(54) Title: DIAGNOSIS AND PROGNOSIS OF GLAUCOMA

(57) Abstract

A glucocorticoid-induced protein, TIGR*, that is produced by cells of the trabecular meshwork can be used to diagnose glaucoma. The TIGR* protein, anti-TIGR* antibodies, and TIGR* encoding sequences also provide a diagnostic for glaucoma and its related diseases.
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TITLE OF THE INVENTION :

5 DIAGNOSIS AND PROGNOSIS OF GLAUCOMA

FIELD OF THE INVENTION :

The present invention is in the fields of diagnostics, and concerns methods and reagents for diagnosing glaucoma and related disorders. This invention was supported with Government funds (NIH EY02477 and NIH EY 08905-02). The Government has certain rights in this invention.

BACKGROUND OF THE INVENTION :

"Glaucomas" are a group of debilitating eye diseases that are the leading cause of preventable blindness in the United States and other developed nations. Primary Open Angle Glaucoma ("POAG") is the most common form of glaucoma. The disease is characterized by the degeneration of the trabecular meshwork, leading to obstruction of the normal ability of aqueous humor to leave the eye without closure of the space (e.g., the "angle") between the iris and cornea (see, Vaughan, D. et al., In: General Ophthalmology, Appleton & Lange, Norwalk, CT, pp. 213-230 (1992)). A characteristic of such obstruction in this disease is an increased intraocular pressure ("IOP"), resulting in progressive visual loss and blindness if not treated appropriately and in a timely fashion.

The disease is estimated to affect between 0.4% and 3.3% of all adults over 40 years old (Leske, M.C. et al., Amer. J. Epidemiol. 113:1843-1846 (1986); Bengtsson, B., Br. J. Ophthalmol. 73:483-487 (1989); Strong, N.P., Ophthal. Physiol. Opt. 12:3-7 (1992)). Moreover, the prevalence of the disease rises with age to over 6% of those 75 years or older (Strong, N.P., Ophthal. Physiol. Opt. 12:3-7 (1992)).
A link between the IOP response of patients to glucocorticoids and the disease of POAG has long been suspected. While only 5% of the normal population shows a high IOP increase (16 mm Hg) to topical glucocorticoid testing, over 90% of patients with POAG show this response. In addition, an open angle glaucoma may be induced by exposure to glucocorticoids. This observation has suggested that an increased or abnormal glucocorticoid response in trabecular cells may be involved in POAG (Zhan, G.L. et al., Exper. Eye Res. 54:211-218 (1992); Yun, A.J. et al., Invest. Ophthalmol. Vis. Sci. 30:2012-2022 (1989); Clark, A.F., Exper. Eye Res. 55:265 (1992); Klemetti, A., Acta Ophthalmol. 68:29-33 (1990); Knepper, P.A., U.S. Patent No. 4,617,299).

The ability of glucocorticoids to induce a glaucoma-like condition has led to efforts to identify genes or gene products that would be induced by the cells of the trabecular meshwork in response to glucocorticoids (Polansky, J.R. et al., In: Glaucoma Update IV, Springer-Verlag, Berlin, pp. 20-29 (1991)). Initial efforts using short-term exposure to dexamethasone revealed only changes in specific protein synthesis. Extended exposure to relatively high levels of dexamethasone was, however, found to induce the expression of related 66 kD and 55 kD proteins that could be visualized by gel electrophoresis (Polansky, J.R. et al., In: Glaucoma Update IV, Springer-Verlag, Berlin, pp.20-29 (1991)). The induction kinetics of these proteins as well as their dose response characteristics were similar to the kinetics of those that were required for steroid-induced IOP elevation in human subjects (Polansky, J.R. et al., In: Glaucoma Update IV, Springer-Verlag, Berlin, pp. 20-29 (1991)). Problems of aggregation and apparent instability or loss of protein in the purification process were obstacles in obtaining a direct protein sequence.


U.S. Patent No. 5,606,043 ("U.S. Patent '043") sets forth an amino acid sequence of a TIGR protein identified by its sequence listing (U.S. Patent '043 SEQ ID NO: 1) and also cDNA nucleic acid sequences (U.S. Patent '043 SEQ ID NO: 2 and SEQ ID NO: 3). The amino acid sequence and cDNA nucleic acid sequences as set forth in this application differ from those sequences as set forth in U.S. Patent '043. In this connection, see the areas set forth in Figures 1A and 1B and labelled as A-L.

**SUMMARY OF THE INVENTION**: The invention concerns a novel peptide sequence (Trabecular Meshwork Induced Glucocorticoid Response* (TIGR*)) discovered to be highly induced by glucocorticoids in the endothelial lining cells of the human trabecular meshwork. The cDNA for this protein, the protein itself, molecules that bind to it, and nucleic acid molecules that encode it, provide improved methods and reagents for diagnosing glaucoma and related disorders, as well as for diagnosing other diseases or conditions, such as cardiovascular, immunological, or other diseases or conditions that affect the expression or activity of the protein. Indeed, the molecules of the present invention may be used to diagnose diseases or conditions which are characterized by alterations in the expression of extracellular proteins. In addition, due to its cellular functions and DNA binding properties, the molecules of
the present invention may be used to diagnose diseases or conditions which are characterized those functions.

In detail, the invention provides a substantially purified TIGR\textsuperscript{*} protein having the sequence of SEQ ID NO:1 residues 1-504 or 15-504.

The invention further provides a nucleic acid molecule that encodes a TIGR\textsuperscript{*} protein, especially a nucleic acid molecule that comprises the sequence of SEQ ID NO:2 or SEQ ID NO:3.

The invention also provides an antibody capable of specifically binding to a TIGR\textsuperscript{*} protein.

The invention also provides a method for diagnosing glaucoma in a patient which comprises determining whether the amount of a TIGR\textsuperscript{*} protein present in the trabecular meshwork of an eye of the patient exceeds the amount of that TIGR\textsuperscript{*} protein present in the trabecular meshwork of an eye of an individual who is not suffering from glaucoma, wherein the detection of an excessive amount of the TIGR\textsuperscript{*} protein is indicative of glaucoma.

The invention also provides a method for quantitatively or qualitatively determining the amount of a TIGR\textsuperscript{*} protein present in the trabecular meshwork of an eye of an individual, and determining whether that amount exceeds the amount of that TIGR\textsuperscript{*} protein present in the trabecular meshwork of an eye of an individual who is not suffering from glaucoma, wherein the detection of an excessive amount of the TIGR\textsuperscript{*} protein is indicative of glaucoma.

The invention further provides for a method for diagnosing glaucoma in a patient which comprises the steps: (A) incubating under conditions permitting nucleic acid hybridization: a marker nucleic acid molecule, the marker nucleic acid molecule capable of specifically hybridizing with a polynucleotide having the sequence of SEQ ID NO:2 or SEQ ID NO:3 or its complement, and a complementary nucleic acid molecule obtained from a cell or a bodily fluid of said patient, where the nucleic acid hybridization between the marker nucleic acid molecule, and the complementary nucleic acid molecule obtained from the patient permits the detection of a polymorphism whose presence is predictive of a
mutation affecting TIGR* Response in the patient; (B) permitting hybridization between the marker nucleic acid molecule and the complementary nucleic acid molecule obtained from the patient; and (C) detecting the presence of the polymorphism, where the detection of the polymorphism is diagnostic of glaucoma.

**BRIEF DESCRIPTION OF THE FIGURE**

Figures 1A and 1B provide the amino acid sequence of the TIGR* protein and the sequence of the cDNA that encodes the TIGR* protein.

**DETAILED DESCRIPTION OF THE INVENTION**

I. Overview of the Invention

As indicated above, the trabecular meshwork has been proposed to play an important role in the normal flow of the aqueous, and has been presumed to be the major site of outflow resistance in glaucomatous eyes. Human trabecular meshwork (HTM) cells are endothelial like cells which line the outflow channels by which aqueous humor exits the eye; altered synthetic function of the cells may involve in the pathogenesis of steroid glaucoma and other types of glaucoma. Sustained steroid treatment of these cells are interesting because it showed major difference when compared to 1-2 day glucocorticoid (GC) exposure, which appears relevant to the clinical onset of steroid glaucoma (1-6 weeks).

Despite decades of research, prior to the present invention, the molecular basis for glaucoma had not been determined (Snyder, R.W. et al., Exper. Eye Res. 57:461-468 (1993); Wiggs, J.L. et al., Genomics 21:299-303 (1994)).

