +
kDa
200
140
100
80
60
50
40
30
20
2HCs +LC
1HC+LC
2HCs

Fig. 12

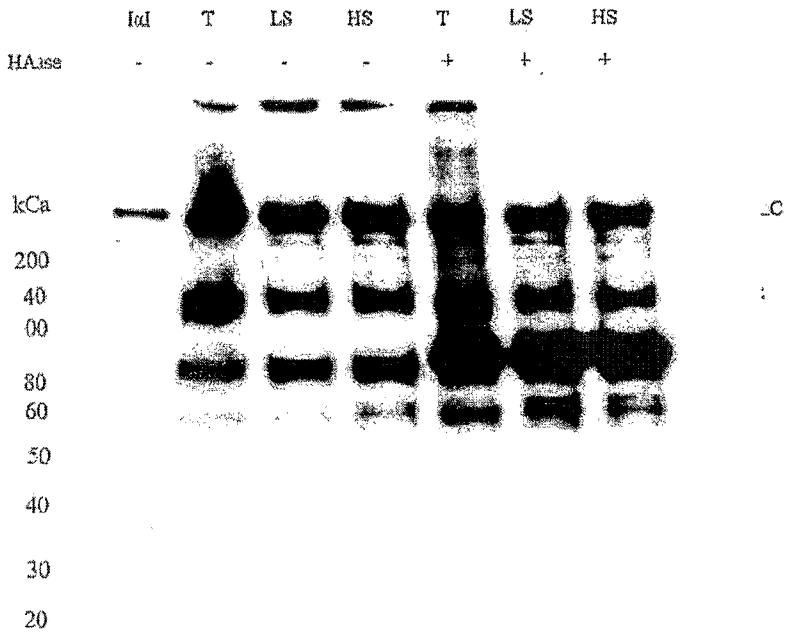


Fig. 13

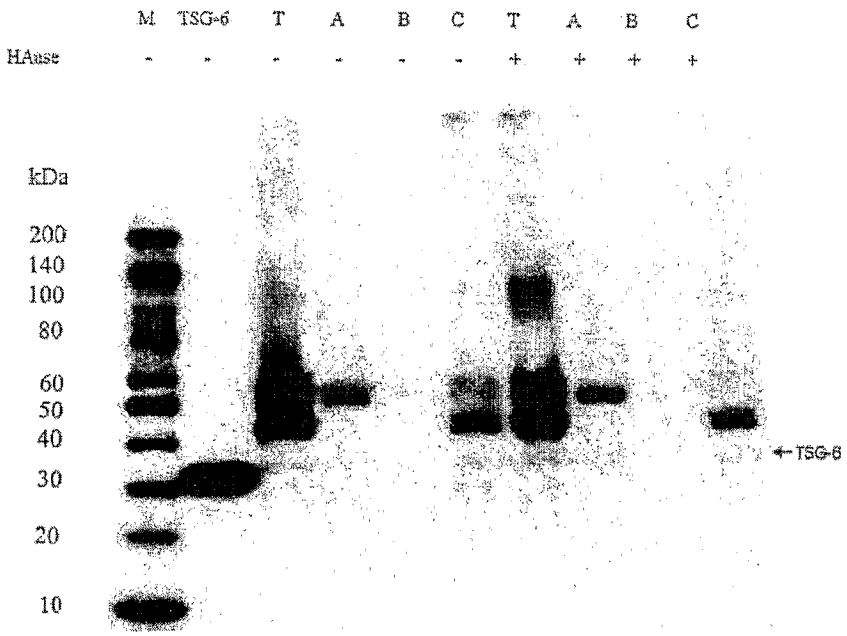


Fig. 14

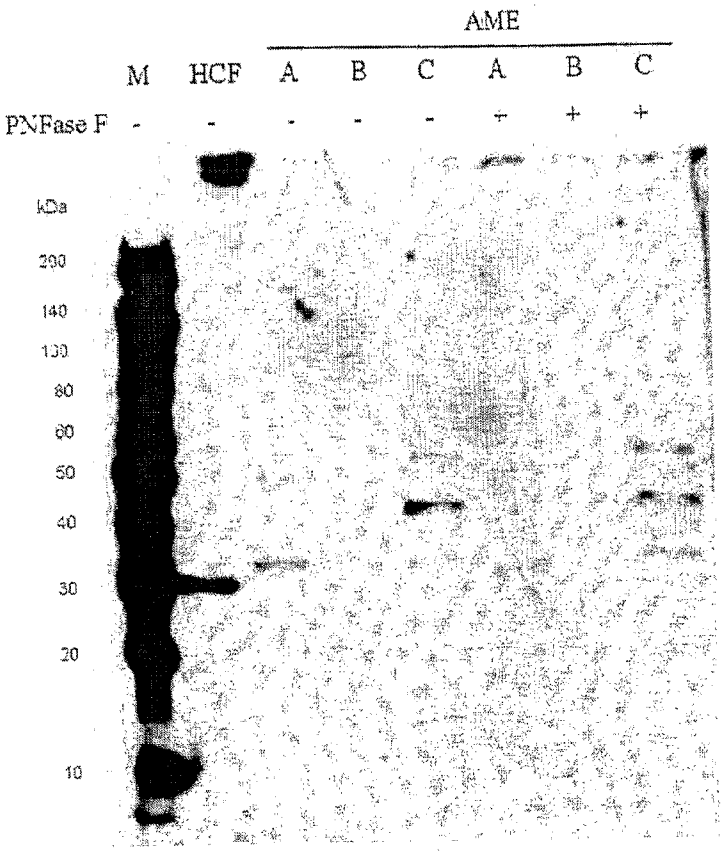


Fig. 15

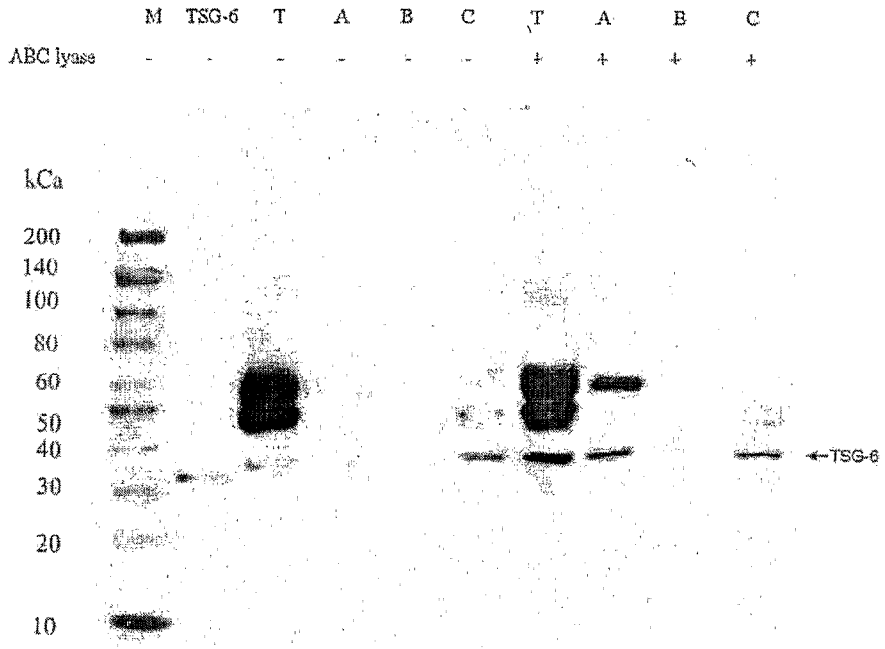

M
TSG-6
T
A
B
C
T
A
B
C
ABC lyase
- - - - - - + + + +
kCa
200
140
100
80
60
50
40
30
20
10
←TSG-6

Fig. 16

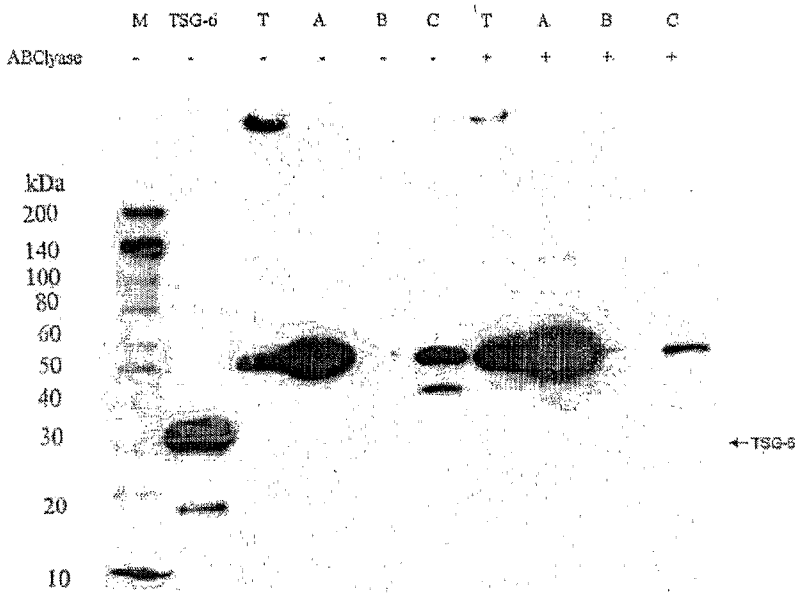

