PHARMACEUTICAL COMPOSITION AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Application No. 61/819,709, filed on 6 May 2013, the entire disclosure of which is incorporated herein by reference.

TECHNOLOGY FIELD

[0002] The present invention relates to pharmaceutical compositions and methods for the treating myopia, inhibiting ocular chondrogenic protein, scleral chondrogenesis and inflammation induced chondrogenesis.

BACKGROUND OF THE INVENTION

[0003] Myopia is due to progressive elongation of the eye and stretching of the ocular tissues. It is an important public health issue, as it affects approximately 25% of the U.S. population, and as high as 80% of the population in some Asian countries. Maculopathy of high myopia has become the leading cause of cataract, glaucoma, retinal detachment, myopic retinal degeneration, visual impairment, and untreatable blindness.

[0004] Optical and laser surgical corrective techniques have been used to alter the refractive state of the myopic eye. These therapies, however, do not address the abnormal elongation of the eye and thus do not treat pathologic changes of high myopia patients.

[0005] There is still a need for a more effective and safe treatment for myopia. The present invention addresses this need.

BRIEF SUMMARY OF THE INVENTION

[0006] Pharmaceutical compositions comprising two anti-chondrogenesis agents are

provided herein. The pharmaceutical compositions are effective in treating myopia, reducing one or more chondrogenic proteins and reducing scleral chondrogenesis.

[0007] Methods for treating myopia, comprising administering an effective amount of non-steroidal anti-inflammatory agent (NSAID) to a subject in need thereof to thereby treat myopia are provided. In one embodiment, the method for treating myopia further comprises administering an effective amount anti-muscarinic agent.

[0008] Methods for reducing one or more ocular chondrogenic proteins, comprising administering an effective amount of one or more anti-chondrogenesis agents to a subject in need thereof to reduce one or more ocular chondrogenic proteins are also provided herein.

[0009] Methods for reducing scleral chondrogenesis, comprising administering an effective amount of one or more anti-chondrogenesis agents to a subject in need thereof to reduce scleral chondrogenesis are also provided herein.

[0010] Methods for reducing inflammation induced chondrogenesis, comprising administering an effective amount of one or more anti-chondrogenesis agents to a subject in need thereof to reduce inflammation induced chondrogenesis in the subject are also provided herein.

[0011] The invention will become more apparent when read with the accompanying figures and detailed description which follow.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] Fig. 1 illustrates schematically a mechanism for myopia.

[0013] Fig. 2 is bar graph illustrating the levels of alpha smooth muscle actin (α-SMA) and collagen type 2 (Col2) mRNA normalized to β-actin expression in scleral stem/progenitor

cells (SSPCs) with or without Transforming growth factor beta (TGF-β) treatment. Data are expressed as fold change over the control sample as determined by the delta-delta Ct method. Bars, SD. * represents statistically significant.

[0014] Fig. 3 is an assembly of images illustrating the expression of α-SMA and Col2 in the sclera of mice with form-deprivation myopia (FDM). Panel A is a photograph of a western blot showing the scleral Col2 and α-SMA expression levels were increased in FDM eyes. Panel B is a bar graph of densitometry analysis showing the levels of scleral Col2 and scleral α-SMA in FDM eyes are significantly higher than that of the control eyes.

[0015] Fig. 4 is a bar graph showing the levels of TGF-β1, TGF-β2 and TGF-β3 mRNA expression in the RPE-choroid complex of the FDM eyes were significantly higher than that of the control eyes.

[0016] Fig. 5A (a western blot analysis) and Fig. 5B (a bar graph) illustrate the expression profiles of Col2 and α-SMA in human SSPC treated with 10 ng/ml TGF-β2, with or without Atropine, Ketorolac and Diclofenac.

[0017] Fig. 6 is a bar graph illustrating the myopia progression rate (Diopter per year) in 11 myopia subjects treated with atropine eye drops and the combined atropine and ketolorac eye drops.

[0018] Fig. 7 is a bar graph illustrating the myopia progression rate of a myopia subject without any treatment, followed by 3 months of ketorolac treatment.

[0019] Fig. 8A is a bar graph illustrating the level of interleukin 6 (IL-6) mRNA normalized to GADPH expression in the choroids of form deprivation myopia (FDM) mice is higher than that of control mice. Fig. 8B is a bar graph illustrating the level of tumor necrosis factor-alpha (TNF-α) in the choroids is higher in the FDM mice than that of control mice.

TNF-α expression is suppressed by ketorolac eye drop.

[0020] Fig. 9A is a bar graph illustrating the suppressive effect of atropine (A), ketorolac (X1) and a pharmaceutical composition comprising atropine and ketorolac on α-SMA expression in SSPC in the presence of TGF-β2 (T2). The pharmaceutical composition comprising atropine and ketorolac has a synergistic effect on α-SMA suppression. Fig. 9B is a bar graph illustrating the suppressive effect of atropine (A), ketorolac (X1) and a pharmaceutical composition comprising atropine and ketorolac on Col2 expression in SSPC in the presence of TGF-β2 (T2). The pharmaceutical composition comprising atropine and ketorolac has a synergistic effect on Col2 suppression.