Although trabecular meshwork cells had been found to induce specific proteins in response to glucocorticoids (see, Polansky, J.R., In: "Schriftenreihe de Adademie der Wissenschaften und der Literatur, Mainz," 307-318 (1993)), efforts to purify the expressed protein were encumbered by insolubility and other problems. Nguyen, T.D. et al. (In: "Schriftenreihe de Adademie der
Wissenschaften und der Literatur, Mainz," 331-343 (1993), herein incorporated by reference) used a molecular cloning approach to isolate a highly induced mRNA species from glucocorticoid-induced human trabecular cells. The mRNA exhibited a time course of induction that was similar to the glucocorticoid-induced proteins. The clone was designated "II.2"

The present invention stems in part from the recognition that the isolated II.2 clone encodes a novel secretory protein that is induced in cells of the trabecular meshwork upon exposure to glucocorticoids. It has been proposed that this protein may become deposited in the extracellular spaces of the trabecular meshwork and bind to the surface of the endothelial cells that line the trabecular meshwork, thus causing a decrease in aqueous flow. Quantitative dot blot analysis and PCR evaluations have shown that the TIGR mRNA exhibits a progressive induction with time whereas other known GC-inductions from other systems and found in HTM cells (metallothionein, alpha-1 acid glycoprotein and alpha-1 antichymotrypsin) reached maximum level at one day or earlier. Of particular interest, the induction level of this clone was very high (4-6% total cellular mRNA) and with control level undetectable without PCR method. Based on studies of 35S methionine cell labeling, the clone has the characteristics recently discovered for the major GC-induced extracellular glycoprotein in these cells, which is a sialenated, N-glycosylated molecule with a putative inositol phosphate anchor. The induction of TIGR RNA approached 4% of the total cellular mRNA. The mRNA increased progressively over 10 days of dexamethasone treatment.

In-situ hybridization using the P₁TIGR clone (P₁TIGR, ATCC No. 97570 American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, USA) shows a sequence or sequences that specifically hybridize to the TIGR* gene located at chromosome 1, q20-26, and most preferably at chromosome 1, q23-24. Clone P₁TIGR comprises human genomic sequences that specifically hybridize to the TIGR* gene cloned into the BamH1

The II.2 clone is 2.0 Kb whereas the Northern blotting shows a band of 2.5 Kb. Although not including a poly A tail, the 3' end of the clone contains two consensus polyadenylation signals. Southern analysis suggested two groups of genomic sequences and two genomic clones were isolated. Study of cyclohexamide treatment in the absence and presence of GC suggest that the induction of TIGR* may involve factors in addition to the GC receptor. The TIGR* gene may be involved in the cellular stress response since it is also induced by stimulants such as H$_2$O$_2$, TPA, glucose and heat; this fact may relate to glaucoma pathogenesis and treatment.

The amino acid sequence of TIGR*, the cDNA sequence that encodes TIGR*, and the 2.0 kb nucleotide sequence that contains this coding region are shown in Figures 1A-1B. The amino acid sequence of TIGR* is shown in Figures 1A-1B (and in SEQ ID NO:1). The nucleotide sequence of SEQ ID NO:2 is that of the TIGR* cDNA molecule that is shown in Figures 1A-1B. The portion of SEQ ID NO:2 that is shown in Figures 1A-1B as encoding the TIGR* protein is presented as SEQ ID NO:3.

The primary structure of TIGR* initiates from an ATG initiation site (SEQ ID NO: 3, nucleotides 1-3; SEQ ID NO:1, residue 1) and includes a 20 amino acid consensus signal sequence immediately downstream from a second ATG (SEQ ID NO: 3, nucleotides 43-45; SEQ ID NO:1, residue 15), indicating that the protein is a secretory protein. The protein contains an N-linked glycosylation site located in the most hydrophilic region of the molecule. The amino terminal portion of the protein is highly polarized and adopts alpha helical structure as shown by its hydropathy profile and the Garnier-Robison structure analysis. In contrast, the protein contains a 25 amino acid hydrophobic region near its carboxy terminus. This region may comprise a GIP anchoring sequence. Thus, the invention concerns two TIGR* proteins: TIGR* and a processed form of TIGR*.
The TIGR* protein also contains 5 putative O-linked glycosylation sites throughout the molecule. "Leucine zipper" regions define a helical structure that permits protein-protein binding to occur (see, generally, Tso, J.Y. et al., PCT Patent Application WO93/11162; Land, K.H. et al., PCT Patent Application WO93/19176). The TIGR* protein contains 7 leucine zipper units. The presence of the zipper regions provides a means for the TIGR* molecules to bind to one another forming macromolecular and possible aggregation. Studies showing the specific binding of this molecule to HTM cells (but not to fibroblast cells) support the notion that it can influence the outflow pathway in HTM tissue to cause the increased intra-ocular pressure that characterizes glaucoma and its related diseases. TIGR* protein has also been successfully expressed using the baculovirus system and Sf9 insect cells. The major recombinant proteins are the two 55 kd cellular proteins encoded by the TIGR* cDNA. Antibodies produced from these protein recognize both the cellular 55 kd proteins and the secreted 66 kd glycosylated form of these proteins in dexamethasome-treated HTM cells and in organ culture systems. In situ analysis of glaucomatous tissue specimens show a high expression level of this protein relative to normal controls.

The presence, induction, and level of the TIGR* secretory protein mirror the onset and kinetics with which glucocorticoids induce glaucoma, and the glucocorticoid-induced expression of this secretory protein comprises the molecular basis for glaucoma and its related diseases. Such an understanding of the molecular basis permits the definition of diagnostic agents for glaucoma and its related diseases.

II. The Preferred Agents of the Invention

As used herein, the term "glaucoma" has its art recognized meaning, and includes both primary glaucomas, secondary glaucomas, and familial (i.e. inherited glaucomas). The methods of the present invention are particularly relevant to the diagnosis of POAG, OAG, juvenile glaucoma, and inherited glaucomas. A disease or condition is said to be related to glaucoma if it possesses or
exhibits a symptom of glaucoma, for example, an increased intraocular pressure resulting from aqueous outflow resistance (see, Vaughan, D. et al., In: General Ophthalmology, Appleton & Lange, Norwalk, CT, pp. 213-230 (1992)). The preferred agents of the present invention are discussed in detail below.

The agents of the present invention are capable of being used to diagnose the presence or severity of glaucoma and its related diseases in a patient suffering from glaucoma (a "glaucomatous patient"). Such agents may be either naturally occurring or non-naturally occurring. As used herein, a naturally occurring molecule may be "substantially purified," if desired, such that one or more molecules that is or may be present in a naturally occurring preparation containing that molecule will have been removed or will be present at a lower concentration than that at which it would normally be found.

The agents of the present invention will preferably be "biologically active" with respect to either a structural attribute, such as the capacity of a nucleic acid to hybridize to another nucleic acid molecule, or the ability of a protein to be bound by antibody (or to compete with another molecule for such binding) Alternatively, such an attribute may be catalytic, and thus involve the capacity of the agent to mediate a chemical reaction or response.

The agents of the present invention comprise nucleic acid molecules, proteins, and organic molecules.

A. Nucleic Acid Molecules

A preferred class of agents of the present invention comprises TIGR* nucleic acid molecules ("TIGR* molecules"). Such molecules may be either DNA or RNA.

In one embodiment, such nucleic acid molecules will encode all or a fragment of TIGR* protein, its "promoter" or flanking gene sequences. As used herein, the term "promoter" is used in an expansive sense to refer to the regulatory sequence(s) that control mRNA production. Such sequences include RNA polymerase binding sites, glucocorticoid response elements, enhancers, etc.
All such TIGR* molecules may be used to diagnose the presence of glaucoma and severity of glaucoma.

Fragment TIGR* nucleic acid molecules may encode significant portion(s) of, or indeed most of, the TIGR* protein. Alternatively, the fragments may comprise smaller oligonucleotides (having from about 15 to about 250 nucleotide residues, and more preferably, about 15 to about 30 nucleotide residues.). Such oligonucleotides may be used as probes of TIGR* mRNA. For such purpose, the oligonucleotides must be capable of specifically hybridizing to a TIGR* nucleic acid molecule. As used herein, two nucleic acid molecules are said to be capable of specifically hybridizing to one another if the two molecules are capable of forming an anti-parallel, double-stranded nucleic acid structure, whereas they are unable to form a double-stranded structure when incubated with a non-TIGR* nucleic acid molecule. A nucleic acid molecule is said to be the "complement" of another nucleic acid molecule if they exhibit complete complementarity. As used herein, molecules are said to exhibit "complete complementarity" when every nucleotide of one of the molecules is complementary to a nucleotide of the other. Two molecules are said to be "minimally complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under at least conventional "low-stringency" conditions. Similarly, the molecules are said to be "complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under conventional "high-stringency" conditions. Conventional stringency conditions are described by Sambrook, J., et al., (In: Molecular Cloning, a Laboratory Manual, 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, New York (1989)), and by Haymes, B.D., et al. (In: Nucleic Acid Hybridization, A Practical Approach, IRL Press, Washington, DC (1985)), both herein incorporated by reference). Departures from complete complementarity are therefore permissible, as long as such departures do not completely preclude the capacity of the molecules to form a double-stranded structure. Thus, in order for
an oligonucleotide to serve as a primer it need only be sufficiently complementary in sequence to be able to form a stable double-stranded structure under the particular solvent and salt concentrations employed.

Apart from their diagnostic uses, such oligonucleotides may be employed to obtain other TIGR* nucleic acid molecules. Such molecules include the TIGR*-encoding nucleic acid molecule of non-human animals (particularly, cats, monkeys, rodents and dogs), fragments thereof, as well as their promoters and flanking sequences. Such molecules can be readily obtained by using the above-described primers to screen cDNA or genomic libraries obtained from non-human species. Methods for forming such libraries are well known in the art. Such analogs may differ in their nucleotide sequences from that of SEQ ID NO:1, because complete complementarity is not needed for stable hybridization. The TIGR* nucleic acid molecules of the present invention therefore also include molecules that, although capable of specifically hybridizing with TIGR* nucleic acid molecules may lack “complete complementarity.”

al., US 4,683,194)) to amplify and obtain any desired TIGR*-encoding DNA molecule or fragment.