M
TSG-6
T
A
B
C
T
A
B
C
ABClyase
- - - - - - + + + +
kDa
200
140
100
80
60
50
40
30
20
10
←TSG-6

Fig. 17

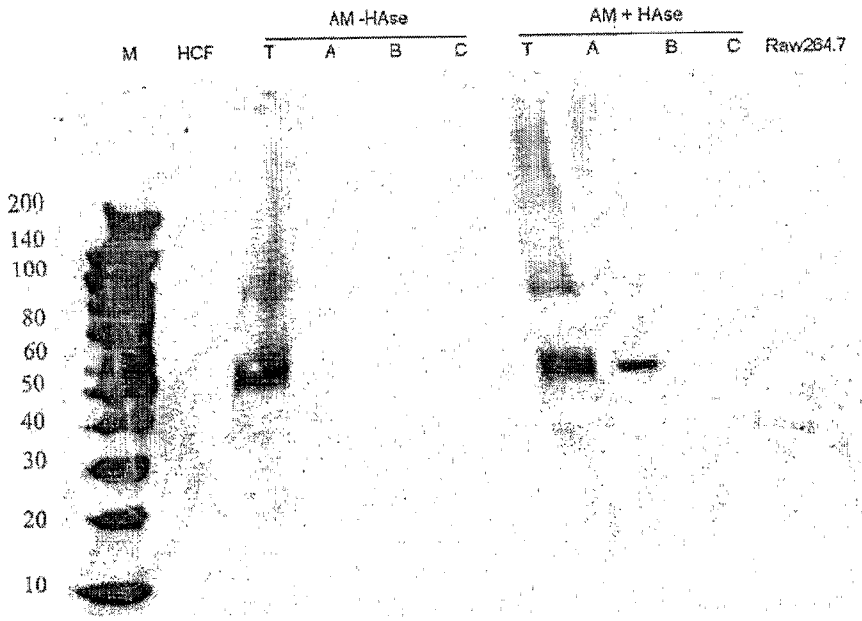

AM -HAse
AM + HAse
M
HCF
T
A
B
C
T
A
B
C
Raw264.7
200
140
100
80
60
50
40
30
20
10

Fig. 18

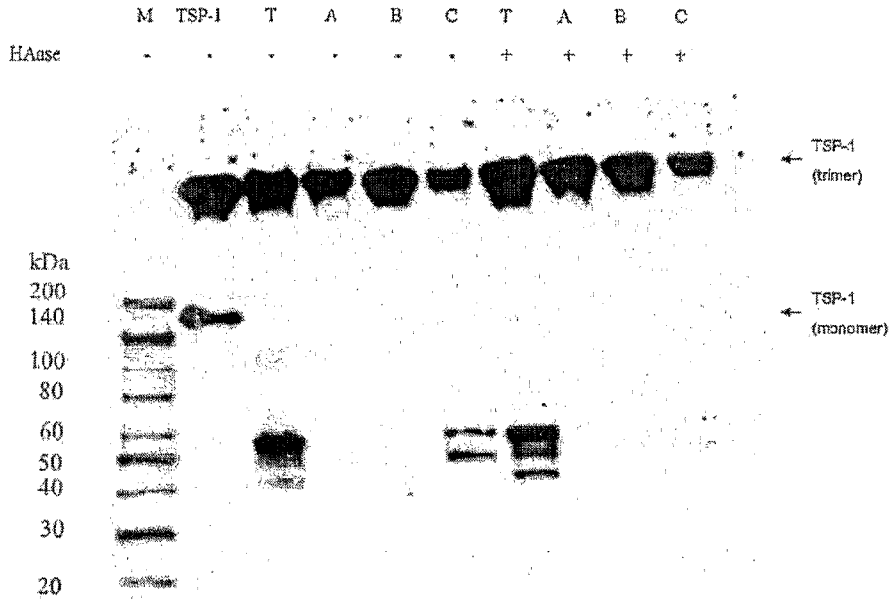

M
TSP-1
T
A
B
C
T
A
B
C
HAase
- - - - - - + + + +
kDa
200
140
100
80
60
50
40
30
20
TSP-1
(trimer)
TSP-1
(monomer)

