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(54) Title: ANTIBODIES TO HTRA1 AND METHODS OF USING THE SAME

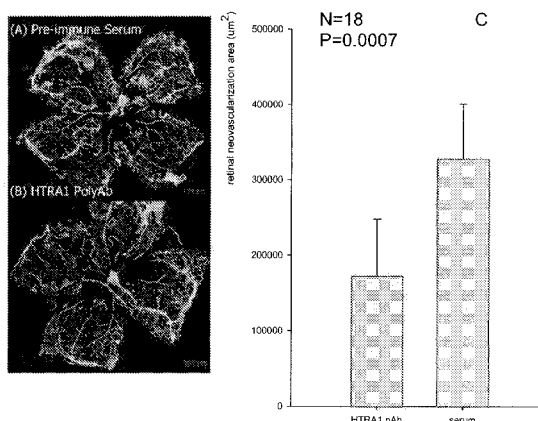


FIG. 7

(57) Abstract: Antibodies to HtrA1 and methods of using such antibodies are described herein. The antibodies described herein are raised against HtrA1 and variants of HtrA1 as defined herein. The antibodies disclosed herein are useful in treating disease or injury of the eye. For example, in one embodiment, HtrA1 antibodies may be used to treat age-related macular degeneration (AMD) in both its wet and dry forms, diabetic retinopathy (DR), retinopathy of prematurity (ROP) or macular edema. In another embodiment, HtrA1 antibodies may be used to treat choroidal or retinal neovascularization associated with or resulting from disease or injury. In another embodiment, the methods of the present invention include use of HtrA1 antibodies to treat ischemia-induced retinal neovascularization resulting from vascular damage, occlusion or leak resulting from disease or injury. The methods described herein include administering a therapeutically-effective amount of one or more HtrA1 antibodies to a subject. Pharmaceutical compositions of antibodies to HtrA1 are also described herein.



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ANTIBODIES TO HTRA1 AND METHODS OF USING THE SAME**SUMMARY**

[0001] Antibodies to HtrA1 and methods of using such antibodies are described herein. The antibodies described herein are raised against HtrA1 and variants of HtrA1 as defined herein. The antibodies may be, for example, monoclonal antibodies, polyclonal antibodies, human antibodies, humanized antibodies, or affinity matured antibodies. In addition, where desired the antibodies described herein may be modified, such as by amino acid substitution, glycosylation modification, or by specific conjugation or formulation techniques as desired to achieve a targeted biological activity or functional characteristic. In particular examples, the antibodies described herein exhibit a minimum threshold reactivity with HtrA1 molecule and inhibit HtrA1 induced neovascularization.

[0002] The antibodies disclosed herein are useful in treating disease or injury of the eye. In one embodiment, the antibodies disclosed herein may be used to treat retinal disease or injury. For example, in one embodiment, HtrA1 antibodies may be used to treat age-related macular degeneration (AMD) in both its wet and dry forms, diabetic retinopathy (DR), retinopathy of prematurity (ROP) or macular edema. In another embodiment, HtrA1 antibodies may be used to treat choroidal or retinal neovascularization associated with or resulting from disease or injury. In another embodiment, the methods of the present invention include use of HtrA1 antibodies to treat ischemia-induced retinal neovascularization resulting from vascular damage, occlusion or leak. In yet another embodiment, the methods of the present invention include use of HtrA1 antibodies to treat a pathological condition associated with vascular leak or edema in the eye. The methods described herein include administering a therapeutically-effective amount of one or more HtrA1 antibodies to a subject.

[0003] Pharmaceutical compositions of antibodies to HtrA1 are also described herein. The pharmaceutical compositions described herein include one or more HtrA1 antibodies in amounts sufficient to allow delivery of therapeutically-effective doses of HtrA1 antibodies to a subject. The pharmaceutical compositions described herein may include pharmaceutically acceptable excipients and may be formulated for delivery via any suitable route and means of administration. In one embodiment,

a pharmaceutical composition for injection or infusion delivery of HtrA1 antibodies is provided.

[0004] Additional aspects and advantages will be apparent from the following detailed description of preferred embodiments, which proceeds with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0005] FIG. 1 shows a fragment of human HtrA1 (or "hHtrA1") (SEQ. ID. NO: 1). The fragment is designated as "HU-142aa" and includes amino acid residues 142-480 of a full-length human HtrA1 (SEQ. ID. NO.: 2), which is designated as "HU-FULL."

[0006] FIG. 2 illustrates the results of a zymography analysis of purified HtrA1 proteins (left) and of an immunoblot analysis (right) of purified HtrA1 under non-reducing (-DTT) and reducing (+DTT) conditions.

[0007] FIG. 3 illustrates the results of an analysis of HtrA1 mRNA expression in AMD. Real-time RT-PCR semiquantitative analysis of HtrA1 RNA levels in blood lymphocytes from three AMD patients with the AA genotype and three normal controls with the GG genotype was conducted. The statistical significance of the differences in expression level was examined using an independent samples t test (SPSS version 13.0): AA:GG (P = 0.02). Each RT-PCR reaction was run twice, and the error bars indicate the 95.0% confidence interval of the mean.

[0008] FIG. 4 shows the results of a western blot analysis of HtrA1 expression in human RPE. HtrA1 protein expression level in AA genotype is 1.7 fold compared to that of GG genotype normalized to β -actin. Significance was examined using an independent samples t-test (SPSS version 13.0): AA:GG (*p=0.049). The error bars indicate the standard deviation of the mean. Mean values are relative to GG expression. GG, n=6; AA, n=4.

[0009] FIG. 5(A) shows the results of a western blot analysis of HtrA1 polyclonal antibody. HtrA1 polyclonal antibody as produced herein recognizes recombinant HtrA1 (a) and HtrA1 in human RPE (c and d). Specificity was confirmed by lack of signal following preabsorption with recombinant HtrA1 protein (e and f), (b) was a negative control consisting of a recombinant protein preparation from bacteria transfected with vector only.

[0010] FIG. 5(B) provides an image showing HtrA1 polyclonal antibody stained on mammalian cells expressing recombinant human HtrA1.

[0011] FIG. 5(C) provides an image showing isolectin staining of retinal blood vessels.

[0012] FIG. 5(D) provides an image showing HtrA1 polyclonal antibody staining of retinal blood vessels.

[0013] FIG. 5(E) is a combination of the images of FIG. 5(C) and FIG. 5(D) with TO-PRO3 staining to show nuclei (blue), which provides an image demonstrating that HtrA1 polyclonal antibody stains pathologic endothelial cells located beyond internal limiting membrane (yellow/bottom arrows) but not intraretinal endothelial cells (white/top and middle arrows). As used in FIG. 5 (C-E), "ONL" refers to the outer nuclear layer, "INL" refers to the inner nuclear layer, "GCL" refers to the ganglion cell layer, and "ILM" refers to the internal limiting membrane.

[0014] FIG. 6(A) – FIG. 6(C) illustrate the results of immunohistochemistry studies of HtrA1 in AMD human donor eyes.

[0015] FIG. 6(A) provides an image showing HtrA1 nonuniformly expressed on drusen, the hallmark of age related macular degeneration, and Bruch's membrane.

[0016] FIG. 6(B) provides an images showing that HtrA1 is not only expressed on drusen, but also on retinal blood vessels, the retinal pigment epithelium (RPE) and internal limiting membrane.

[0017] FIG. 6(C) shows that, in a wet age related macular degeneration donor eye, HTRA1 is expressed on choroidal vessels (white/top arrows) and in choroidal neovascularization (yellow/bottom arrows). Green channel is the staining of HtrA1 polyclonal antibody. Nuclei were counter-stained with propidium iodine (red). The scale bar provided in the figure represents a 20 μ m length.

[0018] FIG. 7 illustrates the results of testing conducted in an OIR mouse model, wherein a polyclonal HtrA1 antibody and a negative control pre-immune serum were administered to the mice.

[0019] FIG. 8 illustrates the results of testing conducted in an OIR mouse model, wherein 8F12 HtrA1 monoclonal antibody and 9F7 HtrA1 monoclonal antibody were administered to the mice.

[0020] FIG. 9 illustrates the results of testing conducted in a CNV mouse model, wherein a polyclonal HtrA1 antibody and a negative control a pre-immune serum were administered to the mice.

[0021] FIG. 10 illustrates the results of testing conducted in CNV mouse model, wherein 8F12 HtrA1 monoclonal antibody and 9F7 HtrA1 monoclonal antibody were administered to the mice.

[0022] FIG. 11 illustrates the association or odds ratios with respect to AMD for HtrA1 rs11200638 and CFH rs1061170. Odds ratios in parentheses were calculated to compare each genotypic combination to the baseline of homozygosity for the common allele at both loci (TT/GG).

DETAILED DESCRIPTION

Definitions

[0023] As used herein, "HtrA1" refers to the HtrA1 fragment (SEQ. ID. NO.: 1) or the full-length HtrA1 (SEQ. ID. NO.: 2) having the deduced amino acid sequences shown in FIG. 1

[0024] "HtrA1 variant" refers to an HtrA1 variant having at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% amino acid sequence identity with the HtrA1 fragment (SEQ. ID. NO.: 1) or the full-length HtrA1 (SEQ. ID. NO.: 2) having the deduced amino acid sequences shown in FIG. 1. Such HtrA1 variants include, for instance, HtrA1 polypeptides wherein one or more amino acid residues are substituted and HtrA1 polypeptides wherein one or more amino acid residues are added, or deleted (i.e., fragments), at the N- or C-terminus of the sequences shown in FIG. 1.

[0025] "Percent (%) amino acid sequence identity" with respect to the HtrA1 sequences (or HtrA1 antibody sequences) identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the HtrA1 sequence (or HtrA1 antibody sequence), after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as, for example, ALIGNTM or Megalign (DNASTAR). All sequence comparison parameters are set by the ALIGN-2 program and do not vary. Those of ordinary skill in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

[0026] "Isolated," when used to describe biomolecules disclosed herein, means a polypeptide, nucleic acid, antibody that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide or antibody, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous materials. Methods for isolation and purification of biomolecules described herein are known and available in the art, and one of ordinary skill in the art can determine suitable isolation and purification methods in light of the material to be isolated or purified. Though isolated biomolecules will typically be prepared using at least one purification step, as it is used herein, "isolated" additionally refers to, for example, polypeptide, antibody, or nucleic acid materials *in-situ* within recombinant cells. In particular, where an HtrA1 polypeptide or HtrA1 antibody as described herein is recombinantly expressed, at least one component of the HtrA1 polypeptide or HtrA1 antibody natural environment will not be present.

[0027] The terms "amino acid" and "amino acids" refer to all naturally occurring L-alpha-amino acids. This definition includes norleucine, ornithine, and homocysteine. Amino acids are identified by either the standard single-letter or standard three-letter designations.

[0028] The terms "antagonist" and "antagonistic" when used herein refer to or describe a molecule which is capable of, directly or indirectly, counteracting, reducing or inhibiting HtrA1 biological activity

[0029] The term "antibody" is used in the broadest sense and specifically covers single anti-HtrA1 monoclonal antibodies (including agonist, antagonist, and neutralizing or blocking antibodies) and anti-HtrA1 antibody compositions with polypeptopic specificity (*e.g.*, compositions including two or more different antibodies exhibiting specificity to different epitopic regions). "Antibody" as used herein encompasses intact or full-length immunoglobulin or antibody molecules, polyclonal antibodies, multispecific antibodies (*i.e.*, bispecific antibodies formed from at least two intact antibodies) as well as immunoglobulin fragments (such as Fab, F(ab')₂, or Fv), provided that such fragments exhibit one or more desired biologic activities or properties, such as, for example, the binding affinity, titer, anti-neovascularization, agonistic or antagonistic properties described herein.

[0030] Antibodies are typically proteins or polypeptides which exhibit binding specificity to a specific antigen. Native antibodies are usually heterotetrameric glycoproteins, composed of two identical light (L) chains and two identical heavy (H) chains. Typically, each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies between the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end. The constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light and heavy chain variable domains (See, Chothia et al., J. Mol. Biol., 186:651-663 (1985); Novotny and Haber, Proc. Natl. Acad. Sci. USA, 82:4592-4596 (1985)). The light chains of antibodies from any vertebrate species can be assigned to one of two clearly distinct types, called kappa and lambda, based on the amino acid sequences of their constant domains. Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG-1, IgG-2, IgG-3, and IgG-4; IgA-1 and IgA-2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called alpha, delta, epsilon, gamma, and mu, respectively.

[0031] "Antibody fragments" comprise a portion of an intact antibody, generally the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', F(ab')₂, and Fv fragments, diabodies, single chain antibody molecules, and multispecific antibodies formed from antibody fragments.

[0032] The term "variable" is used herein to describe certain portions of the variable domains which differ in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not usually evenly distributed through the variable domains of antibodies. It is typically concentrated in three segments called complementarity determining regions (CDRs) or hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of the variable

domains are called the framework (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a β -sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β -sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen binding site of antibodies (See, Kabat, E. A. et al., Sequences of Proteins of Immunological Interest, National Institutes of Health, Bethesda, Md. (1987)).

[0033] The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.

[0034] The monoclonal antibodies herein include chimeric, hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of an anti-HtrA1 antibody with a constant domain (e.g., "humanized" antibodies), or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (e.g., Fab, F(ab')₂, and Fv), provided, again, that such fragments exhibit one or more desired biological activity or property as set out herein (See, e.g. U.S. Pat. No. 4,816,567 and Mage et al., in Monoclonal Antibody Production Techniques and Applications, pp. 79-97 (Marcel Dekker, Inc.: New York, 1987)).

[0035] The modifier "monoclonal," therefore, indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, monoclonal antibodies according to the present description can be produced by the hybridoma method described by Kohler and Milstein, Nature, 256:495 (1975), by the methods set out in Harlow, E and Lane, D, *Antibodies: A laboratory Manual*. 1988 Cold Spring Harbor, NY: Cold Spring Harbor

Laboratory Press, or by recombinant DNA methods, such as the methods described in U.S. Pat. No. 4,816,567. The "monoclonal antibodies" may also be isolated from phage libraries generated using the techniques described in McCafferty et al., Nature, 348:552-554 (1990), for example.

[0036] "Humanized" forms of non-human (e.g., murine) antibodies are specific chimeric immunoglobulins, immunoglobulin chains, or fragments thereof (such as Fv, Fab, Fab', F(ab')₂ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, the humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin.

[0037] A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies known in the art or as disclosed herein. This definition of a human antibody includes antibodies comprising at least one human heavy chain polypeptide or at least one human light chain polypeptide, for example an antibody comprising murine light chain and human heavy chain polypeptides. Human antibodies can be produced using various techniques known in the art. In one embodiment, the human antibody is selected from a phage library, where that phage library expresses human antibodies (Vaughan et al. Nature Biotechnology, 14:309-314 (1996); Sheets et al. PNAS, (USA) 95:6157-6162 (1998)); Hoogenboom and Winter, J. Mol. Biol., 227:381

(1991); Marks et al., J. Mol. Biol., 222:581 (1991)). Human antibodies can also be made by introducing human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks et al., Bio/Technology, 10: 779-783 (1992); Lonberg et al., Nature, 368: 856-859 (1994); Morrison, Nature, 368:812-13 (1994); Fishwild et al., Nature Biotechnology, 14: 845-51 (1996); Neuberger, Nature Biotechnology, 14: 826 (1996); Lonberg and Huszar, Intern. Rev. Immunol., 13:65-93 (1995). Alternatively, the human antibody may be prepared via immortalization of human B lymphocytes producing an antibody directed against a target antigen (such B lymphocytes may be recovered from an individual or may have been immunized in vitro). See, e.g., Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985); Boerner et al., J. Immunol., 147 (1):86-95 (1991); and U.S. Pat. No. 5,750,373.

[0038] An "affinity matured" antibody is one with one or more alterations in one or more CDRs thereof which result an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). Preferred affinity matured antibodies will have nanomolar or even picomolar affinities for the target antigen. Affinity matured antibodies are produced by procedures known in the art. Marks et al. Bio/Technology, 10:779-783 (1992) describes affinity maturation by VH and VL domain shuffling. Random mutagenesis of CDR and/or framework residues is described by: Barbas et al. Proc Nat. Acad. Sci, USA 91:3809-3813 (1994); Schier et al. Gene, 169:147-155 (1995); Yelton et al. J. Immunol., 155:1994-2004 (1995); Jackson et al., J. Immunol., 154(7):3310-9 (1995); and Hawkins et al, J. Mol. Biol., 226:889-896 (1992).

[0039] The term "immunospecific" as used in "immunospecific binding of antibodies" for example, refers to the antigen specific binding interaction that occurs between the antigen-combining site of an antibody and the specific antigen recognized by that antibody.

[0040] "Biologically active" and "desired biological activity" for the purposes herein mean having the ability to modulate HtrA1 activity or HtrA1 activation,

including, by way of example, the function or activation of one or more of the HtrA1 trypsin domain, PDZ domain, a serine protease (SP) domain, insulin-like growth factor binding domain (IGFBP), Kazal-type trypsin (KI) inhibitory domain, Mac25 domain, or the follistatin (FS) domain. In particular, embodiments, when used in conjunction with an antibody as described herein, "biologically active" and "desired biological activity" refer to an ability to directly or indirectly inhibit or block HtrA1 activity or activation, such as by, for example, inhibiting or blocking the activity or activation of one or more of the HtrA1 trypsin domain, PDZ domain, a serine protease (SP) domain, insulin-like growth factor binding domain (IGFBP), Kazal-type trypsin (KI) inhibitory domain, Mac25 domain, or the follistatin (FS) domain.

[0041] As used herein, the terms "treat," "treating," and "treatment" refer to a therapeutic benefit, whereby the detrimental effect(s) or progress of a particular disease, condition, event or injury is prevented, reduced, halted or slowed.