The TIGR* promoter sequence(s) and TIGR* flanking sequences can also be obtained using the SEQ ID NO:2 sequence provided herein. In one embodiment, such sequences are obtained by incubating oligonucleotide probes of TIGR* oligonucleotides with members of genomic human libraries and recovering clones that hybridize to the probes. In a second embodiment, methods of "chromosome walking," or 3' or 5' RACE may be used (Frohman, M.A. et al., Proc. Natl. Acad. Sci. (U.S.A.) 85:8998-9002 (1988); Ohara, O. et al., Proc. Natl. Acad. Sci. (U.S.A.) 86:5673-5677 (1989)) to obtain such sequences.

B. TIGR* Protein and Peptide Molecules

A second preferred class of agents ("TIGR* molecules") comprises the TIGR* protein, its peptide fragments, fusion proteins, and analogs. As used herein, the term "TIGR* protein" refers to a protein having the amino acid sequence of SEQ ID NO:1. TIGR* protein may be produced via chemical synthesis, or more preferably, by expressing TIGR*-encoding cDNA in a suitable bacterial or eukaryotic host. Most preferably, the subsequence of such cDNA that encodes TIGR* may be used for this purpose (SEQ ID NO:3). Alternatively, the entire cDNA (SEQ ID NO:2) shown in Figures 1A-1D may be employed. Suitable methods for expression are described by Sambrook, J., et al., (In: Molecular Cloning, a Laboratory Manual, 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, New York (1989)), or similar texts.

A "TIGR* fragment" is a peptide or polypeptide whose amino acid sequence comprises a subset of the amino acid sequence of TIGR* protein. A TIGR* protein or fragment thereof that comprises one or more additional non-TIGR* peptide regions is a "TIGR* fusion" protein. Such molecules may be derivatized to contain carbohydrate or other moieties (such as keyhole limpet hemocyanin, etc.). As in the case of TIGR* protein, the fragments and fusions of the present invention are preferably produced via recombinant means.
The analogs of the TIGR* molecules comprise TIGR* proteins, fragments or fusions in which non-essential, or non-relevant, amino acid residues have been added, replaced, or deleted. An example of such an analog is the TIGR* protein of non-human species, such as primates, dogs, cats, etc. Such analogs can readily be obtained by any of a variety of methods. Most preferably, as indicated above, the disclosed SEQ ID NO:2 will be used to define a pair of primers that may be used to isolate the TIGR*-encoding nucleic acid molecules from any desired species. Such molecules can be expressed to yield TIGR* analogs by recombinant means.

C. Antibodies Reactive Against TIGR*

One aspect of the present invention concerns antibodies, single-chain antigen binding molecules, or other proteins that specifically bind to TIGR* protein and its analogs, fusions or fragments. Such antibodies are “anti-TIGR* antibodies,” and may be used to diagnose glaucoma and its related diseases. As used herein, an antibody or peptide is said to “specifically bind” to TIGR* if such binding is not competitively inhibited by the presence of non-TIGR* molecules.

Nucleic acid molecules that encode all or part of the TIGR* protein can be expressed, via recombinant means, to yield TIGR* protein or peptides that in turn be used to elicit antibodies that are capable of binding TIGR*. Such antibodies may be used in immunodiagnostic assays of glaucoma. Such TIGR*-encoding molecules, or their fragments may be a “fusion” molecule (i.e. a part of a larger nucleic acid molecule) such that, upon expression, a fusion protein is produced.

The antibodies that specifically bind TIGR* proteins and protein fragments may be polyclonal or monoclonal, and may comprise intact immunoglobulins, of antigen binding portions of immunoglobulins (such as (F(ab')2) fragments, or single-chain immunoglobulins producable, for example, via recombinant means.
Murine monoclonal antibodies are particularly preferred. BALB/c mice are preferred for this purpose, however, equivalent strains may also be used. The animals are preferably immunized with approximately 25 µg of purified TIGR* protein (or fragment thereof) that has been emulsified in a suitable adjuvant (such as TiterMax adjuvant (Vaxcel, Norcross, GA)). Immunization is preferably conducted at two intramuscular sites, one intraperitoneal site, and one subcutaneous site at the base of the tail. An additional i.v. injection of approximately 25 µg of antigen is preferably given in normal saline three weeks later. After approximately 11 days following the second injection, the mice may be bled and the blood screened for the presence of anti-TIGR* antibodies. Preferably, a direct binding ELISA is employed for this purpose.

Most preferably, the mouse having the highest antibody titer is given a third i.v. injection of approximately 25 µg of TIGR* protein or fragment. The splenic leukocytes from this animal may be recovered 3 days later, and are then permitted to fuse, most preferably, using polyethylene glycol, with cells of a suitable myeloma cell line (such as, for example, the P3X63Ag8.653 myeloma cell line). Hybridoma cells are selected by culturing the cells under “HAT” (hypoxanthine-aminopterin-thymine) selection for about one week. The resulting clones may then be screened for their capacity to produce monoclonal antibodies (“mAbs) to TIGR* protein, preferably by direct ELISA.

In one embodiment, anti-TIGR* monoclonal antibodies are isolated using TIGR* fusions, or conjugates, as immunogens. Thus, for example, a group of mice can be immunized using a TIGR* fusion protein emulsified in Freund’s complete adjuvant (approximately 50 µg of antigen per immunization). At three week intervals, an identical amount of antigen is emulsified in Freund’s incomplete adjuvant and used to immunize the animals. Ten days following the third immunization, serum samples are taken and evaluated for the presence of antibody. If antibody titers are too low, a fourth booster can be employed. Polysera capable of
binding TIGR* at 1:5,000 dilution can also be obtained using this method.

In a preferred procedure for obtaining monoclonal antibodies, the spleens of the above-described immunized mice are removed, disrupted, and immune splenocytes are isolated over a ficoll gradient. The isolated splenocytes are fused, using polyethylene glycol with BALB/c-derived HGPRT (hypoxanthine guanine phosphoribosyl transferase) deficient P3x63Ag8.653 plasmacytoma cells. The fused cells are plated into 96 well microtiter plates and screened for hybridoma fusion cells by their capacity to grow in culture medium supplemented with hypoxanthine, aminopterin and thymidine for approximately 2-3 weeks. On average, one out of every \(10^6\) spleen cells subjected to fusion yields a viable hybridoma. A typical spleen yields 5-10 x \(10^7\) spleen cells.

Hybridoma cells that arise from such incubation are preferably screened for their capacity to produce an immunoglobulin that binds to TIGR* protein. An indirect ELISA may be used for this purpose. In brief, the supernatants of hybridomas are incubated in microtiter wells that contain immobilized TIGR* protein. After washing, the titer of bound immunoglobulin can be determined using, for example, a goat anti-mouse antibody conjugated to horseradish peroxidase. After additional washing, the amount of immobilized enzyme is determined (for example through the use of a chromogenic substrate). Such screening is performed as quickly as possible after the identification of the hybridoma in order to ensure that a desired clone is not overgrown by non-secreting neighbors. Desirably, the fusion plates are screened several times since the rates of hybridoma growth vary. In a preferred sub-embodiment, a different antigenic form of TIGR* may be used to screen the hybridoma. Thus, for example, the splenocytes may be immunized with one TIGR* immunogen, but the resulting hybridomas can be screened using a different TIGR* immunogen.

As discussed below, such antibody molecules or their fragments may be used for diagnostic purposes. Where the
antibodies are intended for diagnostic purposes, it may be desirable to derivatize them, for example with a ligand group (such as biotin) or a detectable marker group (such as a fluorescent group, a radioisotope or an enzyme).

The ability to produce antibodies that bind TIGR* molecules permits the identification of mimetic compounds of TIGR*. A "mimetic compound" of TIGR* is a compound that is not TIGR*, or a fragment of TIGR*, but which nonetheless exhibits an ability to specifically bind to anti-TIGR* antibodies. Such molecules can be used to elicit anti-TIGR* antibodies, and thus, may be used to assist the diagnosis of glaucoma and its related diseases.

III. Uses of the Molecules of the Invention in the Diagnosis of Glaucoma and Related Diseases

A particularly desired use of the present invention relates to the diagnosis of glaucoma and its related diseases. As indicated above, methods for diagnosing glaucoma suffer from inaccuracy, or require multiple examinations. The molecules of the present invention may be used to define superior assays for glaucoma.

In a first embodiment, the TIGR* molecules of the present invention are used to determine whether an individual has a mutation in the TIGR* gene, or in regulatory regions or other genes that control or affect the expression of TIGR*, in order to identify individuals who would be predisposed to glaucoma and related diseases.