Fig. 19

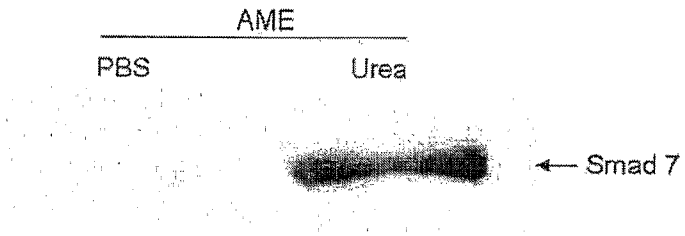


Fig. 20

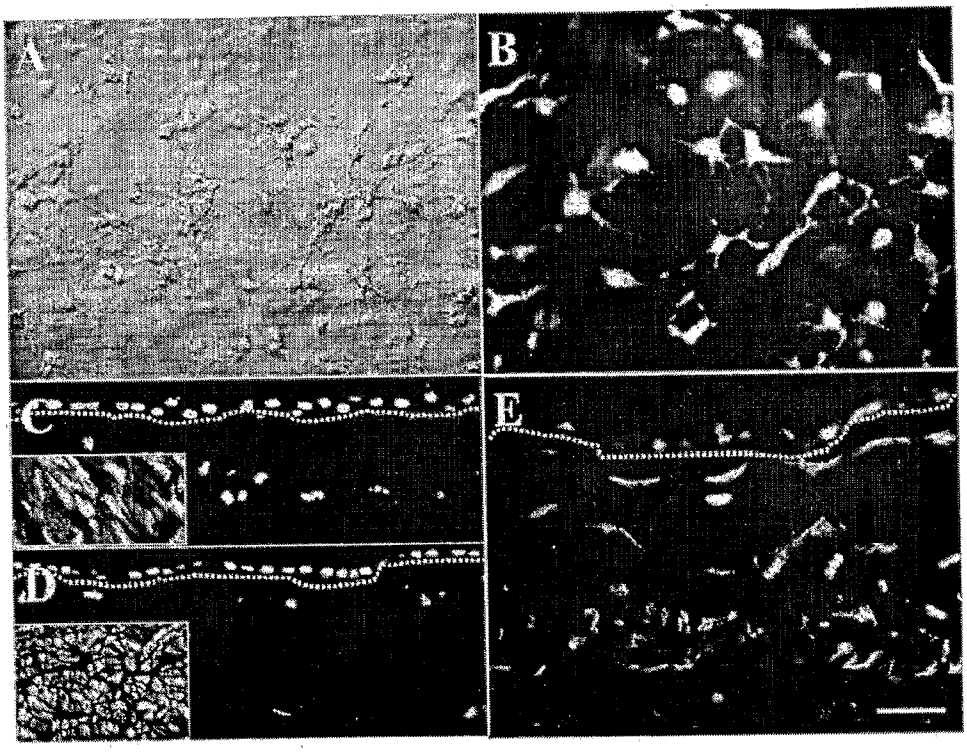


Fig. 21

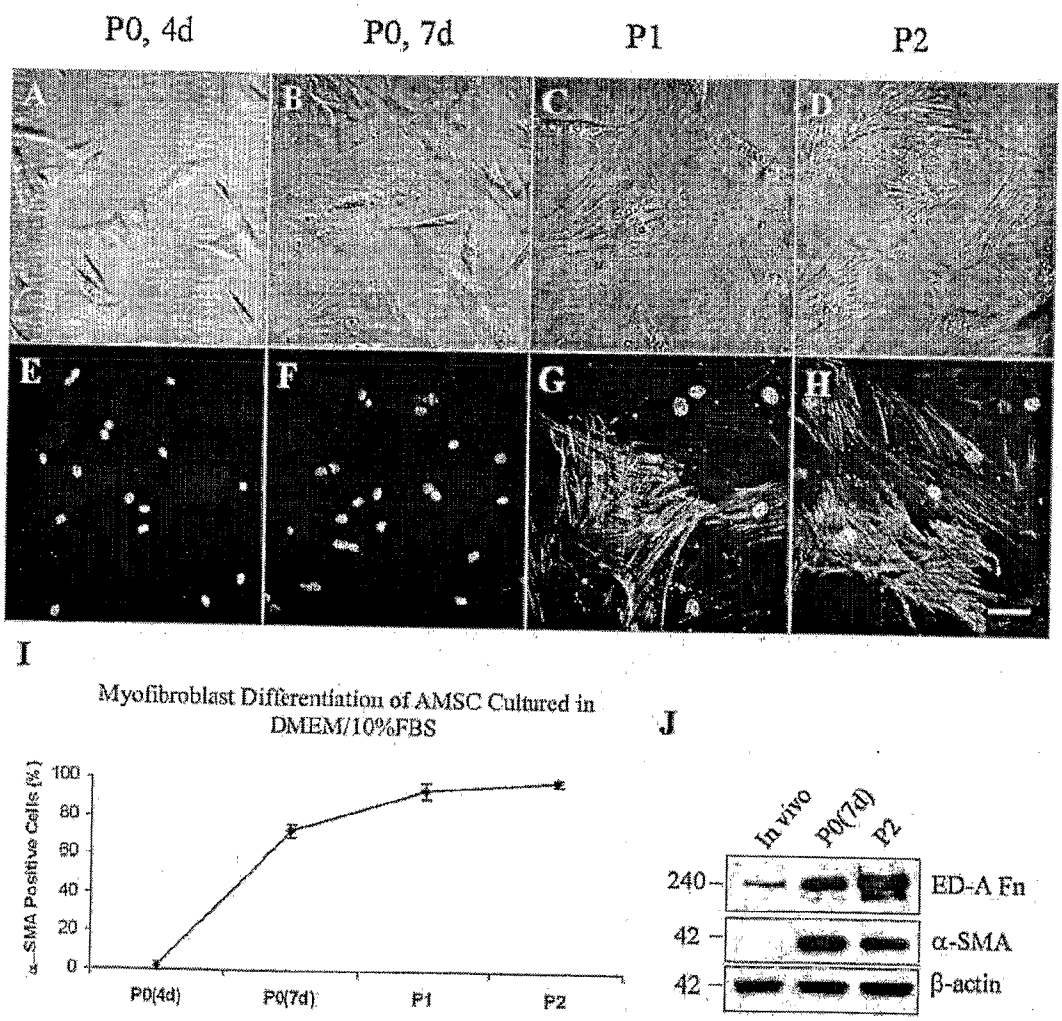

P0, 4d
P0, 7d
P1
P2
A
B
C
D
E
F
G
H
I
Myofibroblast Differentiation of AMSC Cultured in DMEM/10%FBS
α-SMA Positive Cells (%)
100
80
60
40
20
0
P0(4d)
P0(7d)
P1
P2
J
In vivo
P0(7d)
P2
240
42
42
ED-A Fn
α-SMA
β-actin