[0042] "Therapeutically-effective" refers to an amount of a pharmaceutically active substance, including an antibody, capable of treating a particular disease, condition, event or injury.

[0043] The term "subject" refers to an animal or human, preferably a mammal, subject in need of treatment for a given disease, condition, event or injury.

[0044] The terms "pathologic" or "pathologic conditions" refer to any deviation from a healthy, normal, or efficient condition which may be the result of a disease, condition, event or injury.

[0045] As used herein, the terms "ocular tissue(s)" and "tissue(s) of the eye" include, by way of example and not limitation, the retina, including the macula, the retinal pigment epithelium (RPE), the Bruch's membrane, the internal limiting membrane, and the choroid.

Ocular Disease

[0046] The pathologic growth of new blood vessels (neovascularization) in and around the tissues of the eye is associated with a variety of ocular diseases. In particular, hypoxia is known to be the primary stimulus for pathologic neovascularization of the choroid and Bruch's membrane that results in the often catastrophic vision loss associated with diabetic retinopathy, retinopathy of prematurity, and the wet form of AMD. Such ischemia-induced neovascularization is known to be induced or mediated by angiogenic factors such as, for example, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF-2), and

transforming growth factor- β (TGF- β). Ischemia-induced neovascularization need not necessarily be caused by disease. For example, injury or insult to the ocular tissue can lead to hypoxia of ocular tissues and thereby cause ischemia-induced neovascularization that results in loss of vision.

[0047] Where pathologic neovascularization of, for example, the choroid and Bruch's membrane occurs, vision loss and tissue damage is not only caused by the physical intrusion of the new blood vessels, but such vessels tend to be fragile, often leak blood into the surrounding tissue, and can result in macular edema. Macular edema occurs when fluid (e.g., blood), lipids and protein deposits collect on or under the macula, causing the macula to thicken, swell, and/or raise away from its normal place at the back of the eye, further distorting or destroying vision.

Diabetic Retinopathy

[0048] Diabetic retinopathy (DR) is result of vascular damage that can result from poor blood sugar control. Diabetes mellitus is often characterized by reduced blood circulation, and the small blood vessels present in tissues of the eye are especially vulnerable to damage that may result from poor circulation and the resulting over accumulation of sugars present in the blood. For example, diabetes can result in hyperglycemia-induced pericyte death and thickening of the basement membrane and lead to incompetence of the vascular walls of the vessels present in the tissues of the eye. Such damage can change the detrimentally alter the of the blood retinal barrier, which consists of cells that are joined tightly together and work prevent certain substances from entering the tissue of the retina. The vascular damage caused resulting from diabetes mellitus can also lead to localized ischemia of ocular tissues and cause blood vessels present in the tissues of the eye become more permeable. Such localized ischemia and increased vascular permeability both lead to pathologic neovascularization, which ultimately results in the severe vision loss associated with DR.

Retinopathy of Prematurity

[0049] Retinopathy of prematurity (ROP), also known as retrolental fibroplasia (RLF), is an eye disease that affects prematurely born babies. ROP can be mild and may resolve spontaneously. However, the condition may also progress and lead to severe visual impairment, even blindness. Maturation of ocular tissues normally proceeds in-utero, and at term, the tissues of the eye are fully vascularized. However, where an infant is born prematurely the tissues of eye are often not fully

vascularized. ROP is thought to occur when the development of the vasculature of the eye is inhibited or arrested and then proceeds abnormally and in a disorganized fashion, leading to fibrovascular proliferation. Fibrovascular proliferation is the abnormal growth new vessels (neovascularization) that leads to formation of fibrous tissue (i.e., scar tissue) that may contract and cause retinal detachment. Multiple factors can determine whether ROP progresses. For example, the overall health, birth weight, and the stage of ROP at initial diagnosis can all effect either the extent to which the disease will progress or the treatability of the disease once detected.

Age Related Macular Degeneration

[0050] Age-related macular degeneration (AMD) is the leading cause of irreversible blindness in developed countries. AMD is defined as an abnormality of the retinal pigment epithelium (RPE) that leads to overlying photoreceptor degeneration of the macula and consequent loss of central vision. Early AMD is characterized by drusen ($>63\mu\text{m}$) and hyper- or hypo-pigmentation of the RPE. Intermediate AMD is characterized by the accumulation of focal or diffuse drusen ($>120\text{ }\mu\text{m}$) and hyper- or hypo-pigmentation of the RPE. Advanced AMD is associated with vision loss due to either geographic atrophy of the RPE and photoreceptors (dry AMD) or neovascular choriocapillary invasion across Bruch's membrane into the RPE and photoreceptor layers (wet AMD). AMD leads to a loss of central visual acuity, and can progress in a manner that results in severe visual impairment and blindness. Visual loss in wet AMD is more sudden and may be more severe than in dry AMD.

[0051] It is estimated that 1.75 million people in the United States alone suffer from advanced AMD (dry and wet AMD). Also in the United States alone, it is estimated that an additional 7.3 million people suffer from intermediate AMD, which puts them at increased risk for developing the advanced forms of the disease. It is projected that such numbers will increase significantly over the next 10 to 15 years. At this point, patients with wet AMD are treated with laser photocoagulation, photodynamic therapy, or anti-angiogenic therapies to stabilize their disease and prevent further loss of vision. However, there is no treatment or cure for the more prevalent "dry" form of the disease.

[0052] The etiology and pathophysiology of AMD are poorly understood. The pathology is characterized by accumulation of membranous debris underneath the RPE basement membrane, which results in the formation of drusen. Drusen are

small yellowish, extracellular deposits of lipid, protein, and cellular debris, accumulated between the RPE and Bruch's membrane or within Bruch's membrane. Drusen are made of protein and lipid material including complement components and modulators and HtrA1. It is thought that the formation of drusen may be caused by RPE dysfunction or a change in Bruch's membrane composition (*e.g.*, increased lipid deposition and protein cross linking) or the permeability of the Bruch's membrane to nutrients (*e.g.*, impaired diffusion of water soluble plasma constituents across Bruch's membrane).

HtrA1

[0053] HtrA1 is secretory protein that belongs to the mammalian family of HtrA serine proteases. Several members of the HtrA family have been characterized, with each member having a highly conserved trypsin and PDZ domains. Human HtrA1 (hHtrA1) includes a serine protease (SP) domain and its PDZ domain in the C-terminal region of the molecule. hHtrA1 also a signal sequence for secretion, an insulin-like growth factor binding domain (IGFBP), and a Kazal-type trypsin inhibitory domain (KI) in the N-terminal region of the molecule. Together, the IGFBP and KI domains are also known as the Mac25 domain or the follistatin (FS) domain. In addition to these domains, hHtrA1 also contains a linker region between the KI and trypsin domain. It has been reported that hHtrA1 is overexpressed in articular chondrocytes of osteoarthritis patients, suggesting a potential role for HtrA1 in matrix remodeling and inflammatory processes. The PDZ domain of hHtrA1 has also been shown to interact with various extracellular matrix proteins, whereas the signal sequence and linker region have been shown to interact with the transforming growth factor (TGF) family of proteins. A catalytic triad (serine-328, histidine-220, and aspartic acid-250) in the trypsin domain of hHtrA1 is essential for protease activity, and mutation of serine-328 to alanine (SA mutation) has been shown to abrogate the protease activity of hHtrA1.

[0054] Several independent association studies implicated a locus for AMD at chromosome 10q26. To identify the critical gene at this locus, samples from 442 subjects suffering from AMD and samples from 309 control subjects that did not exhibit AMD were genotyped using a panel of 15 single nucleotide polymorphisms (SNP or SNPs) known to be located near SNP rs10490924, identified as a high risk SNP. In agreement with previous reports and as shown in Table 1, rs10490924 was found to have a highly significant association signal ($p=8.1 \times 10^{-8}$ for an additive allele-

dosage model, $OR_{het}=1.35$ (0.99, 1.86), $OR_{hom}=6.09$ (3.27, 11.34); T allele: 39.7% in subjects with AMD versus 24.7% in control subjects). However, of the 15 SNPs analyzed, rs11200638 was determined to be the variant most significantly associated with AMD ($p = 1 \times 10^{-9}$, $OR_{het}=1.86$ (1.35, 2.56), $OR_{hom}=6.56$ (3.23, 13.31), A allele: 40.3% in subjects with AMD versus 25.2% in control subjects). The results of this analysis are provided in FIG. 11. In terms of the significance of the association, the TA haplotype across rs10490924 and rs11200638 was superior to rs10490924 ($p=2.2 \times 10^{-9}$), but inferior to rs11200638.

Table 1

SNP	bp	chi trend	Trend p	ORhet	ORhom	chi dom	dom p	ORdom
rs986960	124082543	0.514100514	0.47336937	0.91 (0.64,1.31)	0.85 (0.55,1.32)	0.409940189	0.522	0.89 (0.64,1.26)
rs1998345	124114296	8.184700313	0.00422452	1.37 (0.99,1.90)	2.02 (1.19,3.43)	6.169103364	0.013	1.48 (1.09,2.02)
rs2901307	124118433	5.05448932	0.02456207	0.79 (0.55,1.14)	0.59 (0.38,0.93)	3.024807559	0.082	0.73 (0.52,1.04)
rs4146894	124145371	16.54941589	4.731E-05	1.76 (1.22,2.53)	2.32 (1.55,3.47)	15.44530314	8.48964E-05	1.96 (1.40,2.74)
rs2421016	124157502	11.09444779	0.00086607	1.82 (1.24,2.66)	2.21 (1.38,3.54)	11.989527	0.000535155	1.91 (1.32,2.77)
rs1045216	124179187	0	1	0.88 (0.64,1.23)	1.12 (0.67,1.87)	0.245867175	0.62	0.92 (0.67,1.26)
rs10490924	124204438	32.1381	8.1359E-08	1.35 (0.99,1.86)	6.09 (3.27,11.34)	15.37210555	8.8007E-05	1.81 (1.35,2.45)
rs3750847	124205411	27.1378	1.8596E-07	1.44 (1.04,1.98)	5.99 (2.98,12.04)	14.60816711	0.000132351	1.82 (1.34,2.47)
rs3750846	124205555	18.64656806	1.5656E-05	1.40 (1.02,1.92)	4.86 (2.32,10.19)	10.3323061	0.001307435	1.65 (1.22,2.24)
rs2014307	124207622	24.36637878	5.9023E-07	0.61(0.44,0.85)	0.23 (0.13,0.41)	14.86771798	0.000115364	0.54 (0.39,0.74)
rs11200638	124210524	37.2931	1.0164E-09	1.86 (1.35,2.56)	6.56 (3.23,13.31)	28.22875977	3.81841E-07	2.21 (1.62,3.01)
rs1049331	124211260	0.808199813	0.36865296	0.71 (0.49,1.03)	0.88 (0.53,1.44)	2.497876271	0.114	0.75 (0.52,1.07)
rs4752700	124227602	1.402298228	0.23633901	0.65(0.45,0.94)	0.85 (0.51,1.41)	4.176008969	0.041	0.69 (0.49,0.99)
rs2300431	124232807	1.6662006	0.1967684	0.74 (0.51,1.08)	0.81 (0.46,1.45)	2.443666776	0.118	0.76 (0.53,1.07)
rs714816	124246335	6.81254399	0.00905169	1.11 (0.80,1.54)	2.26 (1.33,3.84)	2.257131217	0.133	1.27 (0.93,1.73)

[0055] In light of this information, an additional 139 samples were taken from subjects suffering from AMD were genotyped and analyzed for these two variants. The results for both SNPs increased in significance (rs10490924: $p=1.2 \times 10^{-8}$, rs11200638: $p=1.6 \times 10^{-11}$), with variant rs11200638 remaining the best single variant explaining the association ($OR_{het}=1.90$ (1.40, 2.58), $OR_{hom}=7.51$ (3.75, 15.04)). Next an association analyses based on genotypes at both rs11200638 and the CFH

rs1061170 (Y402H) variant at chromosome 1q31 was undertaken. In a global two-locus analysis enumerating all nine two-locus genotype combinations, the association with AMD was significant ($\chi^2=56.56$, 8 df; $p=2.2\times 10^{-9}$). FIG. 11 shows the risk estimates for each two-locus genotype combination compared to the baseline of no risk genotypes (TT at CFHY402H and GG at rs11200638). The association of rs11200638 to AMD was significant when analyzed conditional on the presence of the CFH C risk allele ($p=5.9\times 10^{-8}$). In particular, this conditional analysis indicates an allele-dosage effect such that homozygotes for the A risk allele of rs11200638 are at an increased risk ($OR_{\text{hom}}=7.29(3.18,16.74)$) over that of heterozygotes ($OR_{\text{het}}=1.83(1.25,2.68)$) in all AMD subjects, even when compared to a baseline that includes individuals who carry the risk genotypes at *CFH*. With an allele-dosage model, the estimated population attributable risk (PAR) for rs11200638 is 49.3%. Consistent with an additive effect, the estimated PAR from a joint model with CFH Y402H (that is, for a risk allele at either locus) is 71.4%. Though initial efforts were focused on determining the association of SNP rs11200638 with the wet form of AMD, work has now been conducted with establishes that the presence of the same SNP rs11200638 has also been correlated to an increased risk of the dry form of AMD.

[0056] The SNP rs11200638 is located 512 bp upstream of the transcription start site of the HtrA1 gene (also known as *PRSS11*, *NM_002775*). Using MatInspector to scan putative transcription factor binding sites within this region, a conserved AP2/SRF binding element that is altered by the A risk allele was identified. To investigate the functional significance of the SNP, we used real-time RT-PCR to study the expression levels of HtrA1 mRNA in lymphocytes of four AMD patients carrying the risk allele AA and three normal controls carrying the normal allele GG. The HtrA1 mRNA levels in lymphocytes of AMD patients with the AA genotype were approximately 2.7 fold higher than those in normal controls with the GG genotype (See, FIG. 3). The mean HtrA1 protein level in RPE of four AMD donor eyes with a homozygous AA risk allele were also 1.7 fold higher compared to that of six normal controls with a homozygous GG allele (See, FIG. 4). The data to date, therefore, indicate a trend toward higher expression of HtrA1 when the AA genotype is present.

[0057] Through immunohistochemistry experiments, it has been determined that HtrA1 immunolabeling is present in the drusen of patients suffering from AMD

and that HtrA1 is non-uniformly expressed on drusen (See, FIG. 6). Further, in patients with AMD, the HtrA1 gene is expressed in the retina, internal limiting membrane, and RPE. These findings indicate, therefore, that HtrA1 expression promotes or facilitates both the wet and dry forms of AMD.

[0058] It is thought that HtrA1 is an enzyme that plays a role in the regulation of the degradation of extracellular matrix proteoglycans, and such activity is thought to facilitate access of other degradative matrix enzymes such as collagenases and matrix metalloproteinases to their substrates. Without being bound by a particular theory, it is presently thought that increased expression of HtrA1 associated with the SNP rs11200638 may, for example, contribute to the formation of drusen through increased production of degradation products that result from degradation of extracellular matrix proteoglycans and by increased activity of other degradative matrix enzymes. Again without being bound by a particular theory, such an increase in the presence of degradation products may contribute to pathologic neovascularization, vascular leak, and edema that leads to the wet form of AMD. Alternatively or in addition, without being bound by a particular theory, HtrA1 may contribute to an inflammatory response or physiologic conditions that enable or promote angiogenesis and pathologic neovascularization, even where drusen are not present.

Antibodies to HtrA1

[0059] Antibodies to HtrA1 are described herein. Exemplary antibodies to HtrA1 according to the present description include polyclonal, monoclonal, humanized, human, and affinity matured antibodies. The HtrA1 antibodies contemplated herein also include, for example, antibody fragments, glycosylation variants, antibodies having one or more amino acid substitutions, and antibodies conjugated to one or more chemical entities that allow the antibody to achieve a desired biological activity or functional characteristic. Antibodies as described herein may be agonists, antagonists or blocking antibodies to HtrA1 or an HtrA1 variant.

[0060] In one embodiment, the antibodies to HtrA1 are polyclonal antibodies. Methods of preparing polyclonal antibodies are known to the skilled artisan. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if desired, an adjuvant. Typically, the immunizing agent and/or adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. The immunizing agent may include, for

example, a HtrA1, a variant of HtrA1, or a fusion protein thereof. In addition, it may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins include but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation. The mammal can then be bled, and the serum assayed for HtrA1 antibody titer. If desired, the mammal can be boosted until the antibody titer increases or plateaus. In one embodiment, the immunizing agent used to generate polyclonal antibodies to HtrA1 includes a fragment of human HtrA1 according to SEQ. ID. NO.: 1, the fragment comprising amino acids 141-480 of a full length human HtrA1 molecule, or a variant of such a fragment. In an alternative embodiment, the immunizing agent used to generate polyclonal antibodies to HtrA1 includes a full-length human HtrA1 molecule according to SEQ. ID. NO. 2 or a variant thereof.

[0061] In another embodiment, the HtrA1 antibodies described herein are monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, *Nature*, 256:495 (1975), and by Harlow and Lane, *Antibodies: A laboratory Manual*. 1988 Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized in vitro. The immunizing agent will typically include a desired HtrA1 or a fusion protein thereof. In one embodiment, the immunizing agent used to generate polyclonal antibodies to HtrA1 includes a fragment of human HtrA1 according to SEQ. ID. NO.: 1, the fragment comprising amino acids 141-480 of a full length human HtrA1 molecule, or a variant of such a fragment. In an alternative embodiment, the immunizing agent used to generate polyclonal antibodies to HtrA1 includes a full-length human HtrA1 molecule according to SEQ. ID. NO.: 2 or a variant thereof.