In one sub-embodiment, such an analysis is conducted by determining the presence and/or identity of polymorphisms in the TIGR* gene or its flanking regions which are associated with glaucoma, or a predisposition to glaucoma. The genomes of animals and plants naturally undergo spontaneous mutation in the course of their continuing evolution (Gusella, J.F., Ann. Rev. Biochem. 55:831-854 (1986)).
A "polymorphism" in the TIGR* gene or its flanking regions is a variation or difference in the sequence of the TIGR* gene or its flanking regions that arises in some of the members of a species. The variant sequence and the "original" sequence co-exist in the species' population. In some instances, such co-existence is in stable or quasi-stable equilibrium.

A polymorphism is thus said to be "allelic," in that, due to the existence of the polymorphism, some members of a species may have the original sequence (i.e. the original "allele") whereas other members may have the variant sequence (i.e. the variant "allele"). In the simplest case, only one variant sequence may exist, and the polymorphism is thus said to be di-allelic. In other cases, the species' population may contain multiple alleles, and the polymorphism is termed tri-allelic, etc. A single gene may have multiple different unrelated polymorphisms. For example, it may have a di-allelic polymorphism at one site, and a multi-allelic polymorphism at another site.

The identification of a polymorphism in the TIGR\textsuperscript{*} gene can be determined in a variety of ways. By correlating the presence or absence of glaucoma in an individual with the presence or absence of a polymorphism in the TIGR\textsuperscript{*} gene or its flanking regions, it is possible to diagnose the predisposition of an asymptomatic patient to glaucoma. If a polymorphism creates or destroys a restriction endonuclease cleavage site, or if it results in the loss or insertion of DNA (e.g., a VNTR polymorphism), it will alter the size or profile of the DNA fragments that are generated by digestion with that restriction endonuclease. As such, individuals that possess a variant sequence can be distinguished from those having the original sequence by restriction fragment analysis. Polymorphisms that can be identified in this manner are termed "restriction fragment length polymorphisms" ("RFLPs"). RFLPs have been widely used in human and animal genetic analyses (Glassberg, J., UK patent Application 2135774; Skolnick, M.H. \textit{et al.}, Cytogen. Cell Genet. 32:58-67 (1982); Botstein, D. \textit{et al.}, Ann. J. Hum. Genet. 32:314-331 (1980); Fischer, S.G \textit{et al.} (PCT Application WO90/13668); Uhlen, M., PCT Application WO90/11369)). The role of TIGR\textsuperscript{*} in glaucoma pathogenesis indicates that genetic alterations (e.g., DNA polymorphisms) are associated with the TIGR\textsuperscript{*} gene.

Any of a variety of molecules can be used to identify such polymorphism(s). In one embodiment, the TIGR\textsuperscript{*} cDNA sequence (or a sub-sequence thereof) may be employed as a marker nucleic acid molecule to identify such polymorphism(s). Alternatively, such polymorphisms can be detected through the use of a marker nucleic acid molecule or a marker protein that is genetically linked to (i.e., a polynucleotide that co-segregates with) such polymorphism(s).

In accordance with this embodiment of the invention, a sample DNA is obtained from a patient's cells. In a preferred embodiment, the DNA sample is obtained from the patient's blood. However, any source of DNA may be used. The DNA is subjected to restriction endonuclease digestion. TIGR\textsuperscript{*} is used as a probe in accordance with the above-described RFLP methods. By comparing
the RFLP pattern of the TIGR\* gene obtained from normal and
glaucomatous patients, one can determine a patient’s
predisposition to glaucoma. The polymorphism obtained in this
approach can then be cloned to identify the mutation at the coding
region which alters the protein’s structure or regulatory region of
the gene which affects its expression level. Changes involving
promoter interactions with other regulatory proteins can be
identified by, for example, gel shift assays using HTM cell
extracts, fluid from the anterior chamber of the eye, serum, etc.
Interactions of TIGR\* protein in glaucomatous cell extracts, fluid
from the anterior chamber of the eye, serum, etc. can be compared
to control samples to thereby identify changes in those properties
of TIGR\* that relate to the pathogenesis of glaucoma.

Several different classes of polymorphisms may be
identified through such methods. Examples of such classes
include: (1) polymorphisms present in the TIGR\* cDNA of different
individuals; (2) polymorphisms in non-translated TIGR\* gene
sequences, including the promoter or other regulatory regions of
the TIGR\* gene; (3) polymorphisms in genes whose products
interact with TIGR\* regulatory sequences; (4) polymorphisms in
gene sequences whose products interact with the TIGR\* protein,
or to which the TIGR\* protein binds.

In an alternate sub-embodiment, the evaluation is conducted
using oligonucleotide “probes” whose sequence is complementary
to that of a portion of TIGR\* mRNA. Such molecules are then
incubated with cell extracts of a patient under conditions
sufficient to permit nucleic acid hybridization. For this sub-
embodiment, cells of the trabecular meshworks are preferred. The
detection of double-stranded probe-mRNA hybrid molecules is
indicative of the presence of TIGR\* mRNA; the amount of such
hybrid formed is proportional to the amount of TIGR\* mRNA. Thus,
such probes may be used to ascertain the level and extent of TIGR\*
RNA production in a patient’s cells. Such nucleic acid
hybridization may be conducted under quantitative conditions
(thereby providing a numerical value of the amount of TIGR\* mRNA
present). Alternatively, the assay may be conducted as a
qualitative assay that indicates either that TIGR* mRNA is present, or that its level exceeds a user set, predefined value. In a second embodiment, the above-described "anti-TIGR* antibodies" are employed in an immunodiagnostic assay for glaucoma and its related diseases.

As discussed above, TIGR* protein is secreted into the extracellular matrix of the trabecular meshwork, and thus may circulate systemically in body fluids. This characteristic permits one to assay TIGR* concentrations in blood, lymph, or serum, and to thereby determine whether a patient's TIGR* levels exceed those found in the blood of individuals who are not suffering from glaucoma. Patients found to have abnormally high levels of TIGR* thus may be diagnosed as glaucoma.


The simplest immunoassay involves merely incubating an antibody that is capable of binding to a predetermined target molecule with a sample suspected to contain the target molecule. The presence of the target molecule is determined by the presence, and proportional to the concentration, of any antibody bound to the target molecule. In order to facilitate the separation of target-bound antibody from the unbound antibody initially present, a solid phase is typically employed. Thus, for example the sample can be passively bound to a solid support, and, after incubation with the antibody, the support can be washed to remove any unbound antibody.

In more sophisticated immunoassays, the concentration of the target molecule is determined by binding the antibody to a support, and then permitting the support to be in contact with a sample suspected of containing the target molecule. Target molecules that have become bound to the immobilized antibody can
be detected in any of a variety of ways. For example, the support can be incubated in the presence of a labeled, second antibody that is capable of binding to a second epitope of the target molecule. Immobilization of the labeled antibody on the support thus requires the presence of the target, and is proportional to the concentration of the target in the sample. In an alternative assay, the target is incubated with the sample and with a known amount of labeled target. The presence of target molecule in the sample competes with the labeled target molecules for antibody binding sites. Thus, the amount of labeled target molecules that are able to bind the antibody is inversely proportional to the concentration of target molecule in the sample.

In general, immunoassay formats employ either radioactive labels ("RIAs") or enzyme labels ("ELISAs"). RIAs have the advantages of simplicity, sensitivity, and ease of use. Radioactive labels are of relatively small atomic dimension, and do not normally affect reaction kinetics. Such assays suffer, however, from the disadvantages that, due to radioisotopic decay, the reagents have a short shelf-life, require special handling and disposal, and entail the use of complex and expensive analytical equipment. RIAs are described in *Laboratory Techniques and Biochemistry in Molecular Biology*, by Work, T.S., et al., North Holland Publishing Company, NY (1978), with particular reference to the chapter entitled "An Introduction to Radioimmune Assay and Related Techniques" by Chard, T., incorporated by reference herein. ELISAs have the advantage that they can be conducted using inexpensive equipment, and with a myriad of different enzymes, such that a large number of detection strategies --colorimetric, pH, gas evolution, etc. -- can be used to quantitate the assay. In addition, the enzyme reagents have relatively long shelf-lives, and lack the risk of radiation contamination that attends to RIA use. ELISAs are described in *ELISA and Other Solid Phase Immunoassays* (Kemeny, D.M. *et al.*, Eds.), John Wiley & Sons; NY (1988), incorporated by reference herein.
In an alternative diagnostic format, ocular tissue (obtained, for example by trabeculotomy) may be evaluated in an in situ immunodiagnostic assay for glaucoma and its related diseases.

In such a format, antibodies (especially labeled antibodies) or other TIGR*-binding peptides are incubated in the presence of ocular tissue in order to evaluate the clinical degree and significance of glaucoma in biopsied tissue. The extent, location, or degree of TIGR* in the ocular tissue is determined by staining or other visualization methods. Such information is then compared to the staining pattern obtained from normal or glaucomatous individuals in order to diagnose glaucoma.