Fig. 22

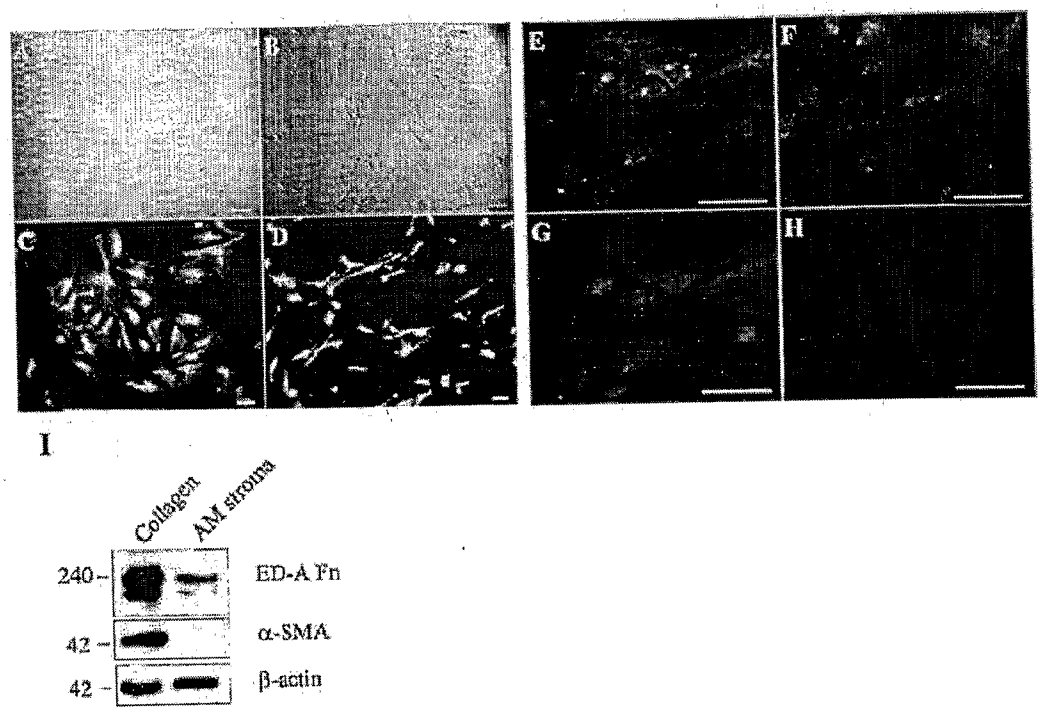


Fig. 23

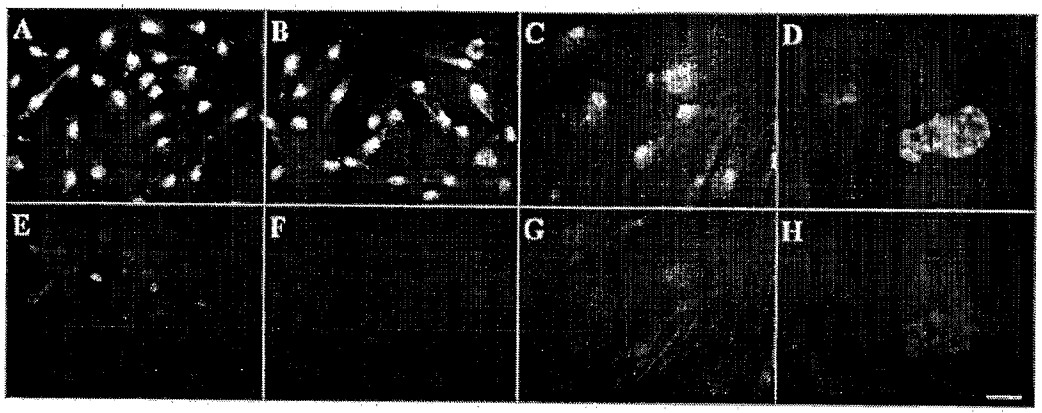


Fig. 24

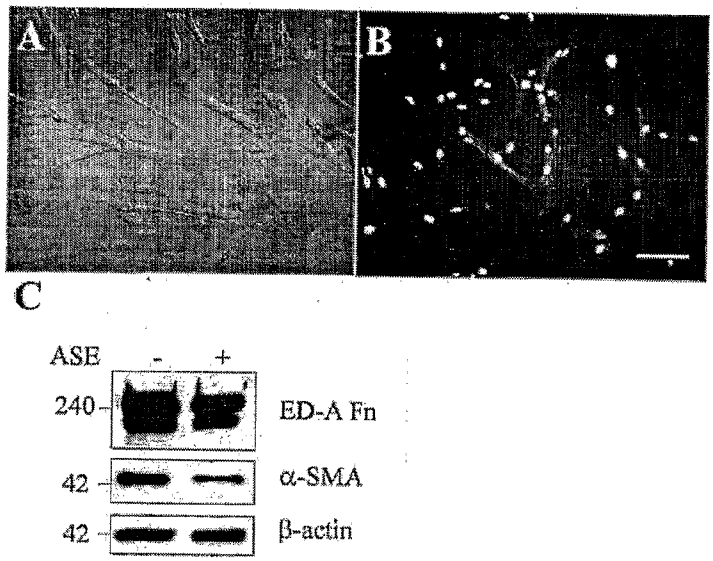


Fig. 25