DETAILED DESCRIPTION

Definitions

[0021] As employed above and throughout the disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings.

[0022] As used herein, the singular forms "a," "an," and "the" include the plural reference unless the context clearly indicates otherwise.

[0023] An "effective amount," as used herein, includes a dose of an anti-chondrogenesis agent that is sufficient to treat or ameliorate at least one symptom of myopia, or to reduce one or more ocular chondrogenic proteins, scleral chondrogenesis or inflammation induced chondrogenesis.

[0024] The term "treating," "treated," or "treatment" as used herein refers to palliative uses or results, and/or slowing or inhibiting the advancement of myopia progression and/or myopia shift index.

[0025] The term "reducing" or "reduce" includes slowing the formation of ocular chondrogenic protein, scleral chondrogenesis, inflammation induced chondrogenesis or myopia progression, or myopia shift, or disassembling the ocular chondrogenic proteins that have already been formed.

[0026] Pharmaceutically acceptable salts of the therapeutic agent of the invention include salts derived from an appropriate base, such as an alkali metal (for example, sodium, and potassium), an alkaline earth metal (for example, calcium, and magnesium), ammonium and NX_4^+ (wherein X is C_1 -C_4 alkyl). Pharmaceutically acceptable salts of an amino group include salts of organic carboxylic acids, such as tartaric, aliphatic, cycloaliphatic, aromatic, heterocyclic, carboxylic and sulfonic classes of organic acids, such as, for example, formic, glucuronic, malic, maleic, fumaric, pyruvic, aspartic, glutamic, benzoic, anthranilic, mesylic, salicylic, hydroxybenzoic, phenylacetic, mandelic, embonic (pamoic), methanesulfonic, ethanesulfonic, benzenesulfonic, pantothenic, toluenesulfonic, 2-hydroxyethanesulfonic, sulfanilic, stearic, algenic, hydroxybutyric, cyclochexylaminosulfonic, galactaric and galacturonic acid and the like, lactobionic, fumaric, and succinic acids; organic sulfonic acids, such as methaniesulfolic, ethanesulfonic, isothionic, benzenylesulfonic and p-toluenesulfonic acids; and inorganic acids such as hydrochloric, hydrobromic, hydroiodic, nitric, carbonic, sulfuric, sulfamic and phosphoric acid and the like. Pharmaceutically acceptable salts of a compound having a hydroxy group consist of the anion of said compound in combination with a suitable cation such as Na^+, NH_4^+ or NX_4^+ (wherein X is, for example, a C_1 - C_4 alkyl group), Ca^{++}, Li^+, Mg^{++}, or, K^+ and zinc or organic salts made from primary, secondary and tertiary amines, cyclic amines, N,N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, ethylenediamine, meglumine (N-methylglucamine) and procaine and the

like. All of these salts may be prepared by conventional means from the corresponding compound by reacting, for example, the appropriate acid or base with the compound in free form.

[0027] The term "myopia" as used herein refers to a condition associated with a refractive error of one or more eyes, wherein light rays entering the eye to focus in front of the retina rather than directly on the retina. The term "myopia" as used herein, encompasses a variety of levels (mild myopia, from 0 to -3 diopters; moderate myopia, from -3 to -5 diopters; and high myopia, from -5 or lower), and types and subtypes of myopia of various etiologies and causes, either known or unknown, including, but not limited to, simple myopia, degenerative myopia, and form deprivation myopia.

[0028] The term "diopter" as used herein includes measurement of how much a corrective lens must bend light to focus the light on the retina to normalize the vision. A lens that can bend parallel light rays to a focal point of 1 meter is said to have a power of 1 diopter (1.00D). A 2-diopters lens can focus light rays at a point 0.5 meters away from itself.

[0029] The term "subject" as used herein typically refers to a human or an animal subjected to the methods described herein. It is to be understood that a subject can be a patient with known or suspected myopia disorder, but subjects without known or suspected myopia disorder, such as research subjects, are also included within the scope of the term "subject."

[0030] All numbers herein may be understood as modified by "about."

Pharmaceutical Composition

[0031] Pharmaceutical compositions for treating myopia, reducing ocular chondrogenic

protein, reducing scleral chondrogenesis or reducing inflammation induced chondrogenesis are provided herein. The pharmaceutical compositions comprising a combination of two anti-chondrogenesis agents preferably by advantageous synergistic effects of the combinations.