Anti-TIGR* antibodies or TIGR* binding molecules may be administered to a patient, and their capacity to bind to TIGR* in vivo may be determined by ocular examination. Significantly, since such a diagnostic test is relatively rapid, immune responses that require significant time, such as the potential eliciting of anti-[anti-TIGR*] antibodies, or the complexing of such antibodies with anti-TIGR* antibodies is not important. In a preferred embodiment, the antibody will be fluorescently labeled, and will be provided to a patient by injection into the patient's circulatory system. The antibody progresses from the circulatory system to the posterior optic chamber. The complexing of the antibody with TIGR* can be monitored using conventional gonioscopy, or by other suitable means. Significantly, such an assay provides both a means to visualize the trabecular meshwork and a means for determining the extent of deposited TIGR* protein in the extracellular matrix.

As discussed above, TIGR* protein exhibits an ability to self-aggregate, due at least in part to the presence of leucine zippers in the molecule. Because small peptide fragments of TIGR* that possess such zipper regions can bind to TIGR*, such peptides may be used as alternatives to anti-TIGR* antibodies in diagnostic assays. The use of such peptides is desirable since the peptides can be modified to possess both lipophilic and hydrophilic groups. The presence of such groups will permit the peptide to traverse the corneal membrane. Thus, such agents may
be provided topically in an eye drop or ointment, and can be used in
the same manner as anti-TIGR* antibodies to effect the diagnosis
of glaucoma. The peptide will desirably be labeled with a
fluorescent group to facilitate detection.

Any suitable peptide fragment of TIGR* may be used for this
purpose, however, it is preferable to use a fragment corresponding
to all or part of SEQ ID NO:1 residues 85-92, 92-99, 131-138,
138-145, 145-152, 152-159, and 159-166. Suitable lipophilic
and hydrophilic groups are known in the art (see, Remington's
Pharmaceutical Sciences), and comprise aliphatic groups, lipids,
etc. (lipophilic groups) and organic acids, esters, ionic groups, etc.
(hydrophilic groups). Such groups can be readily added to the
TIGR* molecules of the present invention by, for example,
derivatizing the side chain groups of appropriate amino acids.

Cysteinyl residues may be reacted with a-haloacetates (and
corresponding amines), such as chloroacetic acid or chloroacet-
amide, to give carboxymethyl or carboxyamidomethyl derivatives.
Cysteinyl residues also are derivatized by reaction with bromotri-
fluoroacetone, a-bromo-b-(5-imidozoyl)propionic acid,
chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl
disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-
chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-
diazole.

Histidyl residues may be derivatized by reaction with
diethylprocarbonate at pH 5.5-7.0 because this agent is relatively
specific for the histidyl side chain. Para-bromophenacyl bromide
also is useful; the reaction is preferably performed in 0.1 M
sodium cacodylate at pH 6.0.

Lysinyl and amino terminal residues may be reacted with
succinic or other carboxylic acid anhydrides. Derivatization with
these agents has the effect of reversing the charge of the lysinyl
residues. Other suitable reagents for derivatizing a-amino-
containing residues include imidoesters such as methyl
picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride;
trinitrobenzenesulfonic acid; O-methylissurea; 2,4 pentanedione;
and transaminase-catalyzed reaction with glyoxylate.
Arginyl residues may be modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedicarboxaldehyde, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high $pK_a$ of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

Carboxyl side groups (aspartyl or glutamyl) may be selectively modified by reaction with carbodiimides (R'-N-C-N-R') such as 1-cyclohexyl-3-(2-morpholinyl)-4-ethyl carbodiimide or 1-ethyl-3-(4 azonia 4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues may be converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

IV. Methods of Administration

The agents of the present invention can be formulated according to known methods to prepare pharmacologically acceptable compositions, whereby these materials, or their functional derivatives, having the desired degree of purity are combined in admixture with a physiologically acceptable carrier, excipient, or stabilizer. Such materials are non-toxic to recipients at the dosages and concentrations employed. The active component of such compositions may be TIGR* protein, TIGR* fusion proteins or fragments of TIGR* protein or analogs or mimetics of such molecules. Where nucleic acid molecules are employed, such molecules may be sense, antisense or triplex oligonucleotides of the TIGR* cDNA or gene.

A composition is said to be "pharmacologically acceptable" if its administration can be tolerated by a recipient patient. An agent is physiologically significant if its presence results in a detectable change in the physiology of a recipient patient.

Suitable vehicles and their formulation, inclusive of other human proteins, e.g., human serum albumin, are described, for

If the composition is to be water soluble, it may be formulated in a buffer such as phosphate or other organic acid salt preferably at a pH of about 7 to 8. If the composition is only partially soluble in water, it may be prepared as a microemulsion by formulating it with a nonionic surfactant such as Tween, Pluronics, or PEG, e.g., Tween 80, in an amount of, for example, 0.04-0.05% (w/v), to increase its solubility. The term "water soluble" as applied to the polysaccharides and polyethylene glycols is meant to include colloidal solutions and dispersions. In general, the solubility of the cellulose derivatives is determined by the degree of substitution of ether groups, and the stabilizing derivatives useful herein should have a sufficient quantity of such ether groups per anhydroglucose unit in the cellulose chain to render the derivatives water soluble. A degree of ether substitution of at least 0.35 ether groups per anhydroglucose unit is generally sufficient. Additionally, the cellulose derivatives may be in the form of alkali metal salts, for example, the Li, Na, K or Cs salts.

Optionally other ingredients may be added such as antioxidants, e.g., ascorbic acid; low molecular weight (less than about ten residues) polypeptides, e.g., polyarginine or tripeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinyl pyrrolidone; amino acids, such as glycine, glutamic acid, aspartic acid, or arginine; monosaccharides, disaccharides, and other carbohydrates including cellulose or its derivatives, glucose, mannose, or dextrins; chelating agents such as EDTA; and sugar alcohols such as mannitol or sorbitol.

Additional pharmaceutical methods may be employed to control the duration of action. Controlled or sustained release preparations may be achieved through the use of polymers to complex or absorb the TIGR* molecule(s) of the composition. The controlled delivery may be exercised by selecting appropriate macromolecules (for example polyesters, polyamino acids,
polyvinyl pyrrolidone, ethylenevinylacetate, methylcellulose, carboxymethylcellulose, or protamine sulfate) and the concentration of macromolecules as well as the methods of incorporation in order to control release.

Sustained release formulations may also be prepared, and include the formation of microcapsular particles and implantable articles. For preparing sustained-release compositions, the TIGR* molecule(s) of the composition is preferably incorporated into a biodegradable matrix or microcapsule. A suitable material for this purpose is a polylactide, although other polymers of poly-(α-hydroxycarboxylic acids), such as poly-D-(−)-3-hydroxybutyric acid (EP 133,988A), can be used. Other biodegradable polymers include poly(lactones), poly(orthoesters), polyamino acids, hydrogels, or poly(orthocarbonates) poly(acetals). The polymeric material may also comprise polyesters, poly(lactic acid) or ethylene vinylacetate copolymers. For examples of sustained release compositions, see U.S. Patent No. 3,773,919, EP 58,481A, U.S. Patent No. 3,887,699, EP 158,277A, Canadian Patent No. 1176565, Sidman, U. et al., Biopolymers 22:547 (1983), and Langer, R. et al., Chem. Tech. 12:98 (1982).

Alternatively, instead of incorporating the TIGR* molecule(s) of the composition into polymeric particles, it is possible to entrap these materials in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatine-microcapsules and poly(methylmethacrylate) microcapsules, respectively, or in colloidal drug delivery systems, for example, liposomes, albumin microspheres, microemulsions, nanoparticles, and nanocapsules or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences (1980).

In an alternative embodiment, liposome formulations and methods that permit intracellular uptake of the molecule will be employed. Suitable methods are known in the art, see, for example, Chicz, R.M. et al. (PCT Application WO 94/04557), Jaysena, S.D. et al. (PCT Application WO93/12234), Yarosh, D.B. (U.S. Patent No. 5,190,762), Callahan, M.V. et al. (U.S. Patent No.
5,270,052) and Gonzalezro, R.J. (PCT Application 91/05771), all herein incorporated by reference.

The pharmaceutical compositions of the present invention may be sterilized, as by filtration through sterile filtration membranes (e.g., 0.2 micron membranes). The compositions may be stored in lyophilized form or as a liquid solution. It will be understood that use of certain of the foregoing excipients, carriers, or stabilizers will result in the formation of salts of the molecules.

The compositions of the present invention can be applied topically as to the skin, or to the cornea. When applied topically, the molecule(s) of the composition may be suitably combined with other ingredients, such as carriers and/or adjuvants. There are no limitations on the nature of such other ingredients, except that they must be pharmaceutically acceptable and efficacious for their intended administration, and cannot degrade the activity of the active ingredients of the composition. Examples of suitable vehicles include ointments, creams, gels, or suspensions, with or without purified collagen. The compositions also may be impregnated into transdermal patches, and bandages, preferably in liquid or semi-liquid form.