[0062] The lymphocytes that produce or are capable of producing HtrA1 antibodies are then fused with an immortalized cell line using a suitable fusing agent,

such as polyethylene glycol, to form a hybridoma cell (See, Goding, *Monoclonal Antibodies: Principles and Practice*, Academic Press, (1986) pp. 59-103). Generally, either peripheral blood lymphocytes (PBLs) are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Typically, rat or mouse myeloma cell lines are employed. The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

[0063] In one embodiment, immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium are selected for preparation of hybridoma cells. For example, murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, CA, and the American Type Culture Collection, Manassas, Va. In one embodiment, as is detailed in the Examples that follow, cells from the myeloma Sp2/0-Ag14 cell line are used to form hybridomas producing the HtrA1 antibodies. Additionally, human myeloma and mouse-human heteromyeloma cell lines may also be used in the formation of hybridomas capable of producing human monoclonal antibodies (See, Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, Marcel Dekker, Inc., New York, (1987) pp. 51-63).

[0064] Once hybridoma cells are prepared, The culture medium in which the hybridoma cells are cultured can be assayed for the presence of monoclonal antibodies directed against HtrA1. The binding specificity of monoclonal antibodies produced by the hybridoma cells can, for example, be determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). Such techniques and assays are known in the art. In addition, the binding affinity of the monoclonal

antibody can, for example, be determined by the Scatchard analysis of Munson and Pollard, *Anal. Biochem.*, 107:220 (1980).

[0065] After desired hybridoma cells are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods (See, Goding, *supra*). Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium or RPMI-1640 medium. Alternatively, the hybridoma cells may be grown *in-vivo* as ascites in a mammal.

[0066] The monoclonal HtrA1 antibodies secreted by the subclones may be isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

[0067] Alternatively, monoclonal HtrA1 antibodies as described herein may also be made by recombinant DNA methods, such as those described in U.S. Pat. No. 4,816,567. DNA encoding the monoclonal antibodies can be isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as, for example, *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences (See, e.g., Morrison, et al., *Proc. Nat. Acad. Sci.* 81, 6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. In that manner, "chimeric" or "hybrid" antibodies that have the binding specificity of an anti-HTRA1 monoclonal antibody according to the present description can be prepared.

[0068] Typically, such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody of the invention, or they are substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody comprising one antigen-combining site having

specificity for HtrA1 and another antigen-combining site having specificity for a different antigen.

[0069] Human, chimeric, hybrid or recombinant anti-HtrA1 antibodies may comprise an antibody having full length heavy and light chains or fragments thereof, such as a Fab, Fab', F(ab')₂ or Fv fragment, a monomer or dimer of such light chain or heavy chain, a single chain Fv in which such heavy or light chain(s) are joined by a linker molecule, or having variable domains (or hypervariable domains) of such light or heavy chain(s) combined with still other types of antibody domains.

[0070] Chimeric or hybrid antibodies also may be prepared in vitro using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate.

[0071] Single chain Fv fragments may also be produced, such as described in Iliades et al., FEBS Letters, 409:437-441 (1997). Coupling of such single chain fragments using various linkers is described in Kortt et al., Protein Engineering, 10:423-433 (1997). A variety of techniques for the recombinant production and manipulation of antibodies are well known in the art.

[0072] In one embodiment, the antibodies according to the present description are humanized HtrA1 antibodies. A humanized HtrA1 antibody will typically have one or more amino acid residues introduced into it from a non-human source. Such non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Often, humanized antibodies are human antibodies in which CDR residues and possibly FR residues are substituted by residues from analogous sites in rodent antibodies. Therefore, humanized HtrA1 antibodies according to the present description are typically chimeric antibodies, wherein less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. Humanized HtrA1 antibodies as contemplated herein may be produced using methods known in the art, such as, for example, the methods described in Jones et al., Nature, 321:522-525 (1986), Riechmann et al., Nature, 332:323-327 (1988), and/or Verhoeyen et al., Science, 239:1534-1536 (1988).

[0073] In another embodiment, the antibodies described herein are human HtrA1 monoclonal antibodies made by a hybridoma method. Cell lines, such as human

myeloma and mouse-human heteromyeloma cell lines, suitable for production of human monoclonal antibodies are known, and are taught, for example, by Kozbor, J. Immunol. 133, 3001 (1984), and Brodeur, et al., Monoclonal Antibody Production Techniques and Applications, pp. 51-63 (Marcel Dekker, Inc., New York, 1987). In another embodiment, human HtrA1 antibodies may be produced using a transgenic animal, such as transgenic mice, capable of producing human antibodies in the absence of endogenous immunoglobulin production. Techniques and transgenic animals suitable for production of human antibodies are described, for example by Jakobovits et al., Proc. Natl. Acad. Sci. USA 90, 2551-2555 (1993), Jakobovits et al., Nature 362, 255-258 (1993), and Mendez et al., Nature Genetics 15: 146-156 (1997)).

[0074] Human antibodies can additionally be produced using the phage display technology described by McCafferty et al., Nature 348, 552-553 (1990). Such technology allows production of human antibodies and human antibody fragments in vitro, from immunoglobulin variable (V) domain gene repertoires from unimmunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous bacteriophage, such as M13 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B-cell. Phage display can be performed in a variety of formats; for their review see, e.g. Johnson, Kevin S. and Chiswell, David J., Current Opinion in Structural Biology 3, 564-571 (1993). Several sources of V-gene segments can be used for phage display. Clackson et al., Nature 352, 624-628 (1991) isolated a diverse array of anti-oxazolone antibodies from a small random combinatorial library of V genes derived from the spleens of immunized mice. A repertoire of V genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self-antigens) can be isolated essentially following the techniques described by Marks et al., J. Mol. Biol. 222, 581-597 (1991), or Griffith et al., EMBO J. 12, 725-734 (1993). In a natural immune response, antibody genes accumulate mutations at a high rate (somatic hypermutation). Some of the changes introduced will confer higher affinity, and B cells displaying high-affinity surface immunoglobulin

are preferentially replicated and differentiated during subsequent antigen challenge. This natural process can be mimicked by employing the technique known as "chain shuffling" (Marks et al., *Bio/Technol.* 10, 779-783 [1992]). In this method, the affinity of "primary" human antibodies obtained by phage display can be improved by sequentially replacing the heavy and light chain V region genes with repertoires of naturally occurring variants (repertoires) of V domain genes obtained from unimmunized donors. This technique allows the production of antibodies and antibody fragments with affinities in the nM range. A strategy for making very large phage antibody repertoires (also known as "the mother-of-all libraries") has been described by Waterhouse et al., *Nucl. Acids Res.* 21, 2265-2266 (1993). Gene shuffling can also be used to derive human antibodies from rodent antibodies, where the human antibody has similar affinities and specificities to the starting rodent antibody. According to this method, which is also referred to as "epitope imprinting", the heavy or light chain V domain gene of rodent antibodies obtained by phage display technique is replaced with a repertoire of human V domain genes, creating rodent-human chimeras. Selection of an antigen results in isolation of human variable domains capable of restoring a functional antigen-binding site, i.e. the epitope governs (imprints) the choice of a partner. When the process is repeated in order to replace the remaining rodent V domain, a human antibody is obtained (see PCT patent application WO 93/06213, published 1 Apr. 1993). Unlike traditional humanization of rodent antibodies by CDR grafting, this technique provides completely human antibodies, which have no framework or CDR residues of rodent origin.

[0075] In certain embodiments, the anti-HtRA1 antibody (including murine, human and humanized antibodies, and antibody variants) is an antibody fragment. Various techniques for the production of antibody fragments are known. For example, antibody fragments can be derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *J. Biochem. Biophys. Methods* 24:107-117 (1992) and Brennan et al., *Science* 229:81 (1985)). Additionally, antibody fragments can be produced directly by recombinant host cells. For example, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., *Bio/Technology* 10:163-167 (1992)). In another embodiment, the $F(ab')_2$ is formed using the leucine zipper GCN4 to promote

assembly of the $F(ab')_2$ molecule. According to another approach, Fv, Fab or $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture.

[0076] The Fab fragments produced in the antibody digestion also contain the constant domains of the light chain and the first constant domain (CH_1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH_1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

[0077] Amino acid sequence variants of the anti-HtrA1 antibodies are prepared by introducing appropriate nucleotide changes into the anti-HtrA1 antibody DNA, or by peptide synthesis. Such variants include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the anti-HtrA1 antibodies of the examples herein. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the humanized or variant anti-HtrA1 antibody, such as changing the number or position of glycosylation sites.

[0078] A useful method for identification of certain residues or regions of the anti-HtrA1 antibody that are preferred locations for mutagenesis is called "alanine scanning mutagenesis," as described by Cunningham and Wells Science, 244:1081-1085 (1989). According to such method, a residue or group of target residues are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with HtrA1 antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to analyze the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed anti-HtrA1 antibody variants are screened for the desired activity.

[0079] Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an anti-HtrA1 antibody with an N-terminal methionyl residue or the antibody fused to an epitope tag. Other insertional variants of the anti-HTRA1 antibody molecule include the fusion to the N- or C-terminus of the anti-HTRA1 antibody of an enzyme or a polypeptide which increases the serum half-life of the antibody.

[0080] Another type of variant is an amino acid substitution variant. These variants have at least one amino acid residue in the anti-HtrA1 antibody molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include the hypervariable regions, but FR alterations are also contemplated.

[0081] Substantial modifications in the biological properties of the antibody are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain.

[0082] Any cysteine residue not involved in maintaining the proper conformation of the humanized or variant anti-HtrA1 antibody also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the antibody to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

[0083] A particularly preferred type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (e.g., a humanized or human antibody). Generally, the resulting variant(s) selected for further development will have improved biological properties relative to the parent antibody from which they are generated. A convenient way for generating such substitutional variants is affinity maturation using phage display. Briefly, several hypervariable region sites (e.g., 6-7 sites) are mutated to generate all possible amino substitutions at each site. The antibody variants thus generated are displayed in a monovalent fashion from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for

their biological activity (e.g., binding affinity) as herein disclosed. In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis can be performed to identify hypervariable region residues contributing significantly to antigen binding. Alternatively, or in addition, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and a desired HtrA1 polypeptide. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

[0084] Glycosylation variants of HtrA1 antibodies are also contemplated herein. Antibodies are glycosylated at conserved positions in their constant regions (See, Jefferis and Lund, *Chem. Immunol.* 65:111-128 (1997); Wright and Morrison, *TibTECH* 15:26-32 (1997)). The oligosaccharide side chains of the immunoglobulins affect the protein's function (See, Boyd et al., *Mol. Immunol.* 32:1311-1318 (1996); Wittwe and Howard, *Biochem.* 29:4175-4180 (1990)), and the intramolecular interaction between portions of the glycoprotein which can affect the conformation and presented three-dimensional surface of the glycoprotein (See, Jefferis and Lund, *supra*; Wyss and Wagner, *Current Opin. Biotech.* 7:409-416 (1996)). Oligosaccharides may also serve to target a given glycoprotein to certain molecules based upon specific recognition structures. For example, it has been reported that in agalactosylated IgG, the oligosaccharide moiety 'flips' out of the inter-CH2 space and terminal N-acetylglucosamine residues become available to bind mannose binding protein (See, Malhotra et al., *Nature Med.* 1:237-243 (1995)). Removal by glycopeptidase of the oligosaccharides from CAMPATH-1H (a recombinant humanized murine monoclonal IgG1 antibody which recognizes the CDw52 antigen of human lymphocytes) produced in Chinese Hamster Ovary (CHO) cells resulted in a complete reduction in complement mediated lysis (CMCL) (See, Boyd et al., *Mol. Immunol.* 32:1311-1318 (1996)), while selective removal of sialic acid residues using neuraminidase resulted in no loss of DMCL. Glycosylation of antibodies has also been reported to affect antibody-dependent cellular cytotoxicity (ADCC). In particular, CHO cells with tetracycline-regulated expression of $\beta(1,4)$ -N-acetylglucosaminyltransferase III (GnTIII), a glycosyltransferase catalyzing formation

of bisecting GlcNAc, was reported to have improved ADCC activity (Umana et al., *Mature Biotech.* 17:176-180 (1999)).

[0085] Glycosylation variants of antibodies are variants in which the glycosylation pattern of an antibody is altered. By altering is meant deleting one or more carbohydrate moieties found in the antibody, adding one or more carbohydrate moieties to the antibody, changing the composition of glycosylation (glycosylation pattern), the extent of glycosylation, etc. Glycosylation variants may, for example, be prepared by removing, changing and/or adding one or more glycosylation sites in the nucleic acid sequence encoding the antibody.

[0086] Glycosylation of antibodies is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

[0087] Addition of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the original antibody (for O-linked glycosylation sites).

[0088] Nucleic acid molecules encoding amino acid sequence variants of the anti-HtrA1 antibody can be prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the anti-HtrA1 antibody.

[0089] The glycosylation (including glycosylation pattern) of antibodies may also be altered without altering the underlying nucleotide sequence. Glycosylation largely

depends on the host cell used to express the antibody. Since the cell type used for expression of recombinant glycoproteins, *e.g.*, antibodies, as potential therapeutics is rarely the native cell, significant variations in the glycosylation pattern of the antibodies can be expected (See, *e.g.*, Hse et al., J. Biol. Chem. 272:9062-9070 (1997)). In addition to the choice of host cells, factors which affect glycosylation during recombinant production of antibodies include growth mode, media formulation, culture density, oxygenation, pH, purification schemes and the like. Various methods have been proposed to alter the glycosylation pattern achieved in a particular host organism including introducing or over expressing certain enzymes involved in oligosaccharide production (See, U.S. Pat. Nos. 5,047,335; 5,510,261 and 5,278,299). Glycosylation, or certain types of glycosylation, can be enzymatically removed from the glycoprotein, for example using endoglycosidase H (Endo H). In addition, the recombinant host cell can be genetically engineered, *e.g.* make defective in processing certain types of polysaccharides. These and similar techniques are well known in the art.

[0090] The glycosylation structure of antibodies can be readily analyzed by conventional techniques of carbohydrate analysis, including lectin chromatography, NMR, Mass spectrometry, HPLC, GPC, monosaccharide compositional analysis, sequential enzymatic digestion, and HPAEC-PAD, which uses high pH anion exchange chromatography to, separate oligosaccharides based on charge. Methods for releasing oligosaccharides for analytical purposes are also known, and include, without limitation, enzymatic treatment (commonly performed using peptide-N-glycosidase F/endo-.alpha.-galactosidase), elimination using harsh alkaline environment to release mainly O-linked structures, and chemical methods using anhydrous hydrazine to release both N- and O-linked oligosaccharides.

[0091] Other modifications of the HtrA1 antibodies are contemplated herein. For example, the antibody may be linked to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, polyoxyalkylenes, or copolymers of polyethylene glycol and polypropylene glycol. The antibody also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (*e.g.*, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively), in colloidal drug delivery systems (*e.g.*, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed, for

example, in Remington's Pharmaceutical Sciences, 16th edition, Oslo, A., Ed., (1980). To increase the serum half life of the antibody, one may incorporate a salvage receptor binding epitope into the antibody (especially an antibody fragment) as described in U.S. Pat. No. 5,739,277, for example. As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (e.g., IgG₁, IgG₂, IgG₃, or IgG₄) that is responsible for increasing the in vivo serum half-life of the IgG molecule.

[0092] The anti-HtrA1 antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as, for example, the methods described in Epstein et al., Proc. Natl. Acad. Sci. USA 82:3688 (1985); Hwang et al., Proc. Natl. Acad. Sci. USA 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556. Liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are typically extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention may be conjugated to the liposomes as described in Martin et al., J. Biol. Chem. 257:286-288 (1982) via a disulfide interchange reaction.

[0093] The antibodies of the invention include "cross-linked" HtrA1 antibodies. The term "cross-linked" as used herein refers to binding of at least two IgG molecules together to form one (or single) molecule. The HtrA1 antibodies may be cross-linked using various linker molecules, preferably the HtrA1 antibodies are cross-linked using an anti-IgG molecule, complement, chemical modification or molecular engineering. It is appreciated by those skilled in the art that complement has a relatively high affinity to antibody molecules once the antibodies bind to cell surface membrane. Accordingly, it is believed that complement may be used as a cross-linking molecule to link two or more anti-HtrA1 antibodies bound to cell surface membrane. Cross-linking of the human anti-HtrA1 antibodies is also described in the Examples using either goat anti-mouse IgG Fc or goat anti-human IgG Fc.

Exemplary Antibodies

[0094] The following embodiments are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

[0095] As described in the Examples below, polyclonal anti-HtrA1 antibodies have been prepared and a number of anti-HtrA1 monoclonal antibodies have been identified and prepared. In one embodiment, the monoclonal antibodies according to this description will have the same biological characteristics as the monoclonal antibodies secreted by the hybridoma cell line(s) referred to in the Examples that follow. The term "biological characteristics" is used to refer to the *in-vitro* and/or *in-vivo* activities or properties of the monoclonal antibody, such as the ability to specifically bind to HtrA1 or to block, inhibit, induce or enhance HtrA1 related activities. By way of example, a blocking antibody may block binding of a ligand to HtrA1 such as, for example, binding of a ligand to the IGFBP domain of the HtrA1 or any other HtrA1 domain to which a ligand may bind. Additionally, by way of example, a blocking antibody may inhibit the activity or function of any one or more of the trypsin domain, PDZ domain, SP domain, KI domain, Mac25 domain, or FS domain.

