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- (54) **DIAGNOSTIC METHOD FOR  
ATHEROSCLEROSIS**
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*Primary Examiner* — Nora M Rooney(74) *Attorney, Agent, or Firm* — Barnes & Thornburg LLP**(57) ABSTRACT**

The invention relates to a method of identifying/monitoring  
active atherosclerotic plaques associated with blood vessel  
walls wherein the plaques comprise activated macrophages  
having accessible binding sites for a ligand. The method  
comprises the steps of administering to a patient being evalu-  
ated for atherosclerosis an effective amount of a composition  
comprising a conjugate of a ligand and a chromophore  
capable of emitting light under predetermined conditions,  
allowing sufficient time for the ligand conjugate to bind to the  
activated macrophages, subjecting the blood vessels to the  
predetermined conditions using a catheter-based device, and  
identifying active plaques by detecting light emitted by the  
chromophore using a catheter-based device or by using an  
external imaging technique. The invention also relates to a  
similar method wherein a chemical moiety capable of emit-  
ting radiation is conjugated to the ligand.

**12 Claims, 11 Drawing Sheets  
(3 of 11 Drawing Sheet(s) Filed in Color)**

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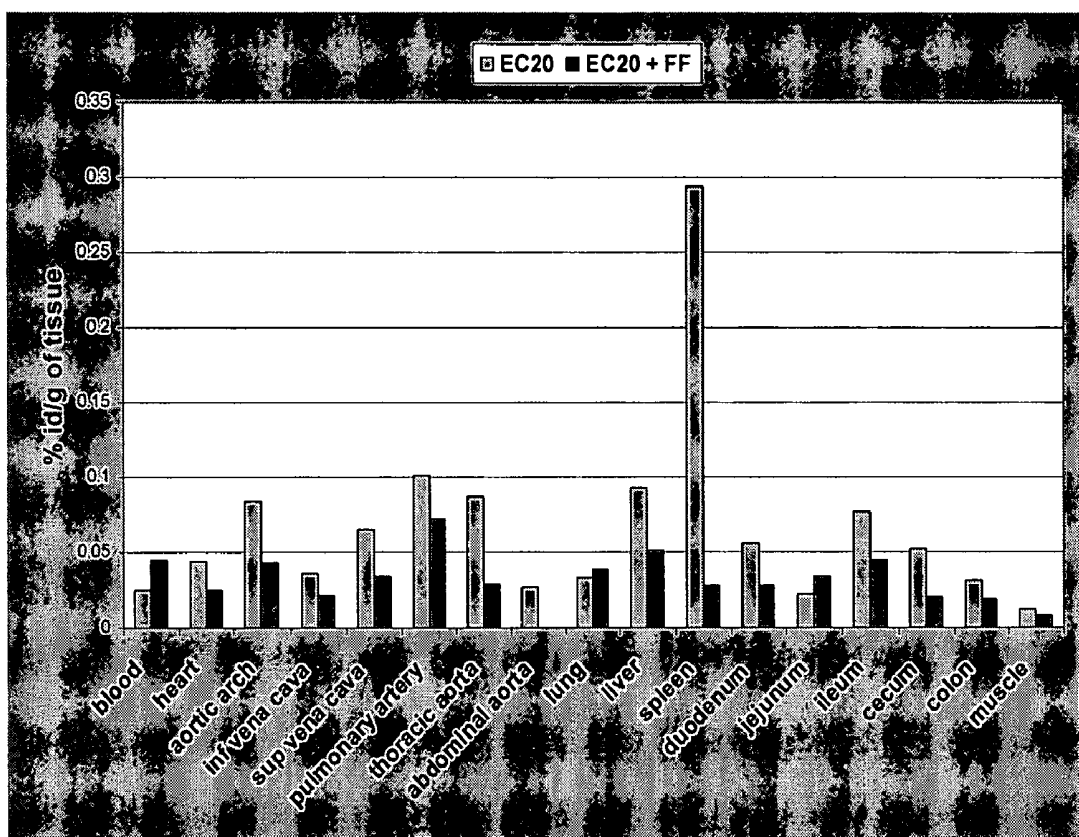