For obtaining a gel formulation, the molecule(s) of the composition formulated in a liquid composition may be mixed with an effective amount of a water-soluble polysaccharide or synthetic polymer such as polyethylene glycol to form a gel of the proper viscosity to be applied topically. The polysaccharide that may be used includes, for example, cellulose derivatives such as etherified cellulose derivatives, including alkyl celluloses, hydroxyalkyl celluloses, and alkylhydroxyalkyl celluloses, for example, methylcellulose, hydroxyethyl cellulose, carboxymethyl cellulose, hydroxypropyl methylcellulose, and hydroxypropyl cellulose; starch and fractionated starch; agar; alginic acid and alginates; gum arabic; pullulan; agarose; carrageenan; dextrins; dextrins; fructans; inulin; mannans; xylans; arabinans; chitosans; glycogens; glucans; and synthetic biopolymers; as well as gums such as xanthan gum; guar gum; locust bean gum; gum arabic;
tragacanth gum; and karaya gum; and derivatives and mixtures thereof. The preferred gelling agent herein is one that is inert to biological systems, non-toxic, simple to prepare, and not too runny or viscous, and will not destabilize the TIGR* molecule(s) held within it. Preferably the polysaccharide is an etherified cellulose derivative, more preferably one that is well defined, purified, and listed in USP, e.g., methylcellulose and the hydroxyalkyl cellulose derivatives, such as hydroxypropyl cellulose, hydroxyethyl cellulose, and hydroxypropyl methylcellulose. Most preferred herein is methylcellulose.

The polyethylene glycol useful for gelling is typically a mixture of low and high molecular weight polyethylene glycols to obtain the proper viscosity. For example, a mixture of a polyethylene glycol of molecular weight 400-600 with one of molecular weight 1500 would be effective for this purpose when mixed in the proper ratio to obtain a paste.

The compositions of the present invention can also be formulated for administration parenterally by injection, rapid infusion, nasopharyngeal absorption (intranasopharangeally), dermoabsorption, or orally. The compositions may alternatively be administered intramuscularly, or intravenously. Compositions for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Carriers, adjuncts or occlusive dressings can be used to increase tissue permeability and enhance antigen absorption. Liquid dosage forms for oral administration may generally comprise a liposome solution containing the liquid dosage form. Suitable forms for suspending liposomes include emulsions, suspensions, solutions, syrups, and elixirs containing inert diluents commonly used in the art, such as purified water. Besides the inert diluents, such compositions can also include wetting agents, emulsifying and suspending agents, or sweetening, flavoring, coloring or perfuming agents.
If methylcellulose is employed in the gel, preferably it comprises about 2-5%, more preferably about 3%, of the gel and the TIGR* molecule(s) of the composition is present in an amount of about 300-1000 μg per ml of gel. The dosage to be employed is dependent upon the factors described above. As a general proposition, the TIGR* molecule(s) of the composition is formulated and delivered to the target site or tissue at a dosage capable of establishing in the tissue a maximum dose that is efficacious but not unduly toxic.

In the most preferred embodiment, the molecules of the invention will be provided to the cornea or surface of the eye, and permitted to adsorb across the cornea into the anterior chamber of the eye. Methods that may be used for accomplishing such ocular drug delivery are described by Zun, L.S. (Emerg. Med. Clin. North. Amer. 6:121 (1988)), Lee, V.H. (J. Ocular Pharmacol. 6:157 (1990)), Ellis, P.P. (In: Ocular Therapeutics and Pharmacology, 7th ed., Mosby, (1987)) and (Vaughan, D. et al., In: General Ophthalmology, Appleton & Lange, Norwalk, CT, pp. 213-230 (1992)).

Most preferably, however, such drug administration will be accomplished by combining effective amounts of the agents of the invention with any of the sustained release ophthalmic delivery systems described by Davis, J.P. et al. (U.S. Patent No. 5,192,535, herein incorporated by reference).

Such preferred sustained release topical ophthalmic medicament delivery systems comprise an aqueous suspension at a pH of from about 3 to about 6.5 and an osmotic pressure of from about 10 to about 400 mOsM containing from about 0.1% to about 6.5% by weight, based on the total weight of the suspension, of a carboxyl-containing polymer prepared by polymerizing one or more carboxyl-containing monoethenically unsaturated monomers and less than about 5% by weight of a crosslinking agent, such-weight percentages of monomers being based on the total weight of monomers polymerized. Desirably the polymer is prepared by suspension or emulsion polymerizing the monomer with the crosslinking agent to a particle size of not more than about 50
μm, preferably not more than about 30 μm, in equivalent spherical diameter. The suspension has an initial viscosity of from about 1,000 to about 30,000 centipoises (cp) and is administrable to the eye in drop form at that initial viscosity. The polymer has average particle size of not more than about 50 μm, preferably not more than about 30 μm, in equivalent spherical diameter. In general, such polymers will range in molecular weight estimated to be about 250,000 to about 4,000,000, and preferably about 500,000 to about 2,000,000.

Aqueous suspensions containing polymer particles prepared by suspension or emulsion polymerization whose average dry particle size is appreciably larger than about 50 μm in equivalent spherical diameter are less comfortable when administered to the eye than suspensions otherwise identical in composition containing polymer particles whose equivalent spherical diameters are, on the average, below about 50 μm. Moreover, above the average 50 μm size, the advantage of substantially increased viscosity after administration is not realized.

The lightly crosslinked suspension is administrable in drop form, upon contact of the lower pH suspension with the higher pH tear fluid of the eye, the suspension is rapidly gellable to a substantially greater viscosity than the viscosity of the suspension as originally administered in drop form. Accordingly, the resulting more viscous gel can remain in the eye for a prolonged period of time so as to release

The polymer of the preferred intra-ocular drug delivery system is preferably prepared from at least about 50% by weight, more preferably at least about 90% by weight, of one or more carboxyl-containing monoethylenically unsaturated monomers. Acrylic acid is the preferred carboxyl-containing, monoethylenically unsaturated monomer, but other unsaturated, polymerizable carboxyl-containing monomers, such as methacrylic acid, ethacrylic acid, b-methylacrylic acid (crotonic acid), cis-a-methylcrotonic acid (angelic acid), trans-a-methylcrotonic acid (tiglic acid), a-butylcrotonic acid, a-phenylacrylic acid, a-benzylacrylic acid, a-cyclohexylacrylic acid, b-phenylacrylic acid
(cinnamic acid), coumaric acid (o-hydroxycinnamic acid), p-hydroxy coumaric acid (umbellic acid), and the like can be used in addition to or instead of acrylic acid.

Such polymers are crosslinked by using a small percentage, i.e., less than about 5%, such as from about 0.5% or from about 0.1% to about 5%, and preferably from about 0.2% to about 1%, based on the total weight of monomers present, of a polyfunctional crosslinking agent. The crosslinking agents of such compositions include non-polyalkenyl polyether difunctional crosslinking monomers such as divinyl glycol; 2,3-dihydroxyhexa-1,5-diene; 2,5-dimethyl-1,5-hexadiene; divinylbenzene; N,N-diallylacrylamide; N,N-diallylmethacrylamide and the like. A preferred crosslinking agent is divinyl glycol. Also included are polyalkenyl polyether crosslinking agents containing two or more alkenyl ether groupings per molecule, preferably alkenyl ether groupings containing terminal H₂C=C< groups, prepared by etherifying a polyhydric alcohol containing at least four carbon atoms and at least three hydroxyl groups with an alkenyl halide such as allyl bromide or the like, e.g., polyallyl sucrose, polyallyl pentaerythritol, or the like; see, e.g., Brown, U.S. Patent No. 2,798,053. Diolefinic non-hydrophilic macromeric crosslinking agents having molecular weights of from about 400 to about 8,000, such as insoluble di- and polyacrylates and methacrylates of diols and polyols, diisocyanate-hydroxyalkyl acrylate or methacrylate reaction products, and reaction products of isocyanate terminated prepolymers derived from polyester diols, polyether diols or polysiloxane diols with hydroxyalkyl methacrylates, and the like, can also be used as the crosslinking agents; see, e.g., Mueller et al. U.S. Patents Nos. 4,192,827 and 4,136,250.

In a preferred method of preparing sustained release topical ophthalmic delivery systems, the foregoing suspensions are prepared and packaged at the desired viscosity of from 1,000 to about 30,000 centipoises, for administration to the eye in drop form. In a preferred delivery method, the foregoing suspensions, containing the medicament, are administered to the eye at the
initial viscosity in drop form to cause the administered suspension, upon contact with the higher pH tear fluid of the eye, to rapidly gel in situ to a substantially greater viscosity than the viscosity of the suspension as originally administered in drop form. The more viscous gel remains in the eye for a prolonged period of time so as to release the medicament, entrapped in the more viscous gel formed in the eye, in sustained fashion.

It may be desirable to replace up to about 40% by weight of the carboxyl-containing monoethylenically unsaturated monomers by one or more non-carboxyl-containing monoethylenically unsaturated monomers containing only physiologically and ophthalmologically innocuous substituents.

The desired osmotic pressure is preferably achieved by using a physiologically and ophthalmologically acceptable salt in an amount of from about 0.01% to about 1% by weight, based on the total weight of the suspensions. A preferred salt is sodium chloride.