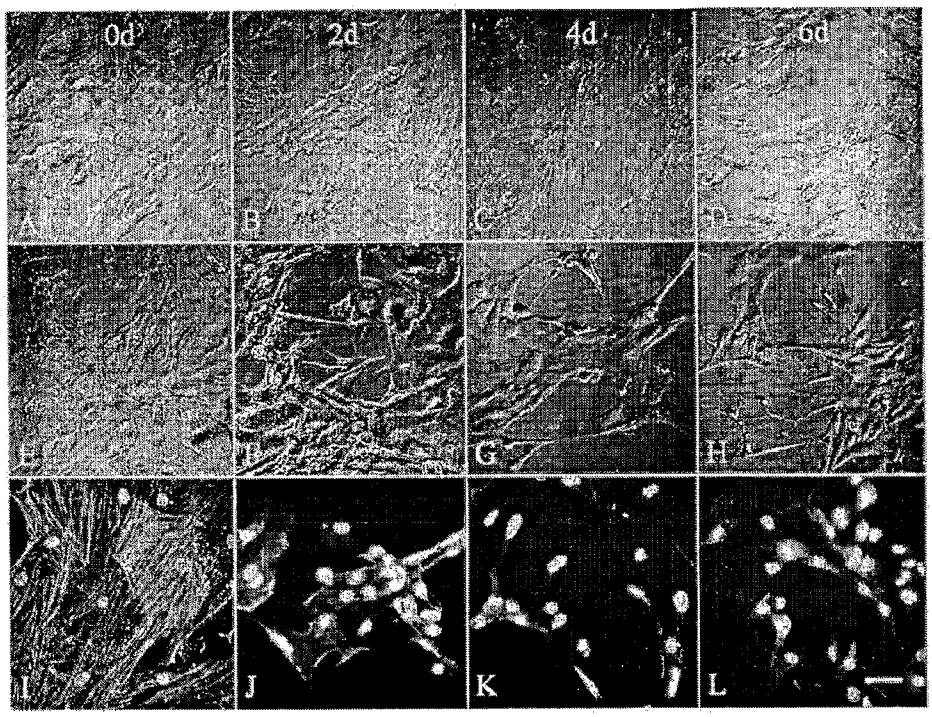


Fig. 26

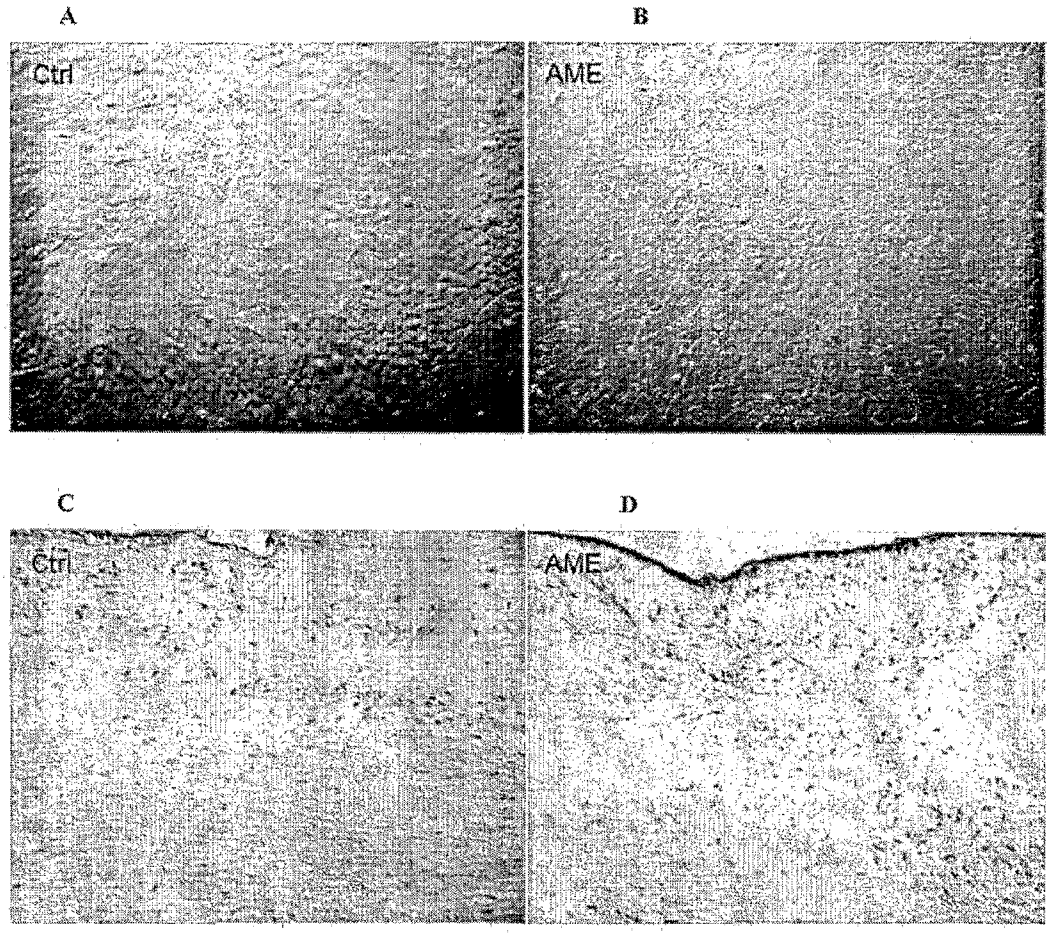
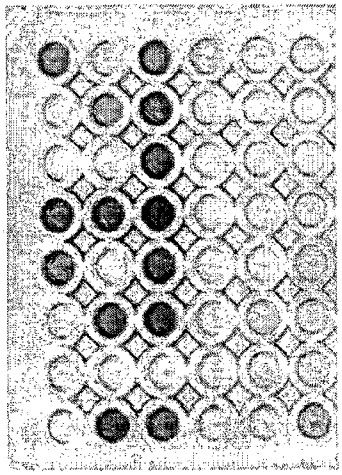
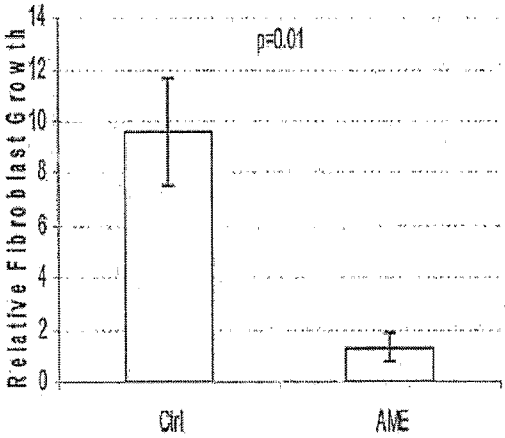