[0096] The HtrA1 antibodies, as described herein, optionally possess one or more desired biological activities or properties. Such HtrA1 antibodies may include, but are not limited to, chimeric, humanized, human, and affinity matured antibodies. As described above, the HtrA1 antibodies may be constructed or engineered using various techniques to achieve these desired activities or properties. In one embodiment, the HtrA1 antibody has an HtrA1 binding affinity of at least 10^5 M^{-1} . In one such embodiment, the HtrA1 antibody is an isolated polyclonal antibody. In another such embodiment, the HtrA1 antibody is an isolated monoclonal antibody. In an alternative embodiment, the HtrA1 antibody has an HtrA1 binding affinity in a range selected from 10^6 M^{-1} to 10^7 M^{-1} , 10^8 M^{-1} to 10^{12} M^{-1} , and 10^9 M^{-1} to 10^{12} M^{-1} . In yet a further alternative embodiment, an HtrA1 antibody as described herein has a binding affinity selected from at least 10^6 M^{-1} , at least 10^7 M^{-1} , at least 10^8 M^{-1} , at least 10^9 M^{-1} , at least 10^{10} M^{-1} , at least 10^{11} M^{-1} , and at least 10^{12} M^{-1} . In one such embodiment, the HtrA1 antibody is an isolated polyclonal antibody. In another such embodiment, the HtrA1 antibody is an isolated monoclonal antibody.

[0097] The binding affinity of the HtrA1 antibody can be determined without undue experimentation by testing the HtrA1 antibody in accordance with techniques known in the art, including Scatchard analysis (See, Munson et al., supra). Embodiments of antibodies exhibiting the binding affinities or ranges of binding affinities set forth herein may include, for example, monoclonal antibodies, polyclonal

antibodies, chimeric antibodies, humanized antibodies, human antibodies, and affinity matured antibodies. Further embodiments of antibodies exhibiting the binding affinities or ranges of binding affinities set forth herein include monoclonal antibodies, polyclonal antibodies, chimeric antibodies, humanized antibodies, human antibodies, and affinity matured antibodies generated using an immunizing agent that includes a fragment of human HtrA1 according to SEQ. ID. NO.: 1, the fragment comprising amino acids 141-480 of a full length human HtrA1 molecule, or a variant of such a fragment, and monoclonal antibodies, polyclonal antibodies, chimeric antibodies, humanized antibodies, human antibodies, and affinity matured antibodies generated using an immunizing agent that includes a full-length human HtrA1 molecule according to SEQ. ID. NO.: 2 or a variant thereof.

[0098] The antibodies disclosed herein also exhibit characteristics related to their ability to immunospecifically bind to HtrA1 epitopes. For example, in one embodiment, the antibodies disclosed herein have the ability to bind to one or more specific epitopes on the HtrA1 molecule. In addition, the amino acid sequences of the antibodies described allow binding of the antibodies described herein to the one or more HtrA1 epitopes. Antibodies disclosed herein also have the ability to competitively inhibit the immunospecific binding of antibodies that recognize an identical or nearly identical epitope on an HtrA1 molecule. In a typical embodiment, as is described and illustrated in Example 5, antibody binding of HtrA1 is evaluated, for example, using an indirect ELISA.

[0099] When evaluated in an indirect ELISA for binding to HtrA1 or a variant thereof, in one embodiment, the antibodies of the present invention exhibit a titer of 1:160,000 or greater. In another embodiment, the antibodies described herein exhibit a titer selected from 1:200,000 or greater, 1:300,00 or greater, 1:400,000 or greater, 1:500,000 or greater, 1:600,000 or greater, 1:700,000 or greater, 1:800,000 or greater, 1:900,000 or greater, and 1:1,000,000 or greater. In still another embodiment, the antibodies described herein exhibit a range of titers selected from 1:200,000 to greater than 1:1,000,000, 1:300,000 to greater than 1:1,000,000, 1:400,000 to greater than 1:1,000,000, 1:500,000 to greater than 1:1,000,000, 1:600,000 to greater than 1:1,000,000, 1:700,000 to greater than 1:1,000,000, 1:800,000 to greater than 1:1,000,000, and 1:900,000 to greater than 1:1,000,000. In yet another embodiment, the antibodies described herein exhibit a range of titers selected from 1:200,000 to 1:300,000, 1:200,000 to 1:400,000, 1:200,000 to

1:500,000, 1:200,000 to 1:800,000, 1:200,000 to 1:900,000, and 1:200,000 to 1:1,000,000. In still a further embodiment, the antibodies described herein exhibit a range of titers selected from 1:200,000 to 1:400,000, 1:200,000 to 1:500,000, 1:400,000 to 1:800,000, 1:400,000 to 1:900,000, 1:400,000 to greater than 1:1,000,000, and 1:800,000 to greater than 1:1,000,000. As used herein, the "titer" of a particular antibody provides an indication of that antibody's capability to bind to HtrA1. In particular, in evaluating and comparing the titer as described herein of different antibodies, antibody solutions exhibiting substantially uniform antibody concentration (e.g., 1mg/ml) are generated for each antibody evaluated. Such solutions are then serially diluted, and the titer of a given antibody refers to the maximum dilution of the initial antibody concentration that, when evaluated in an indirect ELISA under the conditions in Example 5, exhibits an optical density at least approximately twice the optical density of a control well which includes no antibody.

[00100] Embodiments of antibodies exhibiting the titers or ranges of titers set forth herein may include, for example, monoclonal antibodies, polyclonal antibodies, chimeric antibodies, humanized antibodies, human antibodies, and affinity matured antibodies. In yet other embodiments, antibodies exhibiting the titers or ranges of titers set forth herein may include monoclonal antibodies, polyclonal antibodies, chimeric antibodies, humanized antibodies, human antibodies, and affinity matured antibodies generated using an immunizing agent that includes a fragment of human HtrA1 according to SEQ. ID. NO.: 1, the fragment comprising amino acids 141-480 of a full length human HtrA1 molecule, or a variant of such a fragment, and monoclonal antibodies, polyclonal antibodies, chimeric antibodies, humanized antibodies, human antibodies, and affinity matured antibodies generated using an immunizing agent that includes a full-length human HtrA1 molecule according to SEQ. ID. NO.: 2 or a variant thereof.

[00101] In one embodiment, the monoclonal antibodies described herein include isolated monoclonal antibodies having one or more of the same biological characteristics of a monoclonal antibody produced by a hybridoma cell selected from the 8F12, 13D4, 10E7, 9F7, 8G8, 4H5, 7G6, 25A6, 21C9, 14E6, 3E8, 5B12, and 8F11 hybridoma cell lines as produced and described in Example 4 below. Embodiments of such antibodies include, for example, isolated monoclonal antibodies generated using an immunizing agent that includes a fragment of HtrA1

according to SEQ. ID. NO.: 1. Even further, embodiments of such antibodies may exhibit the titers or range of titers and or binding affinities described herein.

[00102] The antibodies described herein have the ability to modulate certain physiological actions or processes. In one embodiment, as is shown in Examples 6-10, the antibodies described herein inhibit one or more activities of HtrA1. In one such embodiment, an antibody according to the present description is biologically active and directly or indirectly inhibits or blocks HtrA1 activity or activation, such as by, for example, directly or indirectly inhibiting or blocking the activity or activation of one or more of the HtrA1 trypsin domain, PDZ domain, a serine protease (SP) domain, insulin-like growth factor binding domain (IGFBP), Kazal-type trypsin (KI) inhibitory domain, Mac25 domain, or the follistatin (FS) domain. In another such embodiment, an antibody according to the present invention inhibits the activity or activation of one or more functions of HtrA1, as described herein, in a manner that inhibits pathologic neovascularization. In one such embodiment, the HtrA1 antibody as described herein inhibits pathologic choroidal neovascularization. In another such embodiment, the HtrA1 antibody inhibits pathologic retinal neovascularization. In another embodiment, an HtrA1 antibody as described herein inhibits choroidal neovascularization in a laser-induced choroidal neovascularization model, wherein the reduction in choroidal neovascularization is selected from at least about 30%, about 40%, about 50%, about 60%, or about 70% or greater relative to a control. In yet another embodiment, an HtrA1 antibody as described herein inhibits retinal neovascularization in an oxygen-induced retinal neovascularization model, wherein the reduction in retinal neovascularization is selected from at least about 30%, about 40%, about 50%, about 60%, or about 70% or greater relative to a control.

[00103] Antibodies to HtrA1 that inhibit neovascularization as described herein may, for example, be isolated monoclonal antibodies, polyclonal antibodies, chimeric antibodies, humanized antibodies, human antibodies, and affinity matured antibodies to HtrA1, isolated monoclonal antibodies, polyclonal antibodies, chimeric antibodies, humanized antibodies, human antibodies, and affinity matured antibodies generated using an immunizing agent that includes a fragment of human HtrA1 according to SEQ. ID. NO.: 1, the fragment comprising amino acids 141-480 of a full length human HtrA1 molecule, or a variant of such a fragment, isolated monoclonal antibodies, polyclonal antibodies, chimeric antibodies, humanized antibodies, human antibodies, and affinity matured antibodies generated using an immunizing agent

that includes a full-length human HtrA1 molecule according to SEQ. ID. NO.: 2 or a variant thereof. In particular embodiments of such antibodies, the antibodies exhibit a titer and/or binding affinity according to the titers, ranges of titers, binding affinities and ranges of binding affinities described herein.

Methods of Using Antibodies to HtrA1

[00104] The HtrA1 antibodies described herein may be used in various methods. For example, HtrA1 antibodies may be employed in methods for treating one or more pathologic conditions in a subject. In each such embodiment, a therapeutically effective amount of HtrA1 antibody is administered to the subject. In another such embodiment, therapeutically effective amounts of two or more HtrA1 antibodies are administered to a subject. In addition to administering a therapeutic amount of one or more HtrA1 antibodies, a therapeutic method according to the present description may further include administration of one or more additional pharmaceutically active agents. Such agents may be chosen from, for example, from one or more agents that modulate the activity of HtrA1, work to inhibit neovascularization, or provide another other desired therapeutic benefit. In one embodiment, the pathologic condition to be treated is neovascularization induced or facilitated by the presence or expression of HtrA1. In another embodiment, the pathologic condition is selected from AMD in its wet form, AMD in its dry form, DR, ROP, macular edema, ischemia-induced neovascularization, and a pathologic condition associated with vascular leak or edema in the eye.

[00105] In one embodiment of the invention, methods for employing the antibody in diagnostic assays are provided. For instance, the antibodies may be employed in diagnostic assays, such as ELISA or western blot assays, to detect expression or over expression of HtrA1 in specific cells and tissues. Various diagnostic assay techniques that are suitable for use with the HtrA1 antibodies described herein are known in the art and include, for example, *in-vivo* imaging assays, *in-vitro* competitive binding assays, direct or indirect sandwich assays, and immunoprecipitation assays conducted in either heterogeneous or homogeneous phases (See, *e.g.*, Zola, Monoclonal Antibodies: A Manual of Techniques, CRC Press, Inc. (1987) pp. 147-158). Where desired, the antibodies used in the diagnostic assays can be labeled with a detectable moiety, with the detectable moiety being capable of directly or indirectly producing a detectable signal. Examples of detectable moieties include radioisotopes, such as ³H, ¹⁴C, ³²P, ³⁵S, or

¹²⁵I, fluorescent or chemiluminescent compounds, such as fluorescein isothiocyanate, rhodamine, or luciferin, and enzymes, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase. Any method known in the art for conjugating the antibody to the detectable moiety may be employed, including, for example, those methods described by Hunter et al., *Nature*, 144:945 (1962); David et al., *Biochemistry*, 13:1014-1021 (1974); Pain et al., *J. Immunol. Meth.*, 40:219-230 (1981); and Nygren, *J. Histochem. and Cytochem.*, 30:407-412 (1982).

[00106] In addition, both *in-vivo* and *in-vitro* models can be used to further explore the therapeutic effects of HtrA1 antibodies according to the present description and to understand the role of the such antibodies in the development and pathogenesis of, for instance, diseases or pathologies associated with neovascularization, wet and dry forms of AMD, DR, ROP, macular edema, ischemia-induced neovascularization, vascular leak or edema in the eye

[00107] For example, as detailed in Examples 6-10, relative to a control, HtrA1 antibodies as described herein significantly reduce neovascularization in animal models of laser-induced choroidal neovascularization and oxygen-induced retinopathy (*i.e.*, oxygen-induced retinal neovascularization). Animal models of choroidal neovascularization and oxygen-induced retinopathy are known, are accepted models of eye diseases associated with neovascularization, and the antibodies described herein are suitable for use in such models. Examples of the application of such models can be seen in, for instance, Saishin et al., *J Cell Physiol.*, 195(2):241-8 (2003); Dejneka et al., *Mol Vis.*, 10:964-72 (2004); and Nambu et al., *Invest Ophthalmol Vis Sci.*, 44(8):3650-5 (2003); Smith et al., *Invest Ophthalmol Vis Sci*, 35(1): 101-11. Additional references to animal models that may be suited for evaluation of the HtrA1 antibodies described herein include, for example, Fu et al., *Hum Mol Genet*, 15;16(20):3411-22 (2007); Karan et al., *Proc Natl Acad Sci USA*, 15;102(11):4164-9 (2005); Kim et al., *Invest Ophthalmol Vis Sci*, 48(10):4407-20 (2007); and Tanaka et al., *Mol Ther*, 13(3):609-16 (2006) .

[00108] The antibody is preferably administered to the subject in a carrier, and in one embodiment, the carrier is a pharmaceutically-acceptable carrier. Carriers that may be used to deliver antibodies as described herein and their formulations are described, for example, in Remington's *Pharmaceutical Sciences*, 16th ed., 1980, Mack Publishing Co., edited by Oslo et al. In addition to a carrier, a pharmaceutically acceptable formulation for delivery of an antibody as described

herein may include, for example, a desired amount a pharmaceutically-acceptable salt or other pharmaceutically acceptable tonicity modifier in order to render the formulation isotonic. Examples of carriers that may be used to provide a pharmaceutical formulation including one or more antibodies as described herein include, but are not limited to, saline, Ringer's solution and dextrose solution. A pharmaceutical formulation of as described herein may exhibit a pH of from about 5 to about 8, and in one such embodiment exhibits a pH of from about 7 to about 7.5. Where desired, a pharmaceutical formulation according to the present description may include a buffer to achieve and maintain a desired pH. Pharmaceutically acceptable buffers useful for achieving a formulation exhibiting a pH suitable for delivery to a subject are well known in the art. Even further, a pharmaceutical formulation as described herein may include a carrier formulated as a sustained release preparation. Sustained release preparations may be formed by, for example, semipermeable polymer matrices containing the antibody, and such matrices may be formed into articles, such as films, particles, or depots, of a desired shape, size or configuration.

[00109] The nature of the carrier, formulation constituents, and even configuration of a pharmaceutical product to be delivered may be adjusted or altered depending upon, for instance, the route of administration and concentration of antibody being administered. For example, where the subject to be treated is a mammal, a pharmaceutical formulation of an antibody as described herein can be formulated for delivery by injection (e.g., intravenous, intraocular, intraperitoneal, subcutaneous, intramuscular, or intraportal injection), or by other methods such as infusion that ensure its delivery to the bloodstream in a therapeutically effective form. An antibody as described herein may also be administered by isolated perfusion techniques, such as isolated tissue perfusion, to exert local therapeutic effects.

[00110] Therapeutically effective dosages and schedules for administering one or more antibodies as described herein may be determined based on the antibody administered, the desired therapeutic effect, the route of administration, and the subject to which the antibody is to be administered. Guidance in selecting appropriate doses for antibody may be found in the literature on therapeutic uses of antibodies, e.g., Handbook of Monoclonal Antibodies, Ferrone et al., eds., Noyes Publications, Park Ridge, N.J., (1985) ch. 22 and pp. 303-357; Smith et al., Antibodies in Human Diagnosis and Therapy, Haber et al., eds., Raven Press, New

York (1977) pp. 365-389. A typical daily dosage of the antibody used alone might range from about 1 µg/kg to up to 100 mg/kg of body weight or more per day, depending on the factors mentioned above. In addition, an antibody as described herein may be administered sequentially or concurrently with the one or more other therapeutic agents.

[00111] In a further embodiment of the invention, there are provided articles of manufacture and kits containing materials useful for treating pathological conditions or detecting or purifying HtrA1. In one such embodiment, the article of manufacture comprises a container having a label. Suitable containers include, for example, bottles, vials, and test tubes. The container may be formed from a variety of materials such as glass or plastic, and holds a composition having an active agent which is effective for treating pathological conditions or for detecting or purifying HtrA1. The active agent in the composition includes an HtrA1 antibody as described herein and, in one embodiment, comprises one or more monoclonal antibody specific for HtrA1. The label on the container may, for example, indicate that the composition included in the container is used for treating one or more pathological conditions or for detecting or purifying HtrA1. In addition, in one embodiment, the label may also indicate directions for either *in vivo* or *in vitro* use, such as for the uses described above. In another embodiment, the article of manufacture is a kit that includes a container having a label as already described and, optionally, a second container comprising one or more carriers, diluents or constituents, such as, for example, a buffer or tonicity modifier. In one embodiment, such a kit further includes one or more other materials desirable from the end user's standpoint, including, for example, filters, needles, syringes, and package inserts with instructions for use.

[00112] The Examples that follow are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. In each instance, unless otherwise specified, standard materials and methods were used in carrying out the work described in the Examples provided.

[00113] All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

EXAMPLE 1 - Expression of HtrA1 and AMD

[00114] As described herein, the SNP rs11200638 is located 512 bp upstream of the transcription start site of the HtrA1 gene (also known as *PRSS11*, *NM_002775*).

Using MatInspector to scan putative transcription factor binding sites within this region, we identified a conserved AP2/SRF binding element that is altered by the A risk allele. To investigate the functional significance of the SNP, a real-time RT-PCR was conducted to study the expression levels of HtrA1 mRNA in lymphocytes of four AMD patients carrying the risk allele AA and three normal controls carrying the normal allele GG. The results of this study are shown in FIG. 3. The HtrA1 mRNA levels in lymphocytes from AMD patients with the AA genotype were approximately 2.7 fold higher than those in normal controls with the GG genotype.