Generally, the dosage needed to provide an effective amount of the composition will vary depending upon such factors as the recipient's age, condition, sex, and extent of disease, if any, and other variables, and can be adjusted and determined by one of ordinary skill in the art. Effective amounts of the compositions of the invention can vary from 0.01-1,000 mg/ml per dose or application, although lesser or greater amounts can be used. For ophthalmic suspensions, the effective amounts will preferably be from about 0.005% to about 10% by weight, and most preferably from about 0.01% to about 5% by weight, based on the total weight of the suspension.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.
EXAMPLE 1
CLONING OF TIGR* cDNA

In order to clone the major DEX-inducible cDNA of HTM cells, a subtraction screening procedure is employed. In such subtractive screening methods, cDNA molecules are created from a complete population of cells and permitted to hybridize with a cDNA library constructed from RNA of different subpopulations of cells in order to identify clones that exhibit differential expression, and that thus reflect mRNA molecules that are induced or repressed in each population (Lamar, E.E. et al., Cell 37:171-177 (1984); Rubenstein, J.L.R. et al., Nucleic Acids Res. 18:4833-4842 (1990); Hedrik, S.M. et al., Science 308:149-153 (1984); Duguid, J.R. et al., Proc. Natl. Acad. Sci. (U.S.A.) 85:5738-5742 (1988); Weiland, I. et al., Proc. Natl. Acad. Sci. (U.S.A.) 87:2720-2724 (1990)).

cDNA is therefore prepared from mRNA of human trabecular meshwork (HTM) cells previously incubated in 100 nM dexamethasone for 10 days, as well as from mRNA of untreated HTM cells. The cDNA library is constructed in lambda ZapII using strain XL-1 (Stratagene, San Diego). Approximately 30-50 µg of mRNA are obtained from 5 x 10^7 dexamethasone treated cells as described by Nguyen, T.D. et al. (In: "Schriftenreihe de Adademie der Wissenschaften und der Literatur, Mainz," 331-343 (1993), herein incorporated by reference).

Two independent screenings of 20,000 phages each are conducted using a differential screening approach. Phages are separately probed with labeled cDNA made from mRNA of untreated cells and of dexamethasone-treated cells for 1 day and 10 days. Clones that exhibit an inducible response (i.e. increased labeling when probed with the dexamethasone treated cDNA relative to control cDNA) are desired candidates for further analysis.

Several cDNA clones are obtained that correspond to mRNA produced in higher amounts in dexamethasone-treated cells. One cDNA clone, corresponding to mRNA that is present in the

The level of these changes was quantitated by both dot-blot and PCR methodology. In the dot-blot analysis, the DNA of the clones are serially diluted and applied to membranes which are then hybridized to labeled total cDNA of control, 1 day or 10 day dexamethasone-treated HTM cells. The dot-blot analysis reveals that the TIGR* mRNA is the major induced species, comprising 3-4% of the total cellular mRNA at day 10. An insignificant level of TIGR* mRNA is detected in the control. The time course of dexamethasone treatment for days 2, 4, 7 and 10 revealed that TIGR* is progressively induced (T.D. Nguyen et al., Invest. Ophthalmol. Vis. Sci. 32:789 (1991)). Cycloheximide studies show that the induction required protein synthesis. Southern analysis and in-situ hybridization show multiple copy numbers of the gene. For PCR amplification analysis, the serial dilution PCR method (Chelly, J.D. et al., Eur. J. Biochem. 187:691-698 (1990); Murphy, L.D. et al., Biochem. 29:10350-10356 (1990); Singer-Sam, J.O. et al., Nucl. Acids Res. 18:1255-1259 (1990)) is modified to maintain the exponential range of the amplification throughout the quantitation procedure to measure and confirm the major and progressive induction of TIGR* mRNA over a 10 day dexamethasone induction period. Quantitative PCR analysis reveals an induction level of about 20 fold compared to the level found in cells treated with dexamethasone for only 1 day.

Northern analysis shows clone TIGR* to be approximately 2.5 kb, and to encode a protein of unique sequence. The induction of the mRNA required protein synthesis and insulin-like growth factor reduced the induction effect by 50%. The TIGR* mRNA induction is not observed in dexamethasone treated fibroblasts, keratinocytes, or ciliary epithelial cells. The pattern of induction in HTM cells is distinguishable from other steroid induced proteins such as metallothionine, alpha1-acid glycoprotein, and TAT which are maximally induced by one day dexamethasone
treatment. In addition to dexamethasone, the TIGR* mRNA was induced in HTM cells exposed to hydrogen peroxide, TPA, or glucose for 3-24 hours. Dexamethasone treatment produced substantial loss in the mRNAs for glucocorticoid receptors and heat shock proteins (e.g., hsp 90 mRNA levels fell approximately 20 fold after 10 days of dexamethasone treatment).

**EXAMPLE 2**

**EXPRESSION OF TIGR**

An ability to express substantial amounts of TIGR* would facilitate the use of this protein for functional assays, and the development of anti-TIGR* antibodies. To achieve such enhanced expression, the PVL1393 baculovirus transfer vector of Invitrogen Corp. is employed. A 2 Kb EcoR1 fragment of the TIGR* cDNA is inserted into the EcoR1 cloning site of PVL1393. PCR and sequencing analysis show that the insert is ligated in the correct orientation into the vector's polyhedrin promoter. Cotransfection of this construct and wild type baculovirus DNA into Sf9 insect cells produce high titers of recombinant protein. The Sf9 insect cell line can be obtained from the American Type Culture Collection, Rockville, MD, US, as deposit accession number ATCC CRL 1711. Methods of using such vectors and cells are described by Summers M.D. *et al.* (In: A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures, Texas Agricultural Experiment Station Bulletin No. 1555 (1987)), and Summers, M.D. (U.S. Patent 5,278,050).

PCR verification of these recombinants show positive signal for the expressed gene. SDS gel analysis of the SF9 transfected cellular proteins show that the new product had a molecular weight of about 55 KDa and amounted to 90-95% of the total protein produced. These values correlate well with the size of the HTM cell protein induced by dexamethasone (DEX) (Polansky, J.R. *et al.*, *Prog Clin Biol Res* 312:113-138, 1989) and the calculated MW from the cDNA sequence isolated. G-150 (Pharmacia) column purification and Edman degradation sequencing of the protein confirm the open reading frame of the TIGR* cDNA.
Studies of the recombinant protein thus suggest (1) that the 55 kD protein exists both in cells and in the medium, (2) that it undergoes oligomerization, (3) phosphorylation, (4) glycosylation, (5) that it is susceptible to metalloprotease, (6) that it exhibits high affinity binding to extracellular matrix and human trabecular meshwork cells, (7) that it exhibits progressive inductions with time in both cell and organ cultures, and (8) that it exhibits high expression in the HTM of glaucomatous patients as compared to normal patients. Significantly, the induction correlated with topical glucocorticoid effects on intra-ocular pressure in patients, and differ from other known glucocorticoid induction patterns which exhibit close to maximal induction at only one day of dexamethasone treatment.

**EXAMPLE 3**

**STRUCTURAL CHARACTERISTICS OF TIGR**

Clone TIGR* is sequenced, and found to comprise a 2.0 kb cDNA molecule (SEQ ID NO:2). The full-length transcript was nearly 2.5 kb as determined by Northern analysis. The cDNA includes two ATG start sites which produces two 55 kD proteins in both HTM and Sf9 cells. The larger protein is 504 amino acids, and is defined by the TIGR* open reading frame (SEQ ID NO:1). The larger protein is due to the unprocessed form of TIGR*; the smaller protein reflects the proteolytic cleavage of the TIGR* signal peptide (the putative cleavage site is located between the Arginine residue at position 32 and the Alanine residue at position 33). The amino terminal sequence of these proteins is verified by amino acid sequencing analysis. The post-translational modification of these proteins also produce a highly glycosylated TIGR* form of about 66 kD.

Structural analysis of the clone demonstrate it encodes a novel extracellular protein of about 55 kD with an N-glycosylation site at SEQ ID NO:1 residues 57-60 and O-glycosylation sites at SEQ ID NO:1 residues 231-232; 232-223; 277-279; 312-313; 404-408; 460-464; 464-466, heparin sulfate binding (SEQ ID NO:1 residues 156-160) and initiation domains (SEQ ID NO:1 residues
233-234, 238-239 and 331-332), 7 consensus leucine zipper units, forming two stretches, one located at SEQ ID NO:1, residues 85-92, and 92-99, and five located at SEQ ID NO:1, residues 131-138, 138-145, 145-152, 152-159, and 159-166), and a potential GIP (guanidyl inositol phosphate) linkage. The 55 kD recombinant protein forms dimer or heteromer in the HTM medium as demonstrated by crosslinking studies, and it could self-aggregate. The recombinant protein had a specific ability to bind trabecular meshwork cells (4.3 x 10^{-9} M and 2.3 x 10^{-8} M) as shown by Skatchard analysis. In contrast, the protein show non-saturable and low affinity binding ability for fibroblasts. The recombinant protein was shown to be a substrate for the 72 kD metalloprotease.