FIG. 1

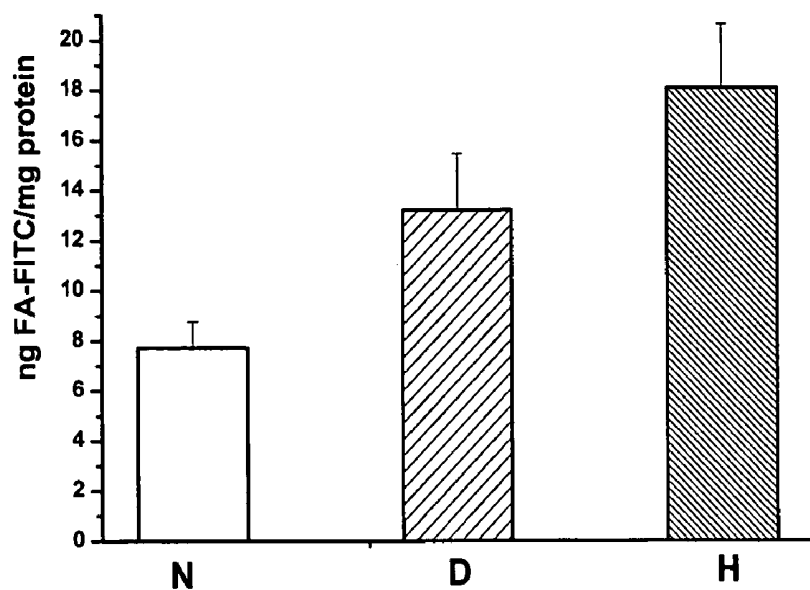


FIG. 2

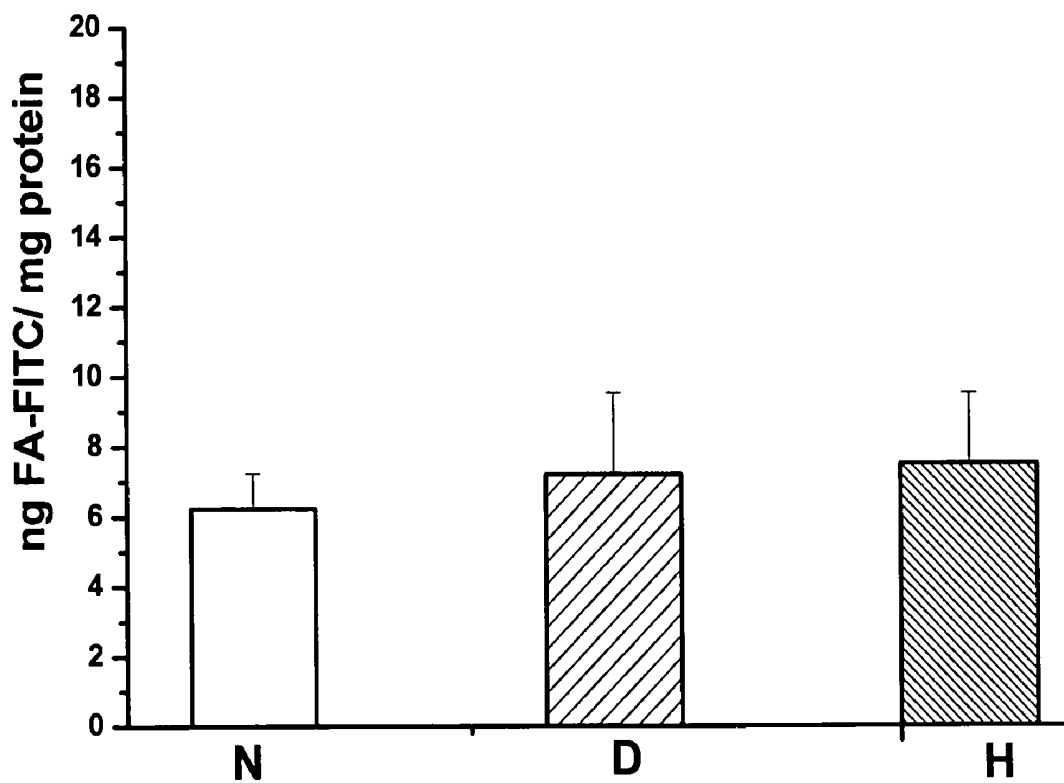