[0032] An anti-chondrogenesis agent is any agent which reduces or slows the process of chondrogenesis. In one embodiment, an anti-chondrogenesis agent in the pharmaceutical composition is a NSAID. In another embodiment, an anti-chondrogenesis agent in the pharmaceutical composition is an anti-muscarinic agent. Non limiting examples of anti-chondrogenesis agent include a microRNA that regulates the expression of lymphoid enhancer-binding factor-1, such as miR-449a (S Paik, et al., miR-449a regulates the chondrogenesis of human mesenchymal stem cells through direct targeting of lymphoid enhancer-binding factor-1, Stem Cells Dev;21(18):3298-308, 2012), a histone deacetylase inhibitor such as valproic acid (FH Paradis et al., Exposure to valproic acid inhibits chondrogenesis and osteogenesis in mid-organogenesis mouse limbs, Toxicol Sci;131(1):234-41, 2013), Nicotine (Y Deng et al., Nicotine-induced retardation of chondrogenesis through down-regulation of IGF-1 signaling pathway to inhibit matrix synthesis of growth plate chondrocytes in fetal rats, Toxicol Appl Pharmacol;269(1):25-33, 2013), bFGF or parathyroid hormone-like peptide (S Weiss et al., Impact of growth factors and PTHrP on early and late chondrogenic differentiation of human mesenchymal stem cells, J Cell Physiol;223(1):84-93, 2010), an agent restricting Leucine (MS Kim, Leucine restriction inhibits chondrocyte proliferation and differentiation through mechanisms both dependent and independent of mTOR signaling, Am J Physiol Endocrinol Metab;296(6):E1374-82, 2009), 17beta-Estradiol (S. Fushimi et al., 17beta-Estradiol inhibits

chondrogenesis in the skull development of zebrafish embryos, Aquat Toxicol;95(4):292-8, 2009), versican (Y Yang et a., The G3 domain of versican inhibits mesenchymal chondrogenesis via the epidermal growth factor-like motifs, J Biol Chem;273(49):33054-63, 1998), SB203580 (a specific inhibitor of p38MAPK, D Kim et al., Alterations in the temporal expression and function of cadherin-7 inhibit cell migration and condensation during chondrogenesis of chick limb mesenchymal cells in vitro, J Cell Physiol; 221(1):161-70, 2009), LiCl (an inhibitor of GSK-3beta, D Kim et al., 2009) or the like.

[0033] In one exemplary embodiment, the pharmaceutical composition includes at least one NSAID and at least one anti-muscarinic agent.

[0034] In certain embodiments, NSAIDs for use in the present invention may be non-selective cyclooxygenase (COX) inhibitors, its derivatives, salts and structural analogues, i.e., compounds that inhibit both COX-1 and COX-2 proteins. Non limiting examples of non-selective COX inhibitors include salicylic acid derivatives (e.g., aspirin, sodium salicylate, choline magnesium trisalicylate, salsalate, diflunisal, sulfasalazine and olsalazine), indole and indene acetic acids (e.g., indomethacin and sulindac), heteroaryl acetic acids (e.g., tolmetin, diclofenac and ketorolac), arylpropionic acids (e.g., ibuprofen, naproxen, flurbiprofen, ketoprofen, fenoprofen, and oxaprozin), anthranilic acids (fenamates) (e.g., mefenamic acid and meclosfenamic acid), enolic acids (e.g., the oxicams, piroxicam and meloxicam) and alkanones (e.g., nabumetone).

[0035] In certain embodiments, NSAIDs for use in the present invention may be selective COX-2 inhibitors, its derivatives, salts and structural analogues. Non limiting examples of selective COX-of selective COX-2 inhibitor include diaryl-substituted furanones (e.g., rofecoxib), diaryl-substituted pyrazoles (e.g., celecoxib), indole acetic acids (e.g., etodolac)

and sulfonanilides (e.g., nimesulide). Further examples of selective COX-2 inhibitor are disclosed in U.S. Pat. No. 6,440,963 and WO 2004/054560, which are incorporated by reference in its entirety.

[0036] Preferred NSAIDs for use in the invention include, but are not limited to, Ketorolac, Diclofenac, Indomethacin, Bromfenac, Nepafenac and Flurbiprofen.

[0037] Examples of anti-muscarinic agent include, but are not limited to, Atropine, Homatropine, Scopolamine, its derivatives, salts, and structural analogues.

[0038] Anti-muscarinic agents may cause side effects of blurred vision and photophobia. These side effects maybe overcome by administering lower dosage of anti-muscarinic agents, in combination with one or more anti-chondrogenesis agent, to achieve the desired therapeutic effect. The observed synergistic effect of a pharmaceutical composition comprising a combination of an anti-muscarinic agent (e.g. atropine) and an NSAID (e.g. ketorolac) may afford effective treatment of myopia wherein one or even all of the lower dosages of the anti-chondrogenesis agents would not be sufficient to have a therapeutic effect when the respective anti-chondrogenesis agent is used in monotherapy.

[0039] The pharmaceutical compositions to be administered according to the methods of some embodiments provided herein can be readily formulated with, prepared with, or administered with, a pharmaceutically acceptable carrier. Such preparations may be prepared by various techniques. Such techniques include bringing into association active components (such as NSAID or anti-muscarinic agent) of the pharmaceutical compositions and an appropriate carrier. In one embodiment, pharmaceutical compositions are prepared by bringing into association active components of the pharmaceutical compositions with liquid carriers, with solid carriers, or with both.