The anti-TIGR* antibodies recognize a 66 kD protein in dexamethasome -treated HTM medium. This protein is shown to be a highly glycosylated form of the 55 kD TIGR* protein. This conclusion is supported by the observation that expression conducted in the presence of tunicamycin shifted production from 66 kD to 55 kD. The 66 kD glycosylated form of TIGR* appears to be a hyaluronate binding protein, since it was found to be capable of binding to hyaluronic acid beads. Such binding proteins are defined by their ability to bind to such beads and to be eluted from the beads in the presence of 4 M guanidine after 0.15 M and 1.5 M NaCl washes.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth and as follows in the scope of the appended claims.
SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA

(ii) TITLE OF THE INVENTION: DIAGNOSIS AND PROGNOSIS OF GLAUCOMA

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Howrey & Simon
(B) STREET: 1299 Pennsylvania Avenue, N.W.
(C) CITY: Washington
(D) STATE: DC
(E) COUNTRY: USA
(F) ZIP: 20004-2402

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Sira, Serge
(B) REGISTRATION NUMBER: 39,445
(C) REFERENCE/DOCKET NUMBER:

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 202 383-6857
(B) TELEFAX: 202 383-6610
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 504 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(x) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Arg Phe Phe Cys Ala Arg Cys Cys Ser Phe Gly Pro Glu Met Pro
1  5  10  15
(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2000 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(3) SEQUENCE DESCRIPTION:
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WHAT IS CLAIMED IS:

1. A substantially purified Trabecular Meshwork Induced Glucocorticoid Response* (TIGR*) protein having the sequence of SEQ ID NO:1 residues 1-504 or of SEQ ID NO:1 residues 15-504.

2. The substantially purified TIGR* protein of claim 1, wherein said protein has the sequence of SEQ ID NO:1 residues 1-504.

3. The substantially purified TIGR* protein of claim 1, wherein said protein has the sequence of SEQ ID NO:1 residues 15-504.

4. A substantially purified nucleic acid molecule that encodes a Trabecular Meshwork Induced Glucocorticoid Response* (TIGR*) protein.

5. The nucleic acid molecule of claim 4 that comprises the sequence of SEQ ID NO:3.

6. The nucleic acid molecule of claim 4 that comprises the sequence of SEQ ID NO:2.

7. A substantially purified Trabecular Meshwork Induced Glucocorticoid Response* (TIGR*) nucleic acid molecule which
comprises an oligonucleotide fragment between about 15 to about 250 nucleotides of the sequence of SEQ ID NO:2 or SEQ ID NO:3.

8. A substantially purified Trabecular Meshwork Induced Glucocorticoid Response* (TIGR*) nucleic acid molecule that specifically hybridizes with a polynucleotide having the sequence of SEQ ID NO:2 or SEQ ID NO:3 or its complement.

9. A substantially purified fragment of a Trabecular Meshwork Induced Glucocorticoid Response* (TIGR*) protein whose amino acid sequences comprise a subset of SEQ ID NO:1 residues 1-504 and which binds TIGR* protein.

10. A method for diagnosing glaucoma in a patient which comprises determining whether the amount of a Trabecular Meshwork Induced Glucocorticoid Response* (TIGR*) protein present in the trabecular meshwork of an eye of said patient exceeds the amount of that TIGR* protein present in the trabecular meshwork of an eye of an individual who does not have glaucoma and is not predisposed to have glaucoma, wherein the detection of an altered amount of said TIGR* protein is indicative of glaucoma.

11. A method for diagnosing glaucoma in a patient under evaluation for suspected glaucoma which comprises assaying the concentration of a molecule, whose concentration is dependent upon the expression of a Trabecular Meshwork Induced
Glucocorticoid Response* (TIGR*) gene, said molecule being present in a sample of cells or bodily fluid of said patient, in comparison to the concentration of that molecule present in a sample of cells or bodily fluid of an individual who does not have glaucoma and is not predisposed to have glaucoma, wherein a concentration of said molecule differs from that found in said individual who does not have glaucoma and is not predisposed to have glaucoma is diagnostic of glaucoma in said patient.

12. A method for diagnosing glaucoma in a patient which comprises the steps:

(A) incubating under conditions permitting nucleic acid hybridization: a marker nucleic acid molecule, said marker nucleic acid molecule capable of specifically hybridizing with a polynucleotide having the sequence of SEQ ID NO:2 or SEQ ID NO:3 or its complement, and a complementary nucleic acid molecule obtained from a cell or a bodily fluid of said patient, wherein nucleic acid hybridization between said marker nucleic acid molecule, and said complementary nucleic acid molecule obtained from said patient permits the detection of a polymorphism whose presence is predictive of a mutation affecting TIGR* level in said patient;

(B) permitting hybridization between said marker nucleic acid molecule and said complementary nucleic acid molecule obtained from said patient; and

(C) detecting the presence of said polymorphism, wherein the detection of said polymorphism is diagnostic of glaucoma.
13. A method for diagnosing steroid sensitivity in a patient which comprises the steps:

(A) incubating under conditions permitting nucleic acid hybridization: a marker nucleic acid molecule, said marker nucleic acid molecule capable of specifically hybridizing with a polynucleotide having the sequence of SEQ ID NO:2 or SEQ ID NO:3 or its complement, and a complementary nucleic acid molecule obtained from a cell or a bodily fluid of said patient, wherein nucleic acid hybridization between said marker nucleic acid molecule, and said complementary nucleic acid molecule obtained from said patient permits the detection of a polymorphism whose presence is predictive of a mutation affecting TIGR* level in said patient;

(B) permitting hybridization between said marker nucleic acid molecule and said complementary nucleic acid molecule obtained from said patient; and

(C) detecting the presence of said polymorphism, wherein the detection of said polymorphism is diagnostic of steroid sensitivity.
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TGC  AGC  TTT  GGG  CCT  GAG  ATG  CCA  GCT  GTC  CAG  CTG  CTG  CTC  CTG  GCC  TGC  CTG  CTG  TGG  
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Ser  Val  Ile  His  Asn  Leu  Gin  Arg  Asp  Ser  Ser  Thr  Gln  Arg  Leu  Asp  Leu  Glu  Ala  Thr

AAA  GCT  CGA  CTC  AGC  TCC  CTG  GAG  AGC  CTC  CTC  CAC  CAA  TTG  ACC  TTG  GAC  CAG  GCT  GCC  
Lys  Ala  Arg  Leu  Ser  Ser  Leu  Glu  Ser  Leu  Leu  His  Gln  Leu  Thr  Leu  Asp  Gln  Ala  Ala

AGG  CCC  [AGG  GAG  ACC  CAG  GAG  GGG  CTG  CAG  AGG  GAG  CTG  GCC  ACC  CTG  AGG  CGG  GAG  CGG]  
Arg  Pro  [Gln  Glu  Thr  Gln  Glu  Gly  Leu  Gin  Arg  Glu  Leu  Gly  Thr  Leu  Arg]  Arg  Glu  Arg

GAC  CAG  CTG  GAA  ACC  CAA  ACC  AGA  GAG  TTG  GAG  ACT  GCC  TAC  AGC  AAC  CTC  CTC  CGA  GAC  
Asp  Gln  Leu  Glu  Thr  Gln  Thr  Arg  Glu  Leu  Glu  Thr  Ala  Tyr  Ser  Asn  Leu  Leu  Arg  Asp

AAG  TCA  GTT  CTG  GAG  GAA  GAG  AAG  CGA  CTA  AGG  CAA  GAA  AAT  GAG  AAT  CTG  GCC  AGG  
Lys  Ser  Val  Leu  Glu  Glu  Glu  Lys  Lys  Arg  Leu  Arg  Gln  Glu  Asn  Glu  Asn  Leu  Ala  Arg

FIG. 1A
FIG. 1D
## INTERNATIONAL SEARCH REPORT

### A. CLASSIFICATION OF SUBJECT MATTER

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<th>C07K14/47</th>
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According to International Patent Classification (IPC) or to both national classification and IPC

### B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database consulted during the international search (name of database and, where practical, search terms used)

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>X</td>
<td>WO 96 14411 A (UNIV CALIFORNIA) 17 May 1996 cited in the application see the whole document, especially examples</td>
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<td>STONE E M ET AL: &quot;Identification of a gene that causes primary open angle glaucoma 'see comments!'&quot; SCIENCE, vol. 275, 31 January 1997, pages 668-670, XP002049424 see the whole document, especially from page 669, left column, line 24</td>
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Further documents are listed in the continuation of box C.

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### Special categories of cited documents:

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### Date of the actual completion of the international search

8 December 1997

### Date of mailing of the international search report

19/12/1997

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-3040, Tx: 31 651 epo nl
Fax (+31-70) 340-3016

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Mandl, B
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<td>EMBL database entry HSU85257; accession number U85257; 2 March 1997; Nguyen T.D. and POLANSKY J. R.: 'Human trabecular meshwork inducible glucocorticoid response protein mRNA, complete cds.' XP002049427 see abstract</td>
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<td>POLANSKY J R ET AL.: &quot;In vitro correlates of glucocorticoid effects on intraocular pressure.&quot; 1991, SPRINGER VERLAG, BERLIN XP000564992 cited in the application see the whole document</td>
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<td>ORTEGO J ET AL: &quot;Cloning and characterization of subtracted cDNAs from a human ciliary body library encoding TIGR, a protein involved in juvenile open angle glaucoma with homology to myosin and olfactomedin.&quot; FEBS LETTERS, vol. 413, no. 2, 18 August 1997, pages 349-353, XP002049426 see the whole document</td>
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## INTERNATIONAL SEARCH REPORT

### Information on patent family members

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