[00115] The mean HtrA1 protein level in RPE of four AMD donor eyes with a homozygous AA risk allele was also analyzed. As shown in FIG. 4, HtrA1 expression level in the AA genotype was 1.7 fold higher compared to that of six normal controls with a homozygous GG allele.

EXAMPLE 2 - *Production of recombinant His-tagged HtrA1 protein*

[00116] To raise antibodies against HtrA1, His-tagged wild type and His-tagged mutant protease (with a point mutation of Ser to Ala (SA) in the trypsin protease domain) proteins were produced using a bacterial expression system and Ni-NTA affinity purification. In order to produce the His-tagged wild type and His-tagged mutant protease proteins, a fragment encoding amino acids 141-480 (SEQ ID NO. 1) of the human HtrA1 protein was amplified from cDNA library. The fragment was amplified with the following PCR primers, HtrA1-L (5'-acgcgtcgacaaaggctgcaccg gccgccgt-3') (SEQ. ID. NO.: 3) and HtrA1-R (5'-ataagaatgcggccgcctatgggtcaatttctt cgg-3') (SEQ. ID. NO.: 4). Using standard techniques, the PCR product was cloned into a pET32a plasmid vector (Novagen) and then digested with *Sa*/I and *Not* I restriction enzymes. The recombinant His-tagged HtrA1 construct was expressed in BL21 bacteria following the IPTG induction. The recombinant proteins were purified from bacterial lysate using a Ni-column (Qiagen) and confirmed by western blot. Purification of HtrA1 proteins indicated that both mutant HtrA1 (SA) and wild type HtrA1 (WT) produced 75 kD dimer and 37 kD monomer (FIG. 2). Moreover, zymography analysis also indicates that the SA mutant does not possess protease activity. In addition, wild type dimer was less active than monomer. Dimer formation of purified HtrA1 proteins was sensitive to reducing condition, indicating that disulfide linkage may be responsible for dimer configuration. The recombinant wild type HtrA1 protein produced according to this example was used to generate rabbit polyclonal antibodies and murine monoclonal antibodies.

EXAMPLE 3 - Generating polyclonal antibody that recognizes HtrA1

[00117] Using the recombinant wild type HtrA1 protein of Example 1, rabbit antibodies were produced using standard methods. Western blot analysis was conducted according to the procedure described in Example 11 and confirmed that the polyclonal antibody produced specifically recognized the immunogen, and immunofluorescence conducted by the process described in Example 10 below showed that the polyclonal antibody recognized HtrA1 expressed in mammalian cells lines. Of significance, it was determined that in murine models of oxygen induced retinopathy, the polyclonal HtrA1 antibody was highly expressed in pathologic retinal vessels when compared to normal blood vessels.

[00118] With reference to FIG. 5(A), the HtrA1 polyclonal antibody was verified by Western blot. HtrA1 polyclonal antibody can recognize recombinant HtrA1 (a) and HtrA1 in human RPE (c and d). Specificity was confirmed by lack of signal following preabsorption with recombinant HtrA1 protein (e and f), (b) was a negative control consisting of a recombinant protein preparation from bacteria transfected with vector only. FIG. 5(B) shows HtrA1 polyclonal antibody stained on mammalian cells expressing recombinant human HtrA1. In FIG. 5(C-E), HtrA1 polyclonal antibody reveals HtrA1 expression in pathologic retinal endothelial cells but not normal retinal vessels in murine model of oxygen-induced retinopathy. In the normal mouse retina there is no blood vessel beyond internal limiting membrane, but in oxygen-induced retinopathy mouse model, proliferative blood vessel grows beyond internal limiting membrane into vitreous cavity (*i.e.*, retinal neovascularization). FIG. 5(E) shows that HtrA1 polyclonal antibody stains pathologic endothelial cells located beyond internal limiting membrane (yellow/bottom arrows) but not intraretinal endothelial cells (white/top and middle arrows). In FIG. 5(C), isolectin staining shows blood vessels, and FIG. 5 (D) shows HtrA1 polyclonal antibody staining. FIG. 5(E) merges the images of 5(C) and 5(D) with TO-PRO3 staining to show nuclei (blue). As used in FIG. 5 (C-E), "ONL" refers to the outer nuclear layer, "INL" refers to the inner nuclear layer, "GCL" refers to the ganglion cell layer, and "ILM" refers to the internal limiting membrane.

[00119] In addition, immunohistochemistry studies of HtrA1 expression in human eyes from AMD donors were conducted. Retinal sections were taken and incubated at 37°C for 30 min. The retinal sections were then washed 3 times with PBS, and non-specific binding sites were blocked with 10% goat serum in PBS for 1 hr at room

temperature. HtrA1 polyclonal antibody and biotin conjugated isolectin B4 (Vector, Burlingame, CA) diluted in PBS with 10% goat serum were applied to the sections at 4°C overnight. After washing 3 times with PBS, the retinal sections were incubated for 1 hr at room temperature with FITC conjugated goat anti mouse IgG (Jackson Immunoresearch, West Grove, PA), Texas red conjugated streptavidin (Vector, Burlingame, CA), and TO-PRO3 iodide (Molecular Probes, Carlsbad, CA). Finally, the sections were washed 3 times with PBS and mounted with Vectashield mounting media (Vector laboratories, Inc., Burlingame, CA) for microscopy. Immunolabeling was visualized using a Zeiss LSM 510 laser scanning confocal microscope (Zeiss, Thornwood, NY).

[00120] The results of the immunohistochemistry studies are shown in FIG. 6. With reference to FIG. 6(A), it was found that HtrA1 is nonuniformly expressed on drusen, the hallmark of dry AMD, and in the Bruch's membrane. In addition, as shown in FIG. 6(B), HtrA1 is not only expressed on drusen, but also on retinal blood vessels and internal limiting membrane. As is shown in FIG. 6(C), in a wet AMD donor eye, HtrA1 is expressed on choroidal vessels (white/top arrows) and in choroidal neovascularization (yellow/bottom arrows). Green channel is the staining of HtrA1 polyclonal antibody. Nuclei were counter-stained with propidium iodine (red). Scale bar shown in FIG. 6 represents a length of 20 μ m.

[00121] These studies indicate that HtrA1 expression plays a role in formation of drusen and that HtrA1 is upregulated in conditions of pathologic neovascularization, potentially accelerating the invasiveness and destructive nature of the new vessels.

EXAMPLE 4 - Generation of hybridomas for production of monoclonal antibodies

[00122] In this example, mouse hybridomas were created using standard techniques for the production of anti-HtrA1 monoclonal antibodies. More particularly, female BALB/c mice were immunized and boosted subcutaneously with 25-50 μ g of the recombinant His-tagged HtrA1 protein of Example 1 according to the schedule described in Harlow, E and Lane, D, *Antibodies: A laboratory Manual*. 1988 Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

[00123] The lymphocytes from the spleens of immunized mice were recovered and fused to myeloma Sp2/0-Ag14 cells, at 1:5 ratio using 50% PEG, to form hybridomas. The supernatant from fused cells was screened for antibody against HtrA1 by indirect ELISA using recombinant GST-tagged HtrA1. The

hybridomas that were identified as producing antibodies for HtrA1 were cloned and cultured to establish a continuous cell line with stable genetic coding.

[00124] Hybridoma cells produced as disclosed herein were implanted via injection into the peritoneal cavity of mice, and monoclonal antibodies were isolated and purified from ascites. Mice peritoneal effusions were obtained and centrifugated at 9600 rpm for 20 minutes. Supernatant pH was measured and adjusted to 6.4-7.0. To the supernatant a two-fold volume of pH4.5, 0.06mol/L sodium acetate solution and caprylic acid 3.3% were added dropwise with agitation. The solution was then centrifuged at 9600rpm for 25 minutes and the supernatant was filtered and measured for pH. To the supernatant, a ten-percent volume of 0.1mol/L PBS was added. After, isovolumic saturated ammonium sulfate was slowly added dropwise, with agitation, to the supernatant. The resulting solution was kept in 4°C overnight, then centrifuged at 11000 rpm for 20min, and the precipitate was dissolved with normal saline. Precipitation and dissolution was repeated 3 times. The solution was then dialyzed with 0.005mol/L PBS for 24 hours and centrifugated with 11000 rpm for 20 min. The supernatant was collected and stored at -20°C.

EXAMPLE 5 - ELISA to compare titers of monoclonal anti-HTRA1 antibodies

[00125] In this example, indirect ELISA was used to estimate and compare the titers of monoclonal antibodies produced and collected from a selection of hybridomas produced according to Example 4. Table 2 provides the measured titers for the HtrA1 monoclonal antibodies produced by the hybridoma cell lines listed therein. The titers provided in Table 2 represent the threshold dilutions at which the monoclonal antibodies produced by the specified hybridomas still provide an optical density in the well that was twice the optical density observed in the control, the control being provided by wells loaded with dilution buffer 1XPBS at pH7.4, 0.05% Tween 20, and 0.1% BSA.

Table 2

CLONE	ELISA TITER
8F12	1:1280000
7E12	1:40000
3E10	1:20000
13D4	1:320000
10E7	1:160000
9F7	1:160000
8G8	1:320000

4H5	1:160000
14A8	1:102400
7G6	1:409600
25A6	1:409600
21C9	1:1280000
13E7	1:102400
24C4	1:102400
3E7	1:102400
14E6	1:819200
3E8	1:1280000
5B12	1:1280000
8F11	1:1280000

[00126] To carry out the ELISA, the wells of a PVC microtiter plate (Corning, Corning, NY) were coated with 100 μ l of 3 μ g/ml recombinant HtrA1 protein diluted in 50 mM carbonate/bicarbonate buffer (pH 9.6) at 4°C overnight. After washing 4 times with 0.05% Tween 20/PBS, the wells were blocked with 10% goat serum in PBS at room temperature for 2 hrs. After blocking, 100 μ l of a 1 mg/ml solution of each the HtrA1 monoclonal antibodies at dilutions ranging from 1:10,000 to 1:1,280,000 in PBS were added with 10% goat serum to each well in triplicate, and incubated at room temperature for 1 hr. After incubation, the plate was washed 4 times, and then each well was incubated with 100 μ l of 1:1000 dilution of horseradish peroxidase (HRP) conjugated goat anti-mouse IgG antibody (Santa Cruz, Santa Cruz, CA) for 1 hr at room temperature. The wells were then washed 6 times and developed with 100 μ l TMB substrate (BIO-RAD, Hercules, CA) for 10 min at 37° C and stopped with 100 μ l of 2 M H₂SO₄. The absorbance (*i.e.*, optical density) in each of the wells was read at 450nm by a Benchmark Plus microplate reader (BIO-RAD, Hercules, CA).

EXAMPLE 6 - *HtrA1 polyclonal antibodies inhibit neovascularization in a mouse model of oxygen-induced retinopathy*

[00127] Intraocular injections of polyclonal HtrA1 antibodies significantly reduced pathologic neovascularization in an animal model of oxygen-induced retinopathy (OIR). More particularly, in a mouse model of OIR, administration of polyclonal HtrA1 antibody resulted in reduced neovascularization when compared to a contralateral control receiving a negative control pre-immune serum. The polyclonal antibody was delivered in solution by intraocular injection, wherein the concentration of antibody was 1 mg/ml and the dose volume delivered was 1 μ l.

[00128] Oxygen-induced retinopathy was induced in mice as described in Smith et al., Invest Ophthalmol Vis Sci, 35(1): 101-11. More specifically, seven day postpartem (P7) pups along with nursing mothers were placed in 75% oxygen, maintained by a Pro-OX oxygen controller (BioSpherix, Redfield, NY). The pups were removed on P12 and given a 1µl intraocular injection of HtrA1 polyclonal antibody in one eye and a 1µl intraocular injection of the pre-immune serum in the contralateral eye. Mice were sacrificed on P17 and perfused via the left ventricle with 1 ml 50 mg/ml FITC-Dextran (Sigma, St. Louis, MO). The mice eyes were enucleated, fixed for 30 minutes in 4% paraformaldehyde, and retinal flat mounts were prepared. The blood vessels in the flat mounts were visualized with either FITC Dextran or lectin staining. The flat mounts were analyzed by Axiovert 200 fluorescence microscopy (Carl Zeiss, Thornwood, NY). Neovascularization was quantified using AxioVision software (Carl Zeiss, Thornwood, NY).

[00129] FIG. 7 shows a retinal flat mount of an eye injected with pre-immune serum as a negative control (A), a retinal flat mount of a contralateral eye injected with HtrA1 polyclonal antibody (B), and the results of a paired t test (C) showing that the area of retinal neovascularization in eyes injected with HtrA1 polyclonal antibody was significantly less than the area of neovascularization seen in contralateral eyes injected with the negative control.

EXAMPLE 7 - *HtrA1 monoclonal antibodies inhibit neovascularization in a mouse model of oxygen-induced retinopathy*

[00130] The OIR model described in Example 6 was repeated. In this instance however, two monoclonal antibodies were administered. The monoclonal antibodies administered were 8F12 HtrA1 monoclonal antibodies and 9F7 monoclonal HtrA1 antibodies. The 8F12 and 9F7 monoclonal antibodies were those produced by the 8F12 and 9F7 hybridoma lines generated according to Example 4 and subject to ELISA analysis according to Example 5. The 8F12 and 9F7 monoclonal antibodies were each delivered by intraocular injection at a dose volume of 1µl where the concentration of antibody was 1 mg/ml.

[00131] FIG. 8 shows a retinal flat mount of an eye injected with 8F12 monoclonal antibody (A), a retinal flat mount of a contralateral eye injected with 9F7 monoclonal antibody (B), and the results of a paired t test (C) showing that the area of retinal neovascularization in eyes injected with 8F12 monoclonal antibody is significantly less than the area of neovascularization seen in contralateral eyes injected with 9F7

monoclonal antibody. These results indicate that monoclonal antibodies exhibiting relatively higher titers as evaluated according to Example 5, may provide relatively higher reduction in retinal neovascularization.

EXAMPLE 8 - *HtrA1 polyclonal antibodies inhibit laser-induced choroidal neovascularization*

[00132] A mouse method for assessing choroidal neovascularization (CNV) was developed. Adult mice (2-3 months old) were subjected to laser-induced disruption of Bruch's. Before doing so, a general anesthetic was introduced in the mice via intraperitoneal injection of a mixture of ketamine hydrochloride and xylazine hydrochloride. Pupils were dilated with 1% tropicamide for photocoagulation. An Iridex OcuLight GL 532 nm laser photocoagulator (Iridex, Mountain View, CA) with slit lamp delivery system was used to disrupt Bruch's membrane at 3 spots at posterior pole of retina with the following parameters: 150mW power, 75um spot size, and 0.1 seconds duration. Production of a bubble at the time of laser treatment, which indicates rupture of Bruch's membrane, was an important factor in obtaining CNV, so only burns in which a bubble produced were included in the study. Immediately after laser treatment and 3 days later, the mice received an intraocular injection of 1µl of a 1 mg/ml solution of the HtrA1 polyclonal antibody in one eye and an intraocular injection of 1µl of a negative control pre-immune serum in the contralateral eye. One week later, the mice were sacrificed and choroidal flat mounts were prepared after fixation. Biotin conjugated isolectin (Sigma, St. Louis, MO) and Texas red conjugated streptavidin (Sigma, St. Louis, MO) were used to stain blood vessels. The flat mounts prepared in this manner were examined by Zeiss LSM 510 confocal microscope (Zeiss, Thornwood, NY) and the area of CNV was measured and quantified by ImageJ (NIH, Bethesda, MD) software.

[00133] FIG. 9 shows a choroidal flat mount of an eye injected with pre-immune serum as a negative control (A), a choroidal flat mount of a contralateral eye injected with HtrA1 polyclonal antibody (B), and the results of a paired t test (C) showing that the area of choroidal neovascularization in eyes injected with HtrA1 polyclonal antibody is significantly less than the area of neovascularization seen in eyes injected with the negative control.

EXAMPLE 9 - *HtrA1 monoclonal antibodies inhibit laser-induced choroidal neovascularization*

[00134] The CNV model described in Example 8 was repeated. In this instance however, two monoclonal antibodies were administered. The monoclonal antibodies administered were 8F12 HtrA1 monoclonal antibodies and 9F7 monoclonal HtrA1 antibodies. The 8F12 and 9F7 monoclonal antibodies were those produced by the 8F12 and 9F7 hybridoma lines generated according to Example 4 and subject to ELISA analysis according to Example 5. The 8F12 and 9F7 monoclonal antibodies were each delivered by intraocular injection at a dose volume of 1 μ l where the concentration of antibody was 1 mg/ml.

[00135] FIG. 10 shows a choroidal flat mount of an eye injected with 9F7 monoclonal antibody (A), a choroidal flat mount of a contralateral eye injected with 8F12 monoclonal antibody (B), and the results of a paired t test (C) showing that the area of choroidal neovascularization in eyes injected with 8F12 monoclonal antibody is significantly less than the area of neovascularization seen in contralateral eyes injected with 9F7 monoclonal antibody. These results indicate that monoclonal antibodies exhibiting relatively higher titers as evaluated according to Example 5, provide relatively higher reduction in choroidal neovascularization.

[00136] The results provided by the OIR and CNV studies detailed in Examples 6-10 herein indicate that HtrA1 antibodies as provided by the present description are biologically active and work to directly or indirectly inhibit or block one or more activity of HtrA1 and, thereby, work to inhibit or block pathologic neovascularization.