Fig 27



A



B

Fig. 28

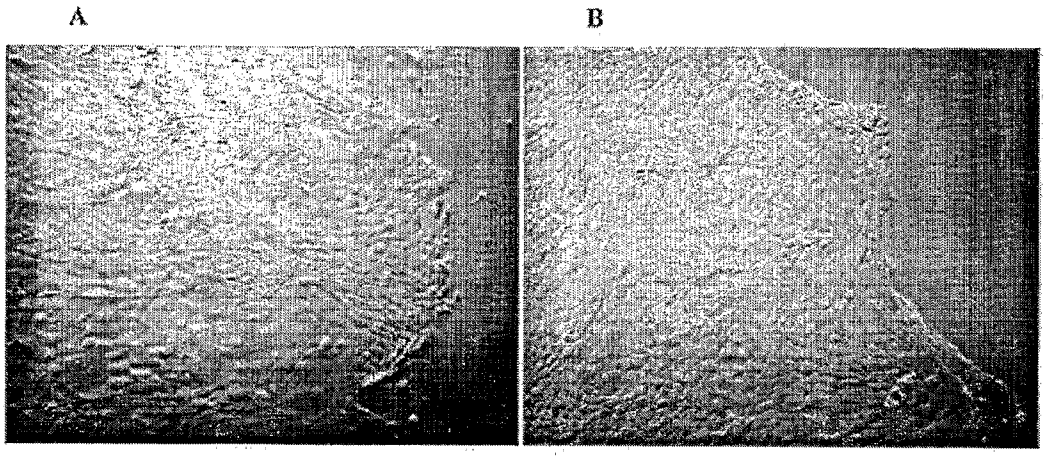


Fig. 29

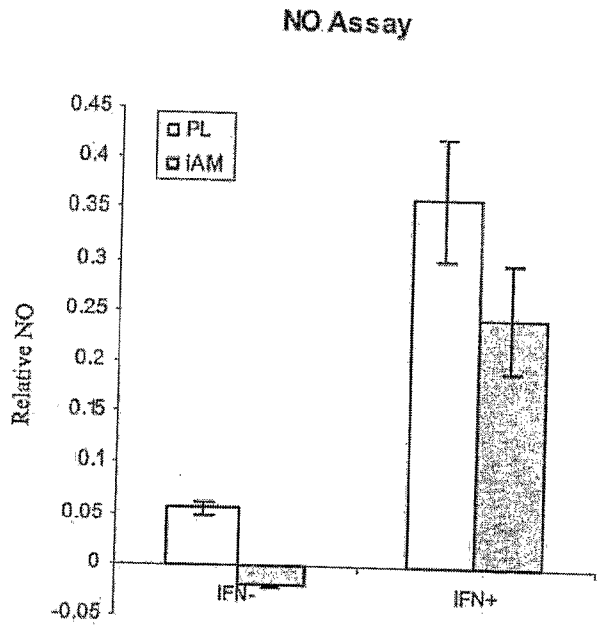

NO Assay
Relative NO
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
PL
iAM
IFN-
IFN+

Fig. 30

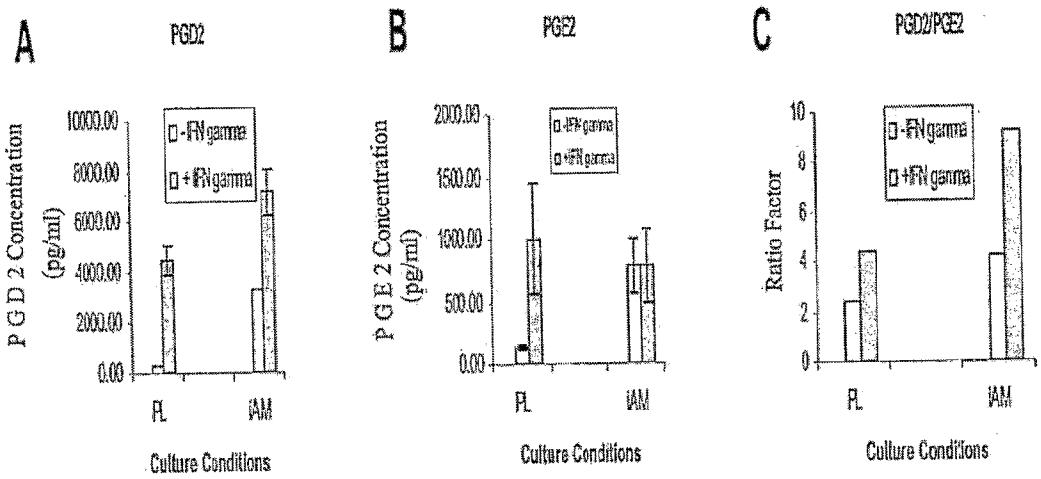

A
PGD2
P G D 2 Concentration (pg/ml)
10000.00
8000.00
6000.00
4000.00
2000.00
0.00
-IFN gamma
+IFN gamma
PL
IAM
Culture Conditions
B
PGE2
P G E 2 Concentration (pg/ml)
2000.00
1500.00
1000.00
500.00
0.00
-IFN gamma
+IFN gamma
PL
IAM
Culture Conditions
C
PGD2/PGE2
Ratio Factor
10
8
6
4
2
0
-IFN gamma
+IFN gamma
PL
IAM
Culture Conditions

Fig. 31

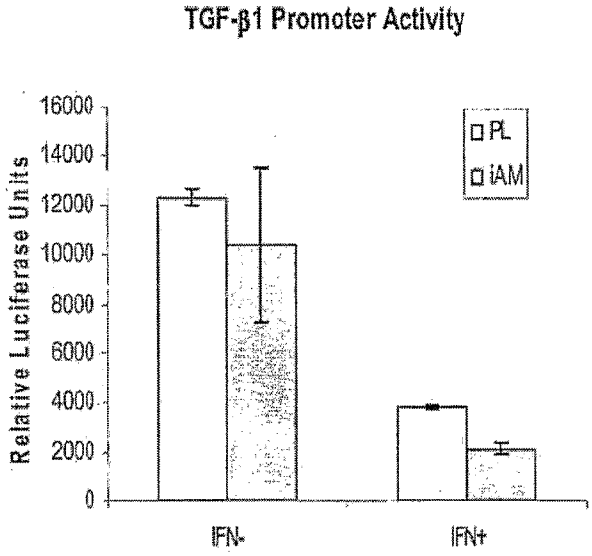

TGF-β1 Promoter Activity
Relative Luciferase Units
16000
14000
12000
10000
8000
6000
4000
2000
0
PL
iAM
IFN-
IFN+