FIG. 3

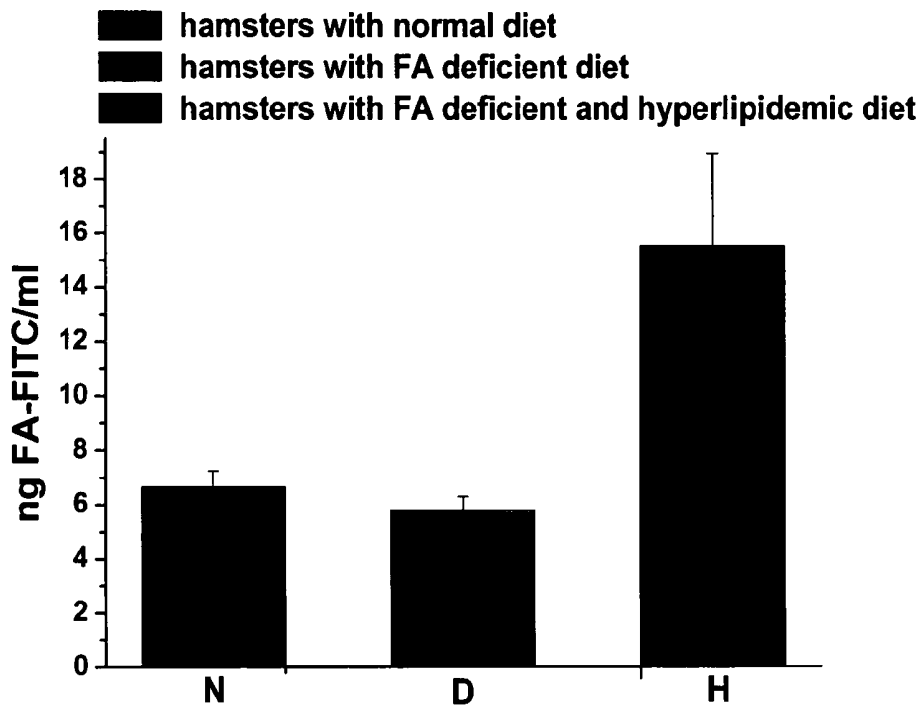


FIG. 4