[0040] The pharmaceutical compositions are administered in an aqueous suspension, an oil emulsion, water in oil emulsion and water-in-oil-in-water emulsion, and in carriers including, but not limited to, creams, gels, liposomes (neutral, anionic or cationic), lipid nanospheres or microspheres, neutral, anionic or cationic polymeric nanoparticles or microparticles, site-specific emulsions, long-residence emulsions, sticky-emulsions, micro-emulsions, nano-emulsions, microspheres, nanospheres, nanoparticles and minipumps, and with various natural or synthetic polymers that allow for sustained release of the pharmaceutical composition including anionic, neutral or cationic polysaccharides and anionic, neutral cationic polymers or copolymers, the minipumps or polymers being implanted in the vicinity of where composition delivery is required.

The pharmaceutical compositions provided herein may optionally include anti-oxidants, buffers, bacteriostatic agents, suspending agents thickening agents, preservatives, co-solvents and viscosity building agents or other therapeutic ingredients. The carrier and other therapeutic ingredients must be acceptable in the sense of being compatible with the other ingredients of the composition and not deleterious to the recipient thereof. Suitable preservatives for ophthalmic preparations include: benzalkonium chloride, thimerosal, chlorobutanol, methyl paraben, propyl paraben, phenylethyl alcohol, edetate disodium, sorbic acid, Onamer M, or other agents known to those skilled in the art. In one embodiment, the preservative is employed at a level of from 0.004% to 0.02%.

[0041] For administration in a non-aqueous carrier, active components of the pharmaceutical compositions provided herein are emulsified with a mineral oil or with a neutral oil such as, but not limited to, a diglyceride, a triglyceride, a phospholipid, a lipid, an oil and mixtures thereof, wherein the oil contains an appropriate mix of polyunsaturated and saturated fatty

acids. Examples include, but are not limited to, soybean oil, canola oil, palm oil, olive oil and myglyol, wherein the number of fatty acid carbons is between 12 and 22 and wherein the fatty acids can be saturated or unsaturated. Optionally, charged lipid or phospholipid is suspended in the neutral oil. A suitable phospholipid is, but is not limited to, phosphatidylserine, which targets receptors on macrophages. The pharmaceutical compositions provided herein are optionally formulated in aqueous media or as emulsions using known techniques.

[0042] The pharmaceutical compositions are administered in an amount effective to reduce ocular chondrogenic protein, reduce scleral chondrogenesis, reduce inflammation induced chondrogenesis or to induce a therapeutic response in an animal, including a human with myopia. The dosage of the pharmaceutical composition administered will depend on the severity of the condition being treated, the particular formulation, and other clinical factors such as weight and the general condition of the recipient and route of administration. In one exemplary embodiment, the amount of the pharmaceutical composition administered corresponds to about 0.001% to about 1% by weight atropine. In another exemplary embodiment, the amount of the pharmaceutical composition administered corresponds to about 0.005%, 0.01%, 0.015%, 0.02%, 0.025%, 0.03%, 0.035%, 0.04%, 0.045%, 0.05%, 0.055%, 0.06%, 0.065%, 0.07%, 0.075%, 0.08%, 0.085%, 0.09%, 0.095%, 0.1% by weight atropine, or any % in between 0.001% and 1% in 0.001% increments. In another exemplary embodiment, the amount of the pharmaceutical composition administered corresponds to about 0.05% to about 1% by weight Ketorolac. In another exemplary embodiment, the amount of the pharmaceutical composition administered corresponds to about 0.1%, 0.2%, 0.25%, 0.3%, 0.35%, 0.4%, 0.45%, 0.5%, 0.55%, 0.6%, 0.65%, 0.7%, 0.75%, 0.8%, 0.85%,

0.9%, 0.95% by weight Ketorolac, or any % in between in 0.01% increments. In another exemplary embodiment, the amount of the pharmaceutical composition administered corresponds to about 0.5% by weight Ketorolac. In another exemplary embodiment, the amount of the pharmaceutical composition administered corresponds to from about 0.01%, 0.025%, 0.05%, 0.1%, 0.15% to about 0.2% of Diclofenac by weight or any % in between in 0.01% increments. Useful dosages of the pharmaceutical compositions provided herein are determined by comparing their *in vitro* activity, and *in vivo* activity in animal models. Methods for the extrapolation of effective dosages in mice, and other animals, to humans are known in the art; for example, see U.S. Pat. No. 4,938,949, which is incorporated by reference herein.

[0043] In accordance with the methods provided herein, the pharmaceutical composition is delivered by any of a variety of routes including, but not limited to, injection (e.g., subcutaneous, intramuscular, intravenous, intra-arterial, intraperitoneal, intradermal, intravitreal); cutaneously; dermally; transdermal; oral(e.g., tablet, pill, liquid medicine, edible film strip); implanted osmotic pumps; suppository, aerosol spray, topical, intra-articular, ocular, nasal inhalation, pulmonary inhalation, impression into skin and electroporation. In one embodiment, the pharmaceutical composition of the present invention can be administered as solution in a suitable ophthalmic vehicle.