EXAMPLE 10 - *Immunocytochemistry analysis of mammalian expressed HtrA1 by monoclonal antibodies*

[00137] Mammalian HtrA1 was exposed to anti-HtrA1 antibodies and analyzed by immunocytochemistry. More specifically, HEK293 cells were seeded onto poly-L-lysine coated four chamber glass slides in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 μ g/ml of streptomycin. The cells were transfected with HTRA1-pcDNA 3.1 recombinant plasmids using fugen 6 (Roche) according to the manufacturer's protocol. Transfected cells were washed twice with phosphate buffer saline (PBS, pH 7.5), and fixed in methanol:acetone (50:50, V/V) for 5 min at 20°C. The fixed cells were washed 2-3 times for 5 min each with PBS, and once with 0.1% triton X-100 in PBS for 5 min. The fixed cells were washed again 2-3 time for 5 min each

with PBS and the slides blocked with 5-10% goat serum for 30 min at room temperature. After incubation with HtrA1 antibody at 1:100 dilution in PBS at room temperature for 2 hrs, the cells were washed with PBS 2-3 times for 5 min each. The slides were exposed to secondary antibody conjugated with FITC for 1 hour at room temperature and then washed with PBS 2-3 times for 5 min each. The slides were mounted with vectashield mounting media (Vector laboratories, Inc., Burlingame, CA) for microscopy.

EXAMPLE 11 - *Western blot analysis of HTRA1 monoclonal antibodies*

[00138] Retinal pigment epithelium (RPE) samples were homogenized according to the method of Laemmli. Approximately 7 ug of protein lysate were resolved with 9% SDS PAGE and transferred to a PVDF membrane (Millipore, Billerica, MA). The non-specific binding sites on the blot were blocked for 2 h at room temperature with 5% skim milk in NaCl/Tris buffer containing 0.05% Tween 20 (TTBS). The membrane was incubated overnight at 4°C with anti-HtrA1 antibody at 1:2000 dilution in TTBS containing 5% milk, and then washed three times with TTBS for 5 min each time. Anti mouse antibody conjugated with HRP (Amersham Biosciences, NJ), 1:4000 dilution in TTBS, was applied to the membrane for 1 hour at room temperature, and the membrane was then washed twice for 5 min each time with TTBS, and once for 5 min with NaCl/Tris buffer. The membrane was developed with an ECL (Amersham Bioscience, NJ) detection kit.

Claims:

1. An antibody to HtrA1, wherein the antibody exhibits a binding affinity to HtrA1 selected from one of 10^5 M^{-1} , a range of 10^6 M^{-1} to 10^7 M^{-1} , a range of 10^8 M^{-1} to 10^{12} M^{-1} , and a range of 10^9 M^{-1} to 10^{12} M^{-1} .
2. The antibody of claim 1, wherein the antibody is a selected from a polyclonal, monoclonal, humanized, human, mouse, and affinity matured antibody.
3. The antibody of claim 2, wherein the immunizing agent used to generate the antibody includes an agent selected from an HtrA1 fragment according to SEQ. ID. NO.: 1, a full-length HtrA1 polypeptide according to SEQ. ID. NO.: 2, or variants thereof.
4. The antibody of claim 3, wherein the antibody exhibits a titer of 1:160,000 or greater.
5. The antibody of claim 3, wherein the antibody exhibits a titer of selected from a titer of 1:200,000 or greater, 1:300,00 or greater, 1:400,000 or greater, 1:500,000 or greater, 1:600,000 or greater, 1:700,000 or greater, 1:800,000 or greater, 1:900,000 or greater, and 1:1,000,000 or greater.
6. The antibody of claim 3, wherein the antibody exhibits a titer selected from a titer ranging from 1:200,000 to greater than 1:1,000,000, 1:300,000 to greater than 1:1,000,000, 1:400,000 to greater than 1:1,000,000, 1:500,000 to greater than 1:1,000,000, 1:600,000 to greater than 1:1,000,000, 1:700,000 to greater than 1:1,000,000, 1:800,000 to greater than 1:1,000,000, and 1:900,000 to greater than 1:1,000,000.
7. The antibody of claim 3, wherein the antibody exhibits a titer selected from a titer ranging from 1:200,000 to 1:300,000, 1:200,000 to 1:400,000, 1:200,000 to 1:500,000, 1:200,000 to 1:800,000, 1:200,000 to 1:900,000, and 1:200,000 to 1:1,000,000.
8. The antibody of claim 3, wherein the antibody exhibits a titer selected from a titer ranging from 1:200,000 to 1:400,000, 1:200,000 to 1:500,000, 1:400,000 to 1:800,000, 1:400,000 to 1:900,000, 1:400,000 to greater than 1:1,000,000, and 1:800,000 to greater than 1:1,000,000.
9. An antibody to HtrA1, wherein the antibody inhibits neovascularization selected from HtrA1-induced neovascularization and ischemia-induced neovascularization.

10. The antibody of claim 9, wherein the antibody exhibits a binding affinity to HtrA1 selected from one of 10^5 M^{-1} , a range of 10^6 M^{-1} to 10^7 M^{-1} , a range of 10^8 M^{-1} to 10^{12} M^{-1} , and a range of 10^9 M^{-1} to 10^{12} M^{-1} .

11. The antibody of claim 10, wherein the antibody is selected from a polyclonal, monoclonal, humanized, human, mouse, and affinity matured antibody.

12. The antibody of claim 11, wherein the immunizing agent used to generate the antibody includes an agent selected from an HtrA1 fragment according to SEQ. ID. NO.: 1, a full-length HtrA1 polypeptide according to SEQ. ID. NO.: 2, or variants thereof.

13. The antibody of claim 12, wherein the antibody exhibits a titer of 1:160,000 or greater.

14.. The antibody of claim 12, wherein the antibody exhibits a titer of selected from a titer of 1:200,000 or greater, 1:300,00 or greater, 1:400,000 or greater, 1:500,000 or greater, 1:600,000 or greater, 1:700,000 or greater, 1:800,000 or greater, 1:900,000 or greater, and 1:1,000,000 or greater.

15. The antibody of claim 12, wherein the antibody exhibits a titer selected from a titer ranging from 1:200,000 to greater than 1:1,000,000, 1:300,000 to greater than 1:1,000,000, 1:400,000 to greater than 1:1,000,000, 1:500,000 to greater than 1:1,000,000, 1:600,000 to greater than 1:1,000,000, 1:700,000 to greater than 1:1,000,000, 1:800,000 to greater than 1:1,000,000, and 1:900,000 to greater than 1:1,000,000.

16. The antibody of claim 12, wherein the antibody exhibits a titer selected from a titer ranging from 1:200,000 to 1:300,000, 1:200,000 to 1:400,000, 1:200,000 to 1:500,000, 1:200,000 to 1:800,000, 1:200,000 to 1:900,000, and 1:200,000 to 1:1,000,000.

17. The antibody of claim 12, wherein the antibody exhibits a titer selected from a titer ranging from 1:200,000 to 1:400,000, 1:200,000 to 1:500,000, 1:400,000 to 1:800,000, 1:400,000 to 1:900,000, 1:400,000 to greater than 1:1,000,000, and 1:800,000 to greater than 1:1,000,000.

18. An antibody to HtrA1, wherein the antibody inhibits neovascularization in an animal model selected from an animal model of oxygen-induced retinopathy and an animal model of laser-induced choroidal neovascularization.

19. The antibody of claim 18, wherein the antibody exhibits a binding affinity to HtrA1 selected from one of 10^5 M^{-1} , a range of 10^6 M^{-1} to 10^7 M^{-1} , a range of 10^8 M^{-1} to 10^{12} M^{-1} , and a range of 10^9 M^{-1} to 10^{12} M^{-1} .

20. The antibody of claim 18, wherein the immunizing agent used to generate the antibody includes an agent selected from an HtrA1 fragment according to SEQ. ID. NO.: 1, a full-length HtrA1 polypeptide according to SEQ. ID. NO.: 2, or variants thereof.

21. The antibody of claim 20, wherein the model is an animal model of laser-induced choroidal neovascularization and the antibody provides a reduction in neovascularization selected from a reduction of at least about 30%, at least about 40%, at least about 50%, at least about 60%, and at least about 70% or greater relative to a control.

22. The antibody of claim 20, wherein the model is an animal model of oxygen-induced retinopathy and the antibody provides a reduction in neovascularization selected from a reduction of at least about 30%, at least about 40%, at least about 50%, at least about 60%, and at least about 70% or greater relative to a control.

23. The antibody of claim 20, wherein the antibody exhibits a titer of selected from a titer of 1:160,000 or greater, 1:200,000 or greater, 1:300,000 or greater, 1:400,000 or greater, 1:500,000 or greater, 1:600,000 or greater, 1:700,000 or greater, 1:800,000 or greater, 1:900,000 or greater, and 1:1,000,000 or greater.

24. The antibody of claim 20, wherein the antibody exhibits a titer selected from a titer ranging from 1:200,000 to greater than 1:1,000,000, 1:300,000 to greater than 1:1,000,000, 1:400,000 to greater than 1:1,000,000, 1:500,000 to greater than 1:1,000,000, 1:600,000 to greater than 1:1,000,000, 1:700,000 to greater than 1:1,000,000, 1:800,000 to greater than 1:1,000,000, and 1:900,000 to greater than 1:1,000,000.

25. The antibody of claim 20, wherein the antibody exhibits a titer selected from a titer ranging from 1:200,000 to 1:300,000, 1:200,000 to 1:400,000, 1:200,000 to 1:500,000, 1:200,000 to 1:800,000, 1:200,000 to 1:900,000, and 1:200,000 to 1:1,000,000.

26. The antibody of claim 20, wherein the antibody exhibits a titer selected from a titer ranging from 1:200,000 to 1:400,000, 1:200,000 to 1:500,000, 1:400,000

to 1:800,000, 1:400,000 to 1:900,000, 1:400,000 to greater than 1:1,000,000, and 1:800,000 to greater than 1:1,000,000.

27. A method of treating pathologic neovascularization in a subject, the method comprising:

administering a therapeutically effective amount of an antibody to HtrA1 to the subject, wherein the immunizing agent used to generate the antibody includes an agent selected from an HtrA1 fragment according to SEQ. ID. NO.: 1, a full-length HtrA1 polypeptide according to SEQ. ID. NO.: 2, or variants thereof.

28. The method of claim 27, wherein administering the antibody of HtrA1 comprises administering an antibody selected from a polyclonal, monoclonal, humanized, human, mouse, and affinity matured antibody.

29. The method of claim 27, wherein said pathologic neovascularization is ischemia-induced neovascularization.

30. The method of claim 27, wherein said pathologic neovascularization is associated with a disease selected from AMD, DR, and ROP.

31. The method of claim 27, wherein said pathologic neovascularization is ischemia-induced neovascularization resulting from injury.

32. The method of claim 27, wherein macular edema or vascular leak is associated with said pathologic neovascularization.

33. The method of claim 27, wherein administering the antibody of HtrA1 comprises administering an antibody to HtrA1 that exhibits a binding affinity to HtrA1 selected from one of 10^5 M^{-1} , a range of 10^6 M^{-1} to 10^7 M^{-1} , a range of 10^8 M^{-1} to 10^{12} M^{-1} , and a range of 10^9 M^{-1} to 10^{12} M^{-1} .

34. The method of claim 27, wherein administering the antibody of HtrA1 comprises administering an antibody to HtrA1 that exhibits a titer of 1:160,000 or greater.

35. The method of claim 27, wherein administering the antibody of HtrA1 comprises administering an antibody to HtrA1 that exhibits a titer of selected from a titer of 1:200,000 or greater, 1:300,000 or greater, 1:400,000 or greater, 1:500,000 or greater, 1:600,000 or greater, 1:700,000 or greater, 1:800,000 or greater, 1:900,000 or greater, and 1:1,000,000 or greater.

36. The method of claim 27, wherein administering the antibody of HtrA1 comprises administering an antibody to HtrA1 that exhibits a titer selected from a titer ranging from 1:200,000 to greater than 1:1,000,000, 1:300,000 to greater than

1:1,000,000, 1:400,000 to greater than 1:1,000,000, 1:500,000 to greater than 1:1,000,000, 1:600,000 to greater than 1:1,000,000, 1:700,000 to greater than 1:1,000,000, 1:800,000 to greater than 1:1,000,000, and 1:900,000 to greater than 1:1,000,000.

37. The method of claim 27, wherein administering the antibody of HtrA1 comprises administering an antibody to HtrA1 that exhibits a titer selected from a titer ranging from 1:200,000 to 1:300,000, 1:200,000 to 1:400,000, 1:200,000 to 1:500,000, 1:200,000 to 1:800,000, 1:200,000 to 1:900,000, and 1:200,000 to 1:1,000,000.

38. The method of claim 27, wherein administering the antibody of HtrA1 comprises administering an antibody to HtrA1 that exhibits a titer selected from a titer ranging from 1:200,000 to 1:400,000, 1:200,000 to 1:500,000, 1:400,000 to 1:800,000, 1:400,000 to 1:900,000, 1:400,000 to greater than 1:1,000,000, and 1:800,000 to greater than 1:1,000,000.

39. A method of treating a ischemia-induced neovascularization in a subject, the method comprising:

administering a therapeutically effective amount of an antibody to HtrA1 to the subject, wherein the immunizing agent used to generate the antibody includes an agent selected from an HtrA1 fragment according to SEQ. ID. NO.: 1, a full-length HtrA1 polypeptide according to SEQ. ID. NO.: 2, or variants thereof.

40. The method of claim 39, wherein administering the antibody of HtrA1 comprises administering an antibody selected from a polyclonal, monoclonal, humanized, human, mouse, and affinity matured antibody.

41. The method of claim 39, wherein said ischemia-induced neovascularization results from injury.

42. The method of claim 39, wherein said ischemia-induced neovascularization is associated with a disease selected from AMD, DR, and ROP.

43. The method of claim 39, wherein macular edema is associated with said ischemia-induced neovascularization.

44. The method of claim 39, wherein administering the antibody of HtrA1 comprises administering an antibody to HtrA1 that exhibits a binding affinity to HtrA1 selected from one of 10^5 M^{-1} , a range of 10^6 M^{-1} to 10^7 M^{-1} , a range of 10^8 M^{-1} to 10^{12} M^{-1} , and a range of 10^9 M^{-1} to 10^{12} M^{-1} .

45. The method of claim 39, wherein administering the antibody of HtrA1 comprises administering an antibody to HtrA1 that exhibits a titer of 1:160,000 or greater.

46. The method of claim 39, wherein administering the antibody of HtrA1 comprises administering an antibody to HtrA1 that exhibits a titer of selected from a titer of 1:200,000 or greater, 1:300,00 or greater, 1:400,000 or greater, 1:500,000 or greater, 1:600,000 or greater, 1:700,000 or greater, 1:800,000 or greater, 1:900,000 or greater, and 1:1,000,000 or greater.

47. The method of claim 39, wherein administering the antibody of HtrA1 comprises administering an antibody to HtrA1 that exhibits a titer selected from a titer ranging from 1:200,000 to greater than 1:1,000,000, 1:300,000 to greater than 1:1,000,000, 1:400,000 to greater than 1:1,000,000, 1:500,000 to greater than 1:1,000,000, 1:600,000 to greater than 1:1,000,000, 1:700,000 to greater than 1:1,000,000, 1:800,000 to greater than 1:1,000,000, and 1:900,000 to greater than 1:1,000,000.

48. The method of claim 39, wherein administering the antibody of HtrA1 comprises administering an antibody to HtrA1 that exhibits a titer selected from a titer ranging from 1:200,000 to 1:300,000, 1:200,000 to 1:400,000, 1:200,000 to 1:500,000, 1:200,000 to 1:800,000, 1:200,000 to 1:900,000, and 1:200,000 to 1:1,000,000.

49. The method of claim 39, wherein administering the antibody of HtrA1 comprises administering an antibody to HtrA1 that exhibits a titer selected from a titer ranging from 1:200,000 to 1:400,000, 1:200,000 to 1:500,000, 1:400,000 to 1:800,000, 1:400,000 to 1:900,000, 1:400,000 to greater than 1:1,000,000, and 1:800,000 to greater than 1:1,000,000.

50. A method of treating HtrA1-induced neovascularization in a subject, the method comprising:

administering a therapeutically effective amount of an antibody to HtrA1 to the subject, wherein the immunizing agent used to generate the antibody includes an agent selected from an HtrA1 fragment according to SEQ. ID. NO.: 1, a full-length HtrA1 polypeptide according to SEQ. ID. NO.: 2, or variants thereof.

51. The method of claim 50, wherein administering the antibody of HtrA1 comprises administering an antibody selected from a polyclonal, monoclonal, humanized, human, mouse, and affinity matured antibody.

52. The method of claim 50, wherein said HtrA1-induced neovascularization is associated with ischemia.

53. The method of claim 52, wherein said HtrA1-induced neovascularization is present in ocular tissue.

54. The method of claim 50, wherein said HtrA1-induced neovascularization is associated with a disease selected from AMD, DR, and ROP.

55. The method of claim 50, wherein macular edema or vascular leak is associated with said HtrA1-induced neovascularization.

56. The method of claim 50, wherein administering the antibody of HtrA1 comprises administering an antibody to HtrA1 that exhibits a binding affinity to HtrA1 selected from one of 10^5 M^{-1} , a range of 10^6 M^{-1} to 10^7 M^{-1} , a range of 10^8 M^{-1} to 10^{12} M^{-1} , and a range of 10^9 M^{-1} to 10^{12} M^{-1} .

57. The method of claim 50, wherein administering the antibody of HtrA1 comprises administering an antibody to HtrA1 that exhibits a titer of 1:160,000 or greater.

58. The method of claim 50, wherein administering the antibody of HtrA1 comprises administering an antibody to HtrA1 that exhibits a titer of selected from a titer of 1:200,000 or greater, 1:300,00 or greater, 1:400,000 or greater, 1:500,000 or greater, 1:600,000 or greater, 1:700,000 or greater, 1:800,000 or greater, 1:900,000 or greater, and 1:1,000,000 or greater.