Fig. 32

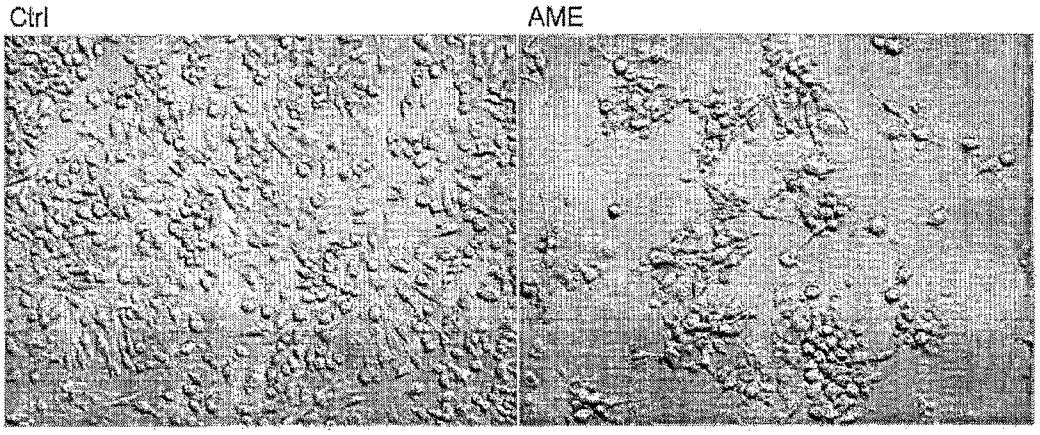


Fig. 33

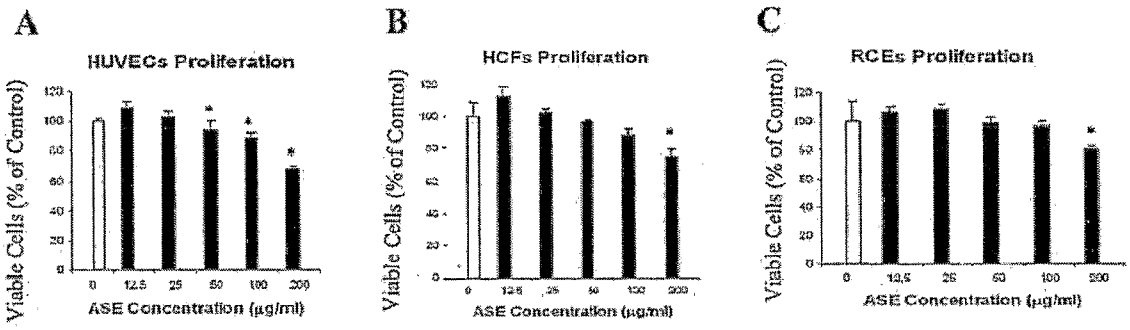

A
HUVECs Proliferation
Viable Cells (% of Control)
ASE Concentration (µg/ml)
B
HCFs Proliferation
Viable Cells (% of Control)
ASE Concentration (µg/ml)
C
RCEs Proliferation
Viable Cells (% of Control)
ASE Concentration (µg/ml)