[0044] In forming the pharmaceutical compositions for topical ocular administration, the combination comprises 0.001% to about 0.005% by weight atropine and 0.1% to about 0.5% by weight Ketorolac solution in water at pH of 4.5 to 8.0, e.g. about 6.9. It is recommended that the solution be topically applied by placing one drop in the affected eye once a day.

[0045] The pharmaceutical composition may be administered in a single dose treatment or

in multiple dose treatments, over a period of time appropriate to the condition being treated. The pharmaceutical composition may conveniently be administered at appropriate intervals, for example, once a day, twice a day, three times a day, once every second day, once every three days or once every week, over a period of at least 3 months, at least 1 year, or until the symptoms and signs of myopia resolved.

Methods for Reducing Scleral Chondrogenesis

[0046] Down regulation of ocular chondrogenic proteins and/or ocular inflammation markers reduces scleral chondrogenesis.

[0047] In one embodiment, the use of an effective amount of one or more anti-chondrogenesis agents or a pharmaceutical composition described herein may alter or reduce the amount of one or more ocular chondrogenic proteins in a subject in need thereof.

[0048] One example of ocular chondrogenic protein is TGF-β. In one embodiment, the TGF-β protein is selected from the group consisting of TGF-β1, TGF-β2 and TGF-β3, all of which are located predominately in the choroid. Another example of ocular chondrogenic protein is α-SMA. Another example of ocular chondrogenic protein is Col2. Both α-SMA and Col2 are located predominately in the sclera.

[0049] In another embodiment, the present invention provides the use of an effective amount of one or more anti-chondrogenesis agents or a pharmaceutical composition described herein for reducing inflammation induced chondrogenesis in a subject in need thereof. The inflammation markers responsible for inducing scleral chondrogenesis include, but are not limited to, IL-6 and TNF-α.

[0050] In yet another embodiment, the present invention provides the use of an effective

amount of one or more anti-chondrogenesis agents or a pharmaceutical composition described herein for reducing scleral chondrogenesis in a subject in need thereof.

[0051] The anti-chondrogenesis agent maybe administered concomitantly or non-concomitantly.

Methods for Treating or Reducing the Severity of Myopia

[0052] Without being bound by any particular theory, it was believed that the expression profiles of ocular inflammation markers and ocular chondrogenic proteins, such as TGF-β, α -SMA and Col2, are correlated with scleral chondrogenesis and myopia. Fig. 1, for example and without limitation, illustrates a mechanism for the development of myopia, wherein increase levels of TGF-βs and inflammatory markers (such as IL-6 and TNF-α) in the choroid lead to the formation of α -SMA and Col2 in the sclera and scleral chondrogenesis. The sclera then undergo remodeling and elongation, followed by the development of myopia.

[0053] The present invention provides methods for treating or reducing the severity of myopia, by administering one or more anti-chondrogenesis agents in an effective amount or the pharmaceutical composition described herein to a myopic subject in need of myopia treatment. The anti- chondrogenesis agent may be administered concomitantly or non-concomitantly. The methods also encompass research methods and uses, including *in vitro* and *in vivo* methods of treating, or inhibiting the progression of myopia in the subject.

[0054] In one embodiment, the method for treating myopia comprises identifying a myopic subject who exhibits side effect to anti-muscarinic agent, and treating said subject with an effective amount of NSAID, without the anti-muscarinic agent or with a lower dose of anti-muscarinic agent (e.g. 0.05% of atropine).

[0055] Embodiments of the present invention are illustrated by the following examples, which are not to be construed in any way as imposing limitations upon the scope thereof. During the studies described in the following examples, conventional procedures were followed, unless otherwise stated. Some of the procedures are described below for illustrative purpose.

Description of Materials and Methods Used in the Examples

[0056] **Mice:** Male wild type C57BL/6 mice (Jackson Labs) were used in the examples. All procedures were performed in accordance with an institutional IACUC approved protocol as well as the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

[0057] **Scleral stem/progenitor cells (SSPCs) isolation and culture:** The SSPCs were isolated and cultured as previously described by CL Tsai et al. (Identification of multipotent stem/progenitor cells in murine sclera. *Invest Ophthalmol Vis Sci* 52:5481-5487, 2011). In brief, sclera from the mouse was obtained and was carefully dissected away from limbus and optic disc under a dissection microscope. After retina and choroid tissues were removed, the scleral tissue was cut into small pieces and digested with 1.5 mg/ml collagenase type I (Worthington Biochemical, Lakewood, USA) and 2 mg/ml of dispase (Roche, Basel, Switzerland) in PBS for 1 h at 37℃ to release individual cells. Individual cells were cultured in α-MEM (Invitrogen, Carlsbad, USA), supplemented with 20% lot-selected FBS (Equitech-Bio, Kerrville, USA), glutamine, penicillin/streptomycin and 100 mM 2-mercaptoethanol (Invitrogen) for 8 to 10 days at 5% CO_2, 37℃.