59. The method of claim 50, wherein administering the antibody of HtrA1 comprises administering an antibody to HtrA1 that exhibits a titer selected from a titer ranging from 1:200,000 to greater than 1:1,000,000, 1:300,000 to greater than 1:1,000,000, 1:400,000 to greater than 1:1,000,000, 1:500,000 to greater than 1:1,000,000, 1:600,000 to greater than 1:1,000,000, 1:700,000 to greater than 1:1,000,000, 1:800,000 to greater than 1:1,000,000, and 1:900,000 to greater than 1:1,000,000.

60. The method of claim 50, wherein administering the antibody of HtrA1 comprises administering an antibody to HtrA1 that exhibits a titer selected from a titer ranging from 1:200,000 to 1:300,000, 1:200,000 to 1:400,000, 1:200,000 to

1:500,000, 1:200,000 to 1:800,000, 1:200,000 to 1:900,000, and 1:200,000 to 1:1,000,000.

61. The method of claim 50, wherein administering the antibody of HtrA1 comprises administering an antibody to HtrA1 that exhibits a titer selected from a titer ranging from 1:200,000 to 1:400,000, 1:200,000 to 1:500,000, 1:400,000 to 1:800,000, 1:400,000 to 1:900,000, 1:400,000 to greater than 1:1,000,000, and 1:800,000 to greater than 1:1,000,000.

62. A method of treating a subject suffering a disease selected from AMD in its wet form, AMD in its dry form, DR, and ROP, the method comprising:

administering a therapeutically effective amount of an antibody to HtrA1 to the subject, wherein the immunizing agent used to generate the antibody includes an agent selected from an HtrA1 fragment according to SEQ. ID. NO.: 1, a full-length HtrA1 polypeptide according to SEQ. ID. NO.: 2, or variants thereof.

63. The method of claim 62, wherein administering the antibody of HtrA1 comprises administering an antibody selected from a polyclonal, monoclonal, humanized, human, mouse, and affinity matured antibody.

64. The method of claim 62, wherein macular edema is associated with said disease.

65. The method of claim 62, wherein administering the antibody of HtrA1 comprises administering an antibody to HtrA1 that exhibits a binding affinity to HtrA1 selected from one of 10^5 M^{-1} , a range of 10^6 M^{-1} to 10^7 M^{-1} , a range of 10^8 M^{-1} to 10^{12} M^{-1} , and a range of 10^9 M^{-1} to 10^{12} M^{-1} .

66. The method of claim 62, wherein administering the antibody of HtrA1 comprises administering an antibody to HtrA1 that exhibits a titer of 1:160,000 or greater.

67. The method of claim 62, wherein administering the antibody of HtrA1 comprises administering an antibody to HtrA1 that exhibits a titer of selected from a titer of 1:200,000 or greater, 1:300,00 or greater, 1:400,000 or greater, 1:500,000 or greater, 1:600,000 or greater, 1:700,000 or greater, 1:800,000 or greater, 1:900,000 or greater, and 1:1,000,000 or greater.

68. The method of claim 62, wherein administering the antibody of HtrA1 comprises administering an antibody to HtrA1 that exhibits a titer selected from a titer ranging from 1:200,000 to greater than 1:1,000,000, 1:300,000 to greater than 1:1,000,000, 1:400,000 to greater than 1:1,000,000, 1:500,000 to greater than

1:1,000,000, 1:600,000 to greater than 1:1,000,000, 1:700,000 to greater than 1:1,000,000, 1:800,000 to greater than 1:1,000,000, and 1:900,000 to greater than 1:1,000,000.

69. The method of claim 62, wherein administering the antibody of HtrA1 comprises administering an antibody to HtrA1 that exhibits a titer selected from a titer ranging from 1:200,000 to 1:300,000, 1:200,000 to 1:400,000, 1:200,000 to 1:500,000, 1:200,000 to 1:800,000, 1:200,000 to 1:900,000, and 1:200,000 to 1:1,000,000.

70. The method of claim 62, wherein administering the antibody of HtrA1 comprises administering an antibody to HtrA1 that exhibits a titer selected from a titer ranging from 1:200,000 to 1:400,000, 1:200,000 to 1:500,000, 1:400,000 to 1:800,000, 1:400,000 to 1:900,000, 1:400,000 to greater than 1:1,000,000, and 1:800,000 to greater than 1:1,000,000.

71. A pharmaceutical formulation for the treatment of neovascularization selected from HtrA1-induced neovascularization and ischemia-induced neovascularization, the formulation comprising:

an antibody to HtrA1, wherein the immunizing agent used to generate the antibody includes an agent selected from an HtrA1 fragment according to SEQ. ID. NO.: 1, a full-length HtrA1 polypeptide according to SEQ. ID. NO.: 2, or variants thereof; and

a pharmaceutically acceptable carrier.

72. The pharmaceutical formulation of claim 71, further comprising at least one additional formulation constituent selected from a diluent, a tonicity modifier, and a buffer.

73. The pharmaceutical formulation of claim 71, wherein the antibody exhibits a binding affinity to HtrA1 selected from one of 10^5 M^{-1} , a range of 10^6 M^{-1} to 10^7 M^{-1} , a range of 10^8 M^{-1} to 10^{12} M^{-1} , and a range of 10^9 M^{-1} to 10^{12} M^{-1} .

74. The pharmaceutical formulation of claim 71, wherein the antibody is a selected from a polyclonal, monoclonal, humanized, human, mouse, and affinity matured antibody.

75. The pharmaceutical formulation of claim 71, wherein the antibody exhibits a titer of 1:160,000 or greater.

76.. The pharmaceutical formulation of claim 71, wherein the antibody exhibits a titer selected from a titer of 1:200,000 or greater, 1:300,00 or greater, 1:400,000 or greater, 1:500,000 or greater, 1:600,000 or greater, 1:700,000 or greater, 1:800,000 or greater, 1:900,000 or greater, and 1:1,000,000 or greater.

77. The pharmaceutical formulation of claim 71, wherein the antibody exhibits a titer selected from a titer ranging from 1:200,000 to greater than 1:1,000,000, 1:300,000 to greater than 1:1,000,000, 1:400,000 to greater than 1:1,000,000, 1:500,000 to greater than 1:1,000,000, 1:600,000 to greater than 1:1,000,000, 1:700,000 to greater than 1:1,000,000, 1:800,000 to greater than 1:1,000,000, and 1:900,000 to greater than 1:1,000,000.

78. The pharmaceutical formulation of claim 71, wherein the antibody exhibits a titer selected from a titer ranging from 1:200,000 to 1:300,000, 1:200,000 to 1:400,000, 1:200,000 to 1:500,000, 1:200,000 to 1:800,000, 1:200,000 to 1:900,000, and 1:200,000 to 1:1,000,000.

79. The pharmaceutical formulation of claim 71, wherein the antibody exhibits a titer selected from a titer ranging from 1:200,000 to 1:400,000, 1:200,000 to 1:500,000, 1:400,000 to 1:800,000, 1:400,000 to 1:900,000, 1:400,000 to greater than 1:1,000,000, and 1:800,000 to greater than 1:1,000,000.

80 A pharmaceutical formulation for the treatment of a disease selected from AMD in its wet form, AMD in its dry form, DR and ROP, the formulation comprising:

an antibody to HtrA1, wherein the immunizing agent used to generate the antibody includes an agent selected from an HtrA1 fragment according to SEQ. ID. NO.: 1, a full-length HtrA1 polypeptide according to SEQ. ID. NO.: 2, or variants thereof; and

a pharmaceutically acceptable carrier.

81. The pharmaceutical formulation of claim 80, further comprising at least one additional formulation constituent selected from a diluent, a tonicity modifier, and a buffer.

82. The pharmaceutical formulation of claim 80, wherein the antibody exhibits a binding affinity to HtrA1 selected from one of 10^5 M^{-1} , a range of 10^6 M^{-1} to 10^7 M^{-1} , a range of 10^8 M^{-1} to 10^{12} M^{-1} , and a range of 10^9 M^{-1} to 10^{12} M^{-1} .

83. The pharmaceutical formulation of claim 80, wherein the antibody is a selected from a polyclonal, monoclonal, humanized, human, mouse, and affinity matured antibody.

84. The pharmaceutical formulation of claim 80, wherein the antibody exhibits a titer of 1:160,000 or greater.

85.. The pharmaceutical formulation of claim 80, wherein the antibody exhibits a titer of selected from a titer of 1:200,000 or greater, 1:300,00 or greater, 1:400,000 or greater, 1:500,000 or greater, 1:600,000 or greater, 1:700,000 or greater, 1:800,000 or greater, 1:900,000 or greater, and 1:1,000,000 or greater.

86. The pharmaceutical formulation of claim 80, wherein the antibody exhibits a titer selected from a titer ranging from 1:200,000 to greater than 1:1,000,000, 1:300,000 to greater than 1:1,000,000, 1:400,000 to greater than 1:1,000,000, 1:500,000 to greater than 1:1,000,000, 1:600,000 to greater than 1:1,000,000, 1:700,000 to greater than 1:1,000,000, 1:800,000 to greater than 1:1,000,000, and 1:900,000 to greater than 1:1,000,000.

87. The pharmaceutical formulation of claim 80, wherein the antibody exhibits a titer selected from a titer ranging from 1:200,000 to 1:300,000, 1:200,000 to 1:400,000, 1:200,000 to 1:500,000, 1:200,000 to 1:800,000, 1:200,000 to 1:900,000, and 1:200,000 to 1:1,000,000.

88. The pharmaceutical formulation of claim 80, wherein the antibody exhibits a titer selected from a titer ranging from 1:200,000 to 1:400,000, 1:200,000 to 1:500,000, 1:400,000 to 1:800,000, 1:400,000 to 1:900,000, 1:400,000 to greater than 1:1,000,000, and 1:800,000 to greater than 1:1,000,000.

89. A method of treating pathologic neovascularization in a subject, the method comprising:

inhibiting at least one function of HtrA1 in said subject.

90. The method of claim 89, wherein inhibiting at least one function of HtrA1 comprises administering a therapeutically effective amount of an agent that inhibits at least one function of HtrA1.

91. The method of claim 90, wherein administering a therapeutically effective amount of an agent comprises administering a therapeutically effective amount of an antibody to HtrA1.

92. A method of treating a disease selected from AMD in its wet form, AMD in its dry form, DR, and ROP in a subject, the method comprising:

inhibiting at least one function of HtrA1 in said subject.

93. The method of claim 92, wherein inhibiting at least one function of HtrA1 comprises administering a therapeutically effective amount of an agent that inhibits at least one function of HtrA1.

94. The method of claim 93, wherein administering a therapeutically effective amount of an agent comprises administering a therapeutically effective amount of an antibody to HtrA1.

HU-FULL	1	MQIPRAALLPLLLLLLAAPASAQLSRAGRSAPLAAGCPDRCEPARCPPQP	50
HU-142aa	1		0
HU-FULL	51	EHCEGGRARDACGCCEVCGAPEGAACGLQEGPCGEGLCQCVVPFGVPASAT	100
HU-142aa	1		0
HU-FULL	101	VERRAQAGLCVCASSEPVCGSDANTYANLCQLRAASRRSERLHRPPVIVL	150
HU-142aa	1	 LHRPPVIVL	9
HU-FULL	151	QRGACGQGQEDPNSLRHKYNFIADVVEKIAPAVVHIELFRKLPFSKREVP	200
HU-142aa	10	 QRGACGQGQEDPNSLRHKYNFIADVVEKIAPAVVHIELFRKLPFSKREVP	59
HU-FULL	201	VASGSGFIVSEDGLIVTNAHVVTNKHVRVKVELKNGATYEAKIKDVDEKAD	250
HU-142aa	60	 VASGSGFIVSEDGLIVTNAHVVTNKHVRVKVELKNGATYEAKIKDVDEKAD	109
HU-FULL	251	IALIKIDHQGKLPVLLLGRSSELRPGEFVVAIGSPFSLQNTVTGTGIVSTT	300
HU-142aa	110	 IALIKIDHQGKLPVLLLGRSSELRPGEFVVAIGSPFSLQNTVTGTGIVSTT	159
HU-FULL	301	QRGGKELGLRNSDMDYIQTDAIINYGNSSGGLVNLDEGIVIGINTLKVTAG	350
HU-142aa	160	 QRGGKELGLRNSDMDYIQTDAIINYGNSSGGLVNLDEGIVIGINTLKVTAG	209
HU-FULL	351	ISFAIPSDKIKKFLTESHDRQAKGKAITKKKYIGIRMMSLTSSKAKELKD	400
HU-142aa	210	 ISFAIPSDKIKKFLTESHDRQAKGKAITKKKYIGIRMMSLTSSKAKELKD	259
HU-FULL	401	RHRDFPDVISGAYIIEVIPDTPAEAGGLKENDVIISINGQSVVSANDVSD	450
HU-142aa	260	 RHRDFPDVISGAYIIEVIPDTPAEAGGLKENDVIISINGQSVVSANDVSD	309
HU-FULL	451	VIKRESTLNMVVRGNEDIMITVIPEEIDP	480
HU-142aa	310	 VIKRESTLNMVVRGNEDIMITVIPEEIDP	339

FIG. 1

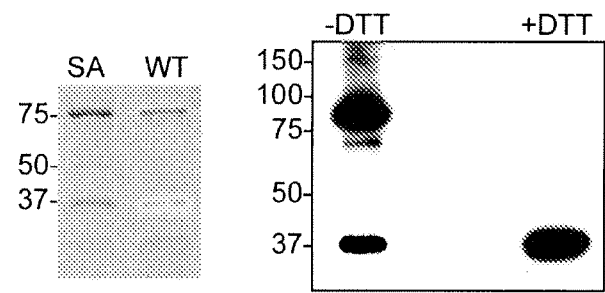


FIG. 2

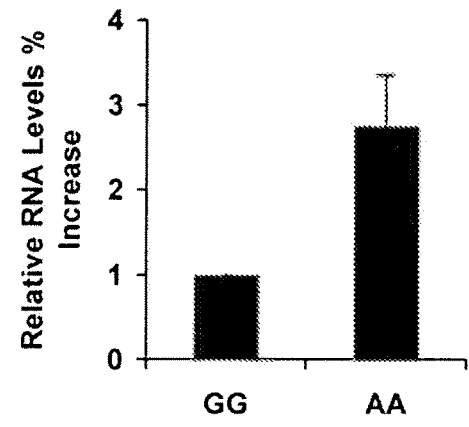
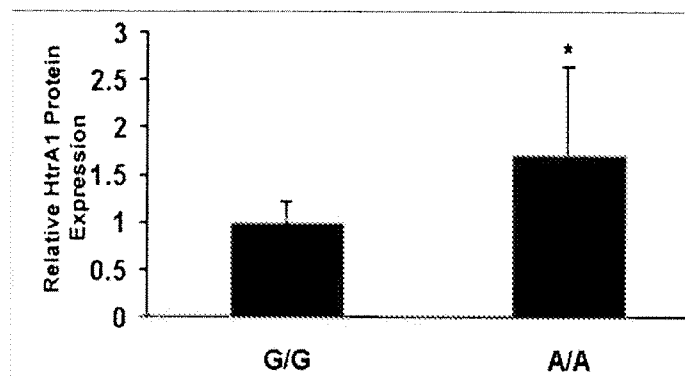