Fig. 34

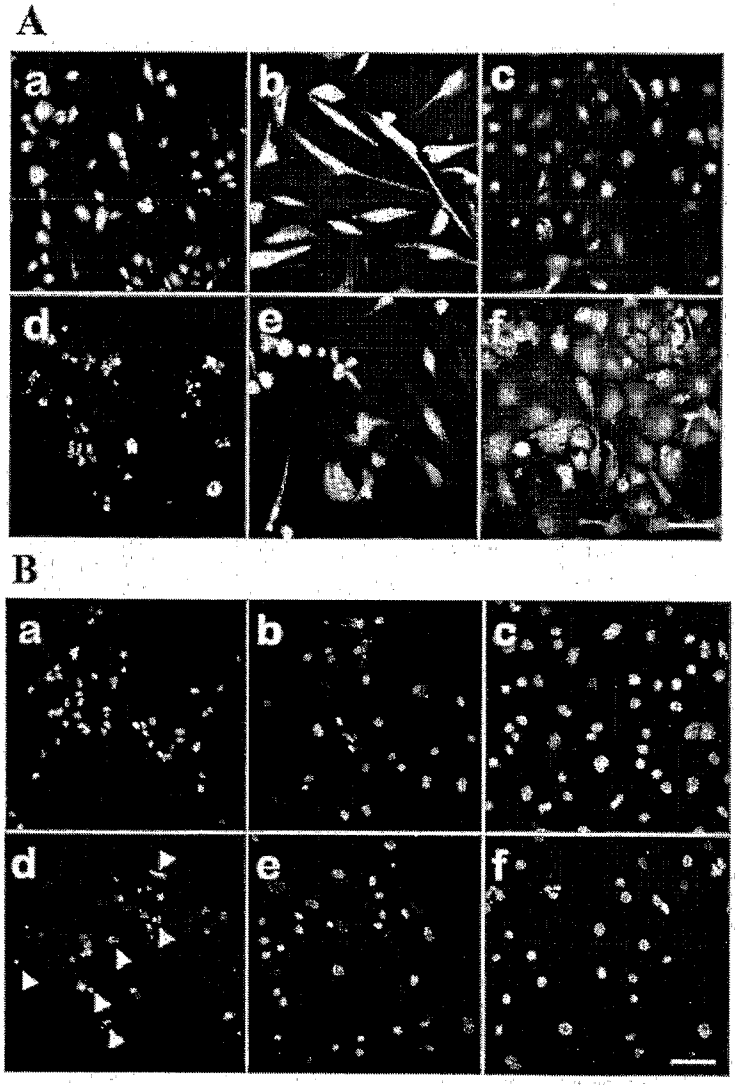


Fig. 35

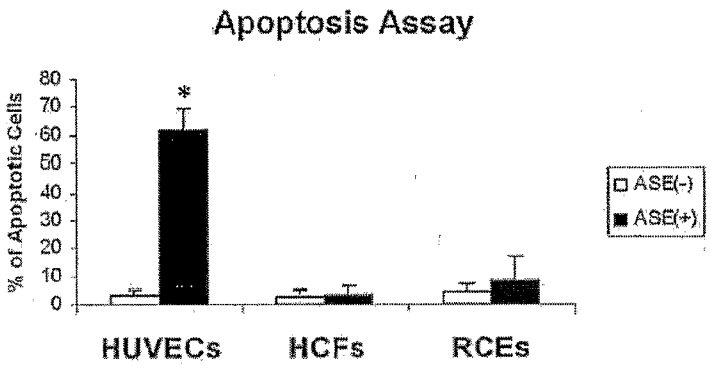

Apoptosis Assay
% of Apoptotic Cells
80
70
60
50
40
30
20
10
0
*
HUVECs
HCFs
RCEs
ASE(-)
ASE(+)

Fig. 36

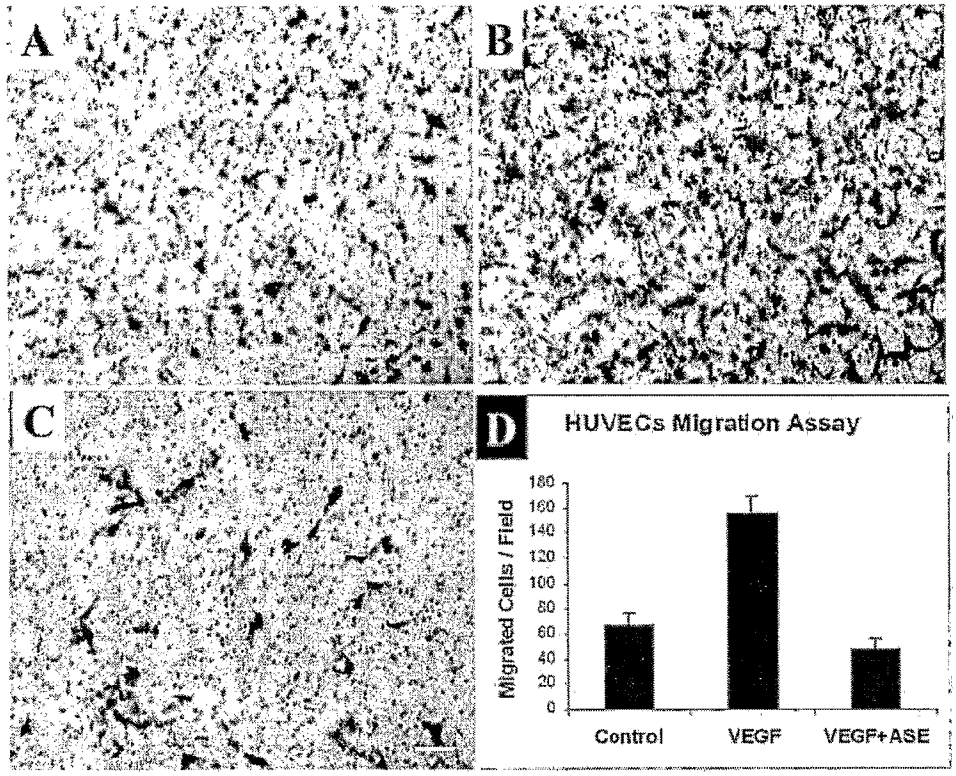

A
B
C
D
HUVECs Migration Assay
Migrated Cells / Field
180
160
140
120
100
80
60
40
20
0
Control
VEGF
VEGF+ASE

Fig. 37

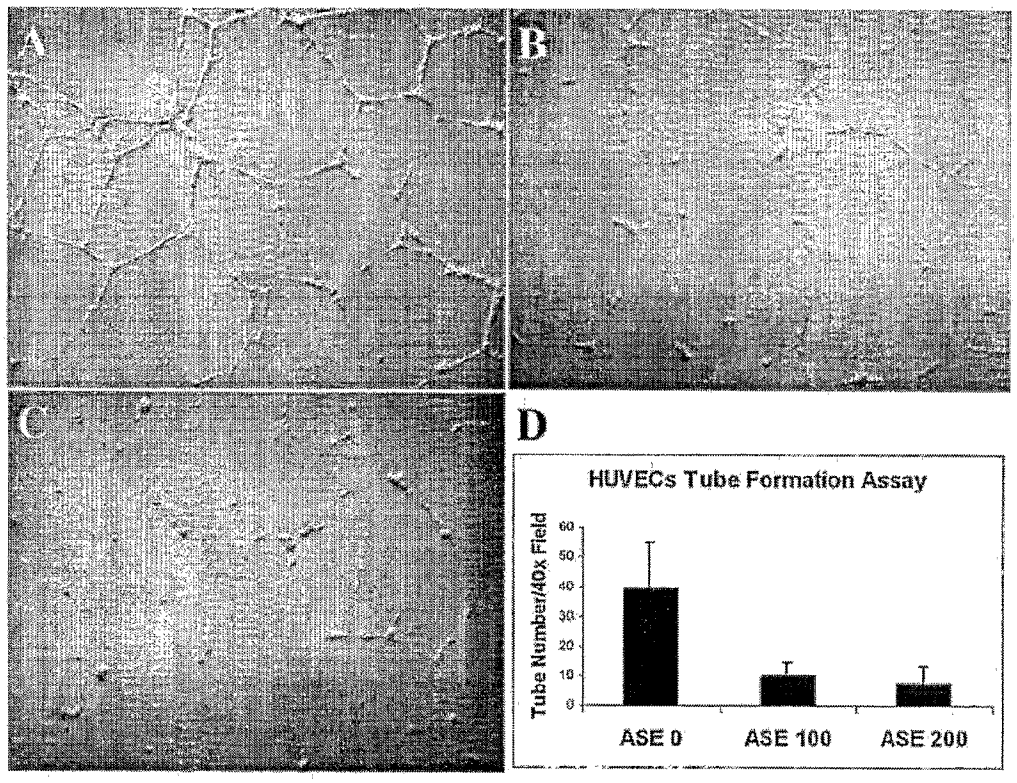

A
B
C
D
HUVECs Tube Formation Assay
Tube Number/40x Field
60
50
40
30
20
10
0
ASE 0
ASE 100
ASE 200

Fig. 38