[0058] **TGF-β treatment:** Different concentrations of TGF-β2 were added into 12-wells of SSPCs. After 24 hrs, the images of SSPC morphology were recorded. The total RNA was

extracted for further analysis. A chamber slide culture for the immunofluorescence study was performed under the same condition.

[0059] Induction of chondrogenic differentiation. At semiconfluence, SSPCs were trypsinized and counted to make aliquots of 2×10^5 cells in 2 ml growth medium which were spun down at 500g for 10 min to obtain the pellet. The pellets were incubated at 37°C, under 5% CO2. Within 12–24 h of incubation, the cells formed an essentially spherical aggregate that did not adhere to the walls of the tube. Culture medium was added with 10 ng/ml TGF-β2 and the medium was changed at 2 to 3 day intervals. The pellets were then harvested at 4 weeks. Subsequently, they were washed twice in PBS, fixed in 4% paraformaldehyde for 3 h at room temperature and prepared for paraffin embedment. Eight μm thick sections were obtained for immunohistochemistry,

[0060] Immunohistochemistry and immunofluorescence study: Immunohistochemistry and immunofluorescence studies were performed to demonstrate the presence of α -SMA protein and Col2 during chondrogenesis. For immunohistochemistry, paraffin sections were treated with a 20% blocking goat serum for 30 min, then incubated with primary antibodies which were rabbit IgG anti-SMA mAb at1:200 dilution (Abcam, Temecula, CA) and mouse IgG2a anti-type II collagen mAbb at 1:100 dilution (Abcam, Temecula, CA) at 4°C overnight. The sections were then treated with horseradish peroxidase (HRP)-conjugated secondary antibodies at 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour. The DAB reagent (diaminobenzidine tetrahydrochloride) was subsequently used to detect the immunoactivity. For immunofluorescence, cryostat sections and rehydrated paraffin sections were treated with blocking serum, incubated with primary antibody, reacted with the corresponding fluorescein-isothiocyanate-conjugated secondary antibody, and finally

evaluated by fluorescence microscopy.

[0061] Real time PCR: Total RNA from SSPCs or the choroid tissue in each eye was isolated using Trizol (Invitrogen, Carlsbad, CA) accordingly to the manufacture's protocol. qRT-PCR analysis was carried out using the iScript one-step RT-PCR kit with SYBR Green (Bio-Rad, Hercules, USA) on an ABI PRISM 7900 HT sequence detection system (Applied Biosystems, Foster City, USA), according to the manufacturer's instructions. Primers used for the experiment were: *α-SMA* (Forward primer: 5′- ATGCCTCTGGACGTACAACTG-3′, Reverse primer: 5′- CGGCAGTAGTCACGAAGGAAT-3′), *Col2* (Forward primer: 5′- GTCCTTCTGGCCCTAGAGGT-3′, Reverse primer: 5′- TGTTTCTCCTGAGCGTCCA-3′), *β-actin* (Forward primer: 5′- CATTGCTGACAGGATGCAGA-3′, Reverse primer: 5′- CTGATCCACATCTGCTGGAA-3′), and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (Forward primer: 5′- AACTTTGGCATTGTGGAAGG-3′, Reverse primer: 5′- ACACATTGGGGGTAGGAACA-3′). *GAPDH* and *β-actin* served as controls. Ct values of the control gene were subtracted from those of *α-SMA* and *Col2* to provide a semiquantitative analysis, and fold change relative to no treatment was assessed.

[0062] Deprived myopia of mice. On the day of the experiment (postnatal day [P] 21~24), C57BL/6J mice were anesthetized by intraperitoneal injection of ketamine (90 mg/kg) and xylazine (10 mg/kg), and the diffuser eye patches were sutured to the skin around the right eye whereas the left eye served as a control. The hemispherical plastic diffuser eye patches were made from caps of 0.5-mL PCR plastic tubes. The mice were recovered and monitored on a warming pad until they were fully mobile. Deprived myopia mice were housed in transparent plastic cages under 12 hours of light (200 ± 15 lux horizontal illuminance) and 12 hours of darkness for 21 days. A spectral-domain optical coherence tomography was used for

ocular biometric measurement before and after form-deprived myopia induction.

Western Blot Analysis: The total protein from the sclera was extract by using RIPA protein extraction buffer. After homogenization of scleral tissue, the sample was centrifuged and the supernatant was collected. The protein concentration of each sample was measured using a BCA ™ protein Assay Kit (Bio-Rad). Scleral protein samples were standardized and electrophoresed on 10% SDS-PAGE gel, then transferred to a polyvinylidene fluoride transfer membrane (Immun-Blot PVDF Membrane, BIO-RAD) at 21 V for 1 h. Membranes were blocked for 1 h at room temperature with 5% dry milk in PBS with 0.1% Tween and incubated at 4°C overnight with primary antibodies. Membranes were washed and incubated with 1:10,000 goat anti-mouse or anti-rabbit IgG antibodies conjugated to horseradish peroxidase (Santa Cruz) for 1 h at room temperature and washed again. Membranes were developed by chemiluminescence with the reagent Lumigen TMA-6 (GE Healthcare UK limited, Buckinghumshire, UK) and images were captured with the LAS-4000 imaging system (Fujifilm, Tokyo, Japan). Protein bands were quantified using ImageJ software.