FIG. 3

**FIG. 4**

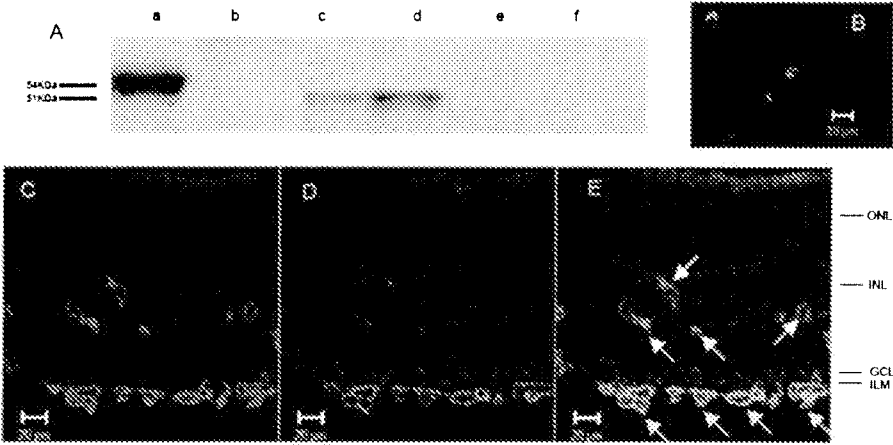


FIG. 5

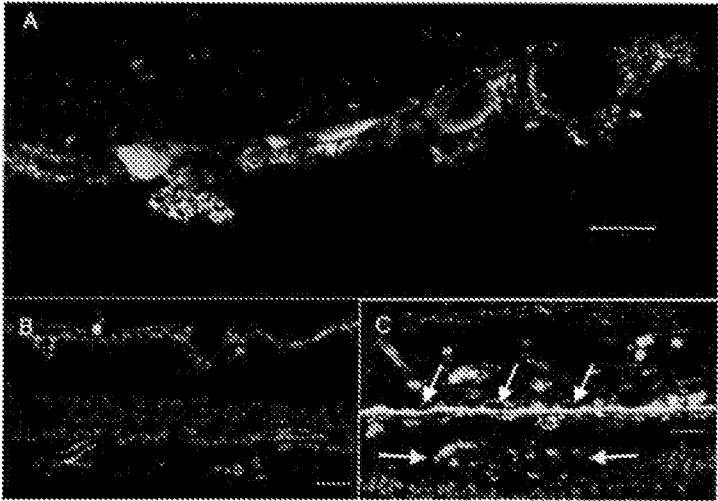


FIG. 6

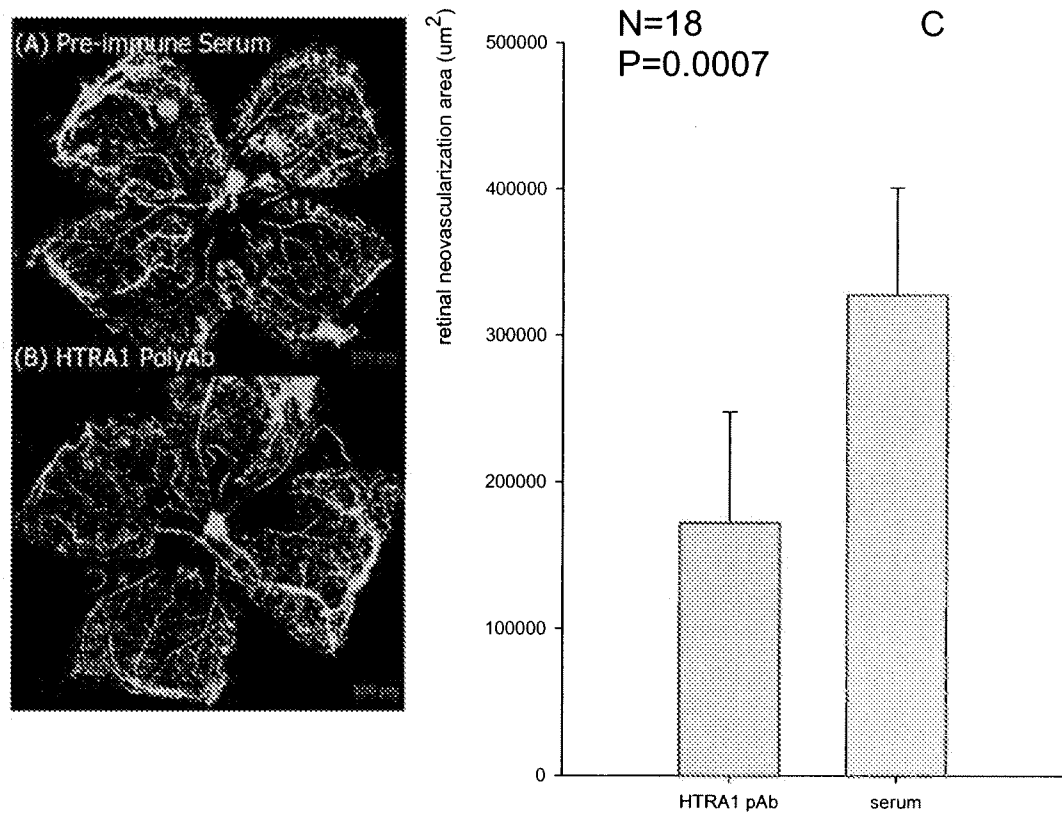
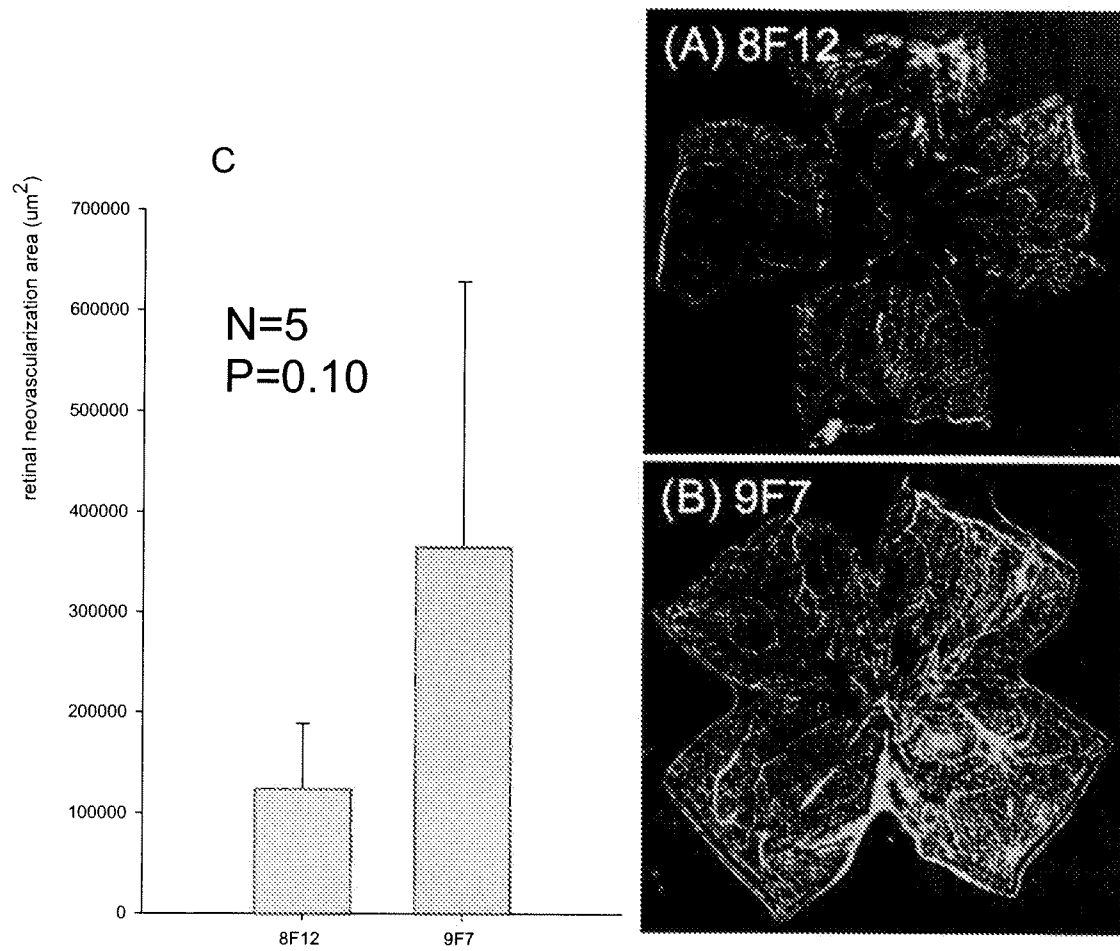
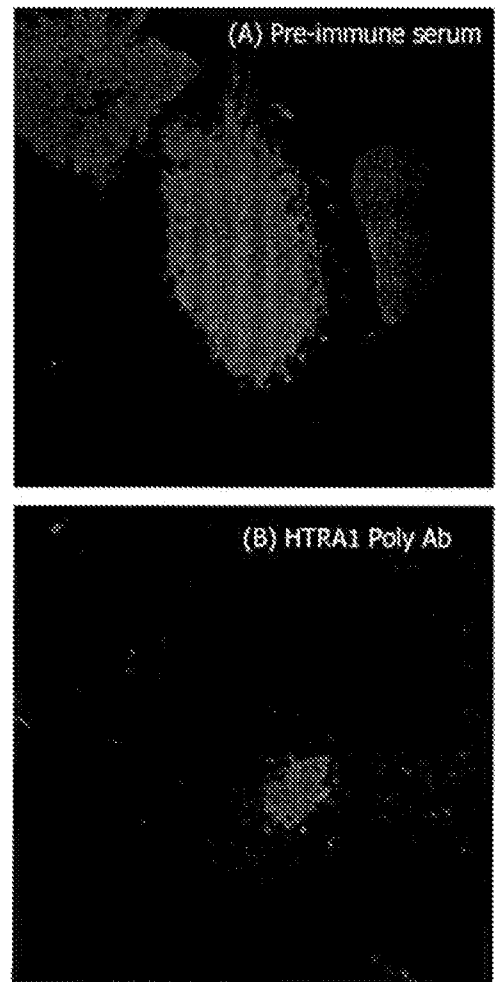
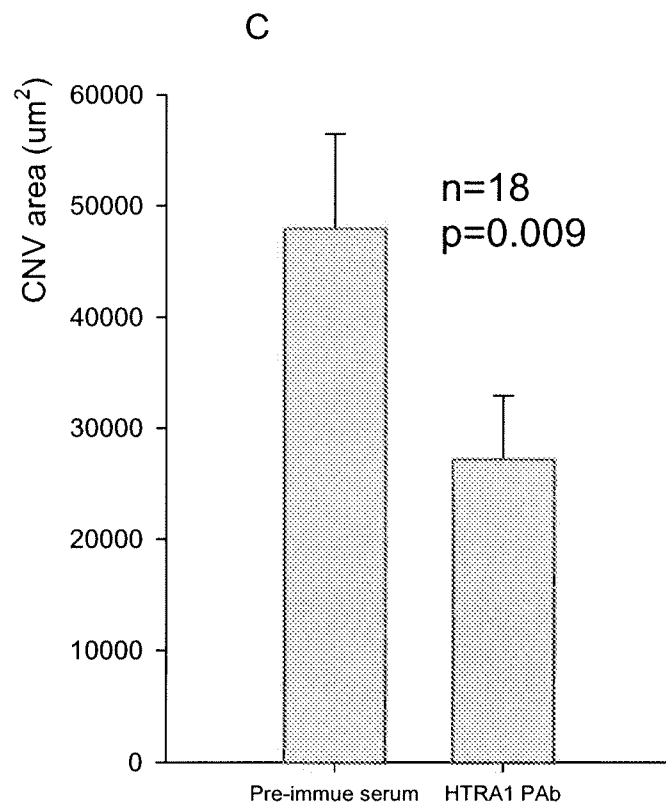


FIG. 7



**FIG. 9**

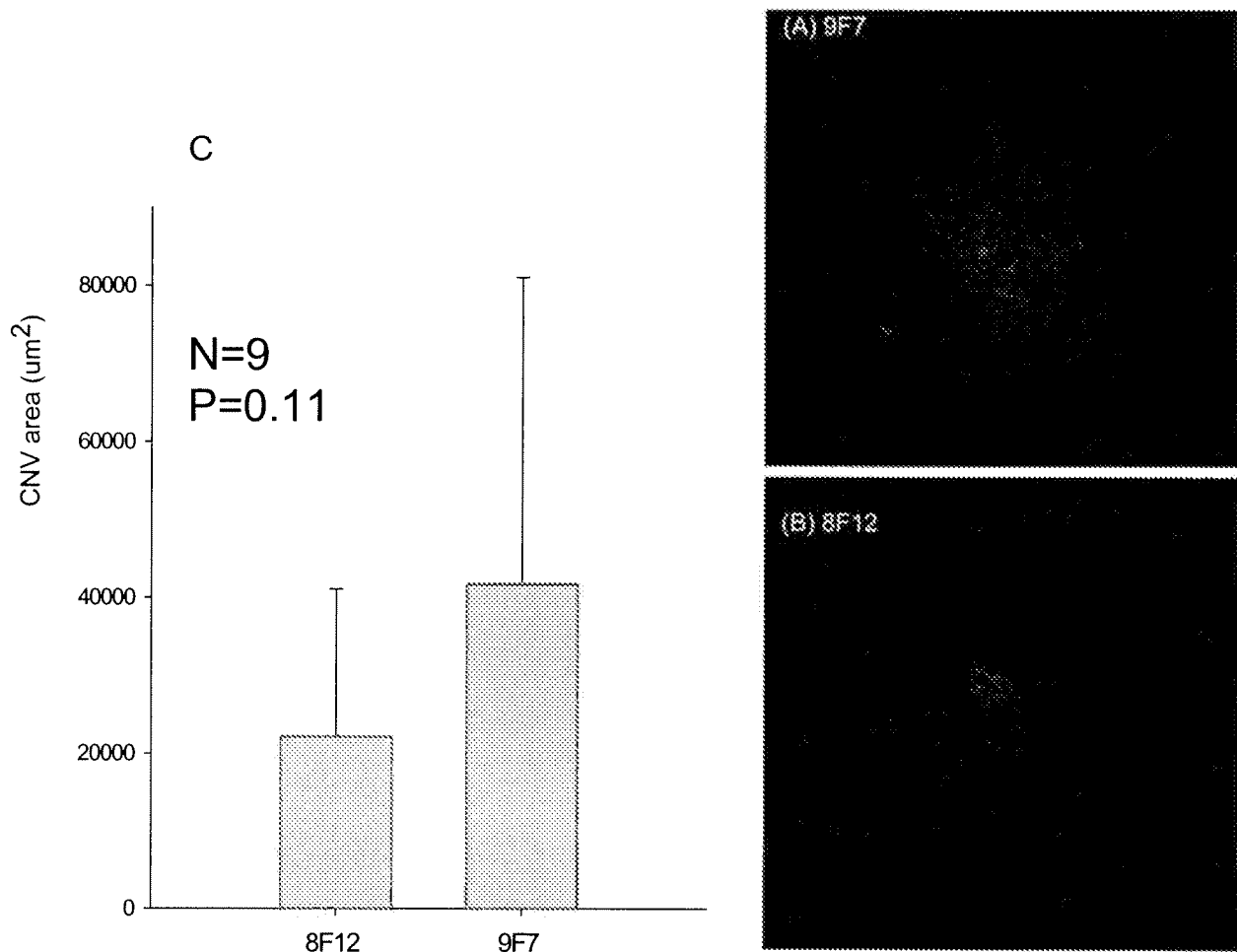


FIG. 10

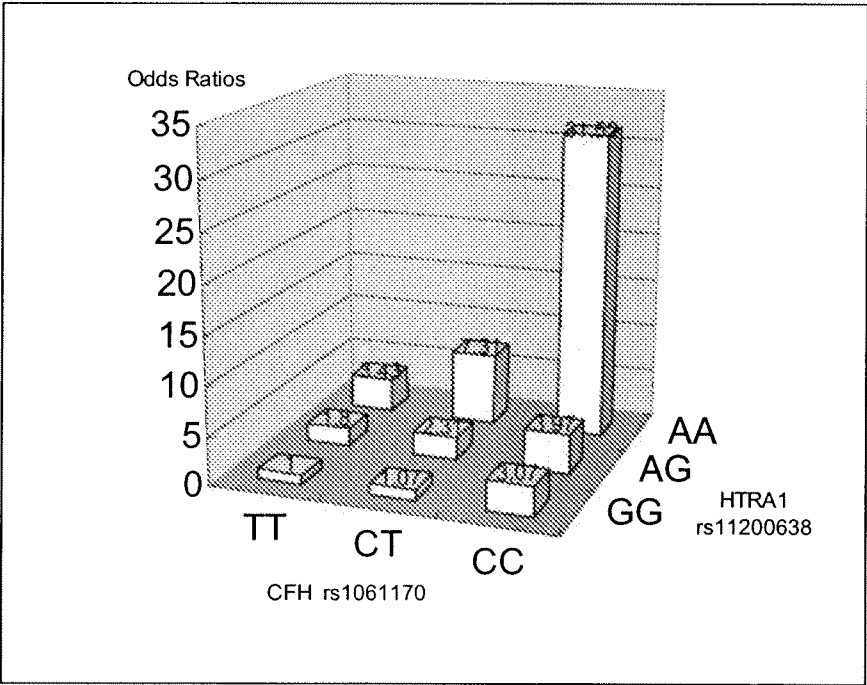


FIG. 11

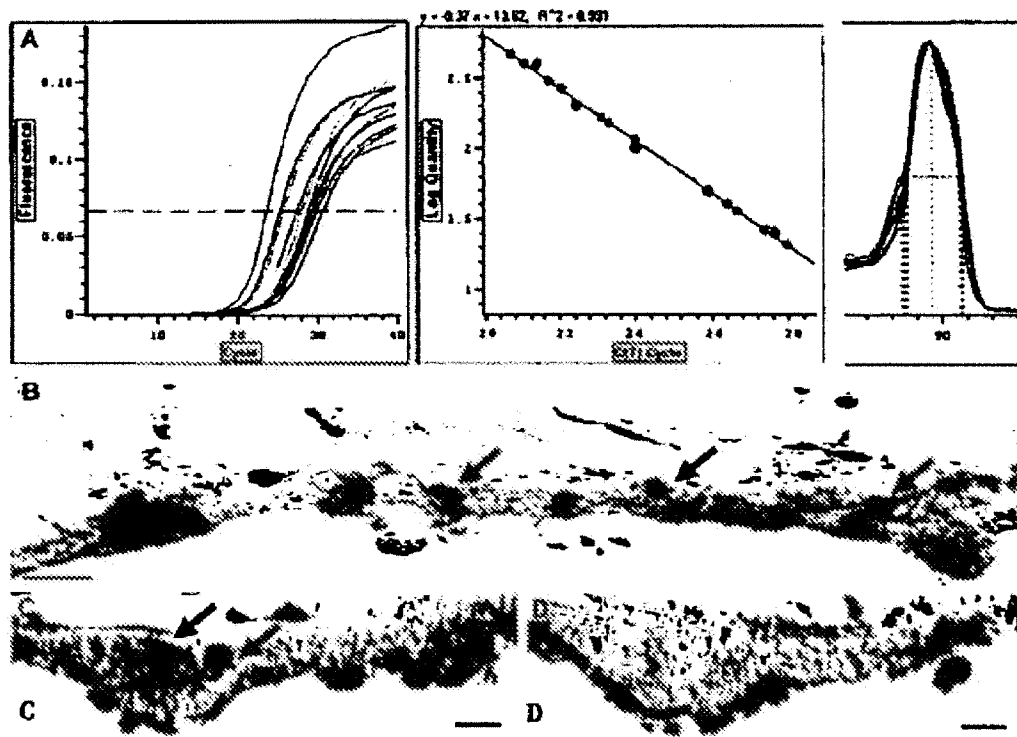


Fig. 12