Statistical analysis:

[0063] For *in vitro* studies, the statistical significant was calculated by ANOVA test with Bonferroni post hoc test. For *in vivo* studies, the statistical significance was calculated by analysis of variance (the paired t-test). Statistical significance was defined as a p value less than 0.05.

EXAMPLES

Example 1: SSPC Morphology Change After TGF-β treatment

[0064] An in vitro study of the SSPC morphology change after TGF-β treatment was performed. SSPC was incubated with TGF-β2 treatment (0.1-10 ng/ml) for 24hrs as previously described. Microscopy study shows without TGF-β2 treatment or at a low concentration of TGF-β2 treatment (0.1ng/ml), many SSPCs had thin spindle shape and showed a widened phenotype. In addition, the cytoskeleton filaments of SSPCs were not prominent. After exposing to a higher concentration of TGF-β2 (1 to 10 ng/ml), SSPCs became broad with prominent cytoskeletal filaments. Immunofluorescence microscopy showed an increased number of α-SMA positive SSPCs (with prominent intracellular α-SMA filament staining) after treatment with 10 ng/ml of TGF-β2.

Example 2: Effect of TGF-β treatment on α-SMA and Col2 expression

[0065] An in vitro study of the effect of TGF-β treatment on α-SMA and Col2 expression was performed using SSPC and 3-D pellets of SSPCs. SSPCs and 3-D pellets of SSPC were treated with various concentrations of TGF-β2, as previously described.

[0066] Total mRNA was analyzed to determine whether there was any alteration in *α-SMA* and *Col2* gene expression after 0.1 to 10 ng/ml of TGF-β2 treatment for 24 hrs. Fig. 2 shows there is a statistically significant increase in *α-SMA* and *Col2* gene expressions after TGF-β2 treatment using quantitative Real Time-PCR analysis, in a dose dependent manner ($p<0.0001$ and $=0.011$ respectively).

[0067] SSPC pellets were cultured in control medium and medium containing 10ng/ml of TGF-β2 (TM-pellets) for 4 weeks. Histological analysis showed that most SSPC were located in the midperipheral and peripheral area which surrounded the central matrix tissue in TM-pellets. Immunohistochemical analysis showed Col2 was expressed in the local, mid-

peripheral area of TM-pellets whereas α-SMA expression was more extensive within the TM-pellets, especially in mid-peripheral area and peripheral area. In contrast, Col2 andα-SMA expressions were less in the control group.

Example 3: The Expression of Col2 and α-SMA in Sclera of FDM Mice

[0068] An in vivo study evaluating the expression of Col2 and α-SMA in the sclera was evaluated using FDM Mice. FDM was induced in the right eye of the mouse as described previously and the left eye served as a control. The differences between the two eyes of each mouse in axial length was not significant at the beginning (p=0.378). By Day 21, form deprived eyes had myopia with an axial length of 3055±39μm which was significantly longer than the contralateral control eyes (3015±40μm, p<0.001)

[0069] Fig. 3 shows after 21 days of visual deprivation, the expressions of Col2 and α-SMA were higher in the sclera of FDM eyes using western blot analysis. Fig. 3A and 3B show the expressions of Col2 and α-SMA in FDM eyes were significantly higher than contralateral control eyes in the same mouse (P=0.021 for Col2 and (p=0.042 for α-SMA). Immunostaining shows Col2 expression was higher in the scleral region of the FDM eyes than in the control eyes, whereas α-SMA expression was greater in the scleral (close to the choroid side) and choroid areas of FDM eyes in comparison to control eyes.

Example 4: The Expression of TGF-β mRNA Levels in the Choroid of FDM Mice

[0070] An in vivo study evaluating the expression of TGF-β in the choroid was performed using FDM Mice. FDM was induced in mice as previously described.

[0071] The relative expressions of TGF-β1, TGF-β2 and TGF-β3 mRNA in the choroids of FDM mice were significantly higher than contralateral control choroids (2.98, 4.44 and 3.86

fold change, p=0.042, 0.045 and 0.041, respectively, Fig.4).

Example 5: The Effect of Anti-muscarinic Agent and NSAID on Col2 and α-SMA Expression

[0072] An in vitro study examining the effect of an anti-muscarinic agent and NSAID on Col2 and α-SMA expressions was performed using human SSPC. SSPCs were treated with 1mM of atropine, 5mM of ketorolac and 1mM of diclofenac in the presence of TGF-β2 (10ng/ml), as previously described.

[0073] Fig. 5A and 5B show the expressions of Col2 and α-SMA were suppressed by atropine, ketorolac and diclofenac, in the presence of TGF-β2.

Example 6: Treating Myopia Subjects With a Pharmaceutical Composition Comprising Anti-muscarinic Agent and NSAID

[0074] A clinical study of 11 myopia patients using atropine and a pharmaceutical composition comprising atropine and ketorolac was conducted.

[0075] 11 myopic patients received atropine treatment for at least a year, with the dose of atropine ranged from 0.005% to 1% weight atropine per unit dose (about 0.05 to 0.5ml). Each affected eye was given one drop (about 0.05 to 0.5ml) of atropine ophthalmic solution at night. During the atropine treatment, the average myopia progression rate for these 11 myopic patients was -0.9 Diopter/year.

[0076] Subsequently, these 11 myopic patients received a pharmaceutical composition comprising atropine and ketorolac for at least 3 months. The dose of atropine ranged from about 0.005% to about 1% weight atropine per unit dose (about 0.05 to 0.5ml) and the dose of ketorolac ranged from about 0.25% to about 0.5% weight ketorolac per unit dose (0.5ml).

Each affected eye was given one drop (about 0.05 to 0.5ml) of combined atropine with ketorolac ophthalmic solution at night. During the combined atropine with ketorolac treatment, the average myopia progression rate for these 11 myopia patients reduced to -0.38 Diopter/year (Fig. 6).

Example 7: Treating a Myopia Subject With NSAID

[0077] A myopic patient could not tolerate the side effects of atropine and was given NSAID to treat his myopia. The dose of ketorolac was about 0.5% weight ketorolac per unit dose (about 0.05 to 0.5ml) and the affected eye was given one drop (about 0.05 to 0.5ml) of ketorolac ophthalmic solution at night.

[0078] The average myopia progression rate for this patient was -0.78 Dioptor per year in right eye and -0.91 Diopter per year in left eye without any treatment. After three months of NSAID treatment, there was no myopia progression in both eyes (Fig. 7).

Example 8: The Expression of Inflammation Markers in Choroids of FDM Mice

[0079] An in vivo study evaluating the expression of inflammation markers in the choroid was evaluated using FDM Mice. FDM was induced in the right eye of the mouse as described previously and the left eye served as a control.

[0080] Fig. 8A shows the level of IL-6 by real-time PCR in the choroids of FDM eye was higher than that of control eye. Fig. 8b shows the level of TNF-α by real-time PCR in the choroids of FDM eye was higher than that of control eye. The level of TNF-α was suppressed by administering one ketorolac eye drop once a day to the FDM eye.

Example 9: Treatment of SSPC with a Pharmaceutical Composition Comprising Atropine and Ketorolac Reduced α-SMA and Col2

[0081] An in vitro study of the effect of a pharmaceutical composition comprising Atropine and Ketorolac on α-SMA and Col2 expressions was performed using SSPCs. SSPCs were treated with 10 *n*g/ml of TGF-β2, as previously described.

[0082] Fig. 9A shows the expression of α-SMA increased in the presence of TGF-β2 (T2) but reduced with 0.5mM of atropine (A), 0.25mM of Ketorolac (X1), and a pharmaceutical composition comprising Atropine and Ketorolac, in the presence of TGF-β2.

[0083] Fig. 9B shows the expression of Col2 increased in the presence of TGF-β2 (T2) but reduced with 0.5mM of atropine (A), 0.25mM of Ketorolac (X1), and a pharmaceutical composition comprising Atropine and Ketorolac, in the presence of TGF-β2.

[0084] The results show that a pharmaceutical composition comprising Atropine and Ketorolac has a synergistic effect in α-SMA and Col2 reduction.

CLAIMS

1. A pharmaceutical composition, comprising:

about 0.001 % to about 1% by weight atropine; and

about 0.01% to about 1% by weight nonsteroidal anti-inflammatory drug (NSAID).

2. The pharmaceutical composition of claim 1, wherein said atropine is about 0.005% to about 0.05% by weight.

3. The pharmaceutical composition of claim 1, wherein the NSAID is selected from Ketorolac, Diclofenac, Indomethacin, Bromfenac, Nepafenac, Flurbiprofen, or combination thereof.

4. The pharmaceutical composition of claim 3, wherein the NSAID is about 0.05% to about 1% by weight Ketorolac.

5. The pharmaceutical composition of claim 3, wherein the NSAID is about 0.01% to about 0.2% by weight diclofenac.

6. Use of a pharmaceutical composition comprising about 0.05% to about 1% by weight Ketorolac in the manufacture of a medicament for the treatment of myopia.

7. Use of a pharmaceutical composition comprising about 0.01% to about 0.2% by weight diclofenac in the manufacture of a medicament for the treatment of myopia.

8. The use of claim 6 or 7, further comprising about 0.001% to about 1% by weight atropine.

9. The use of claim 6 or 7, further comprising about 0.005% to about 0.05% by weight atropine.