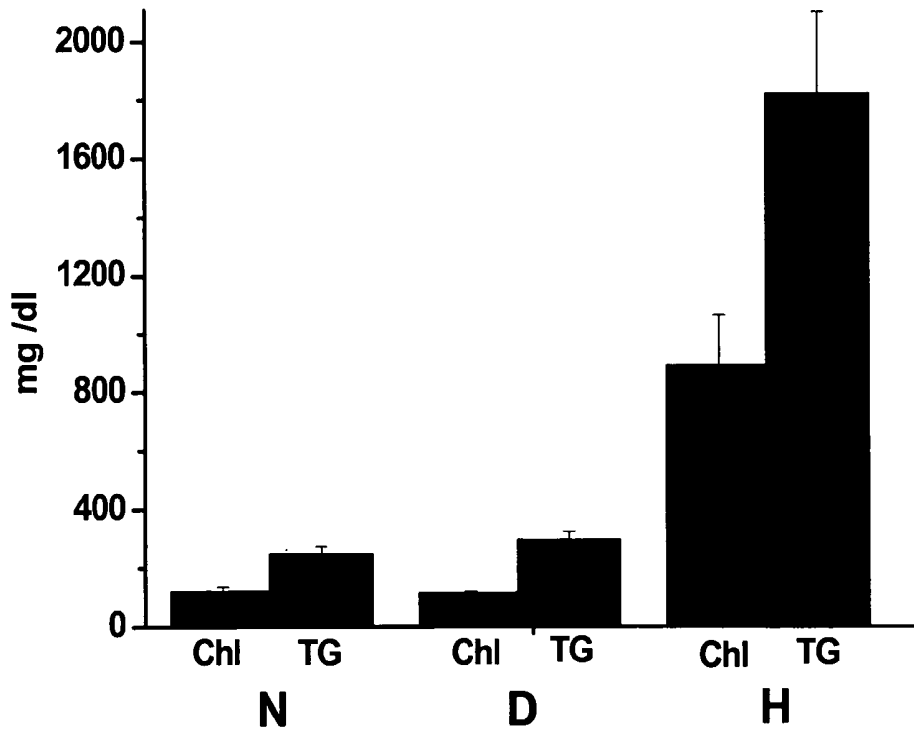


FIG. 5

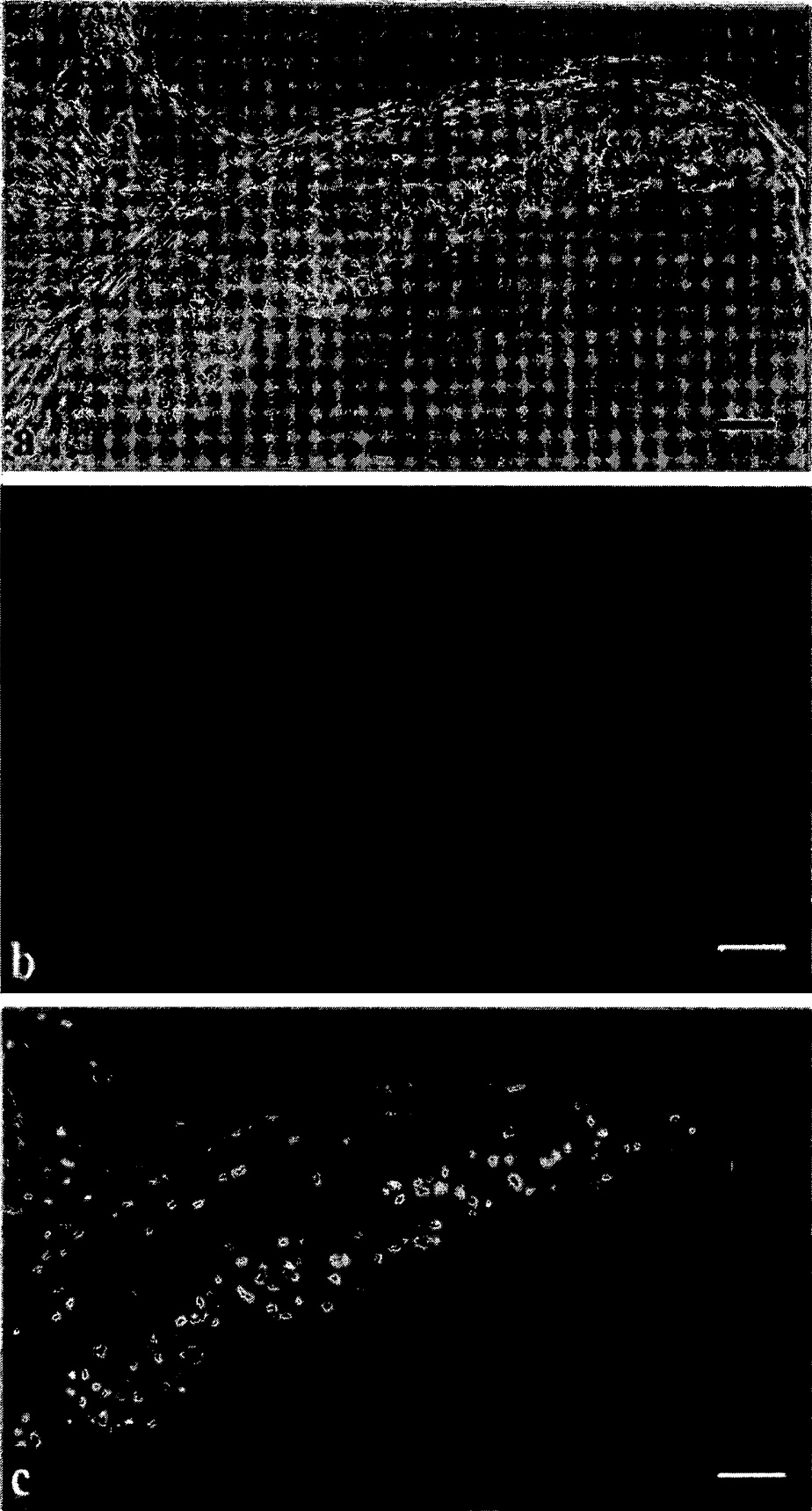


FIG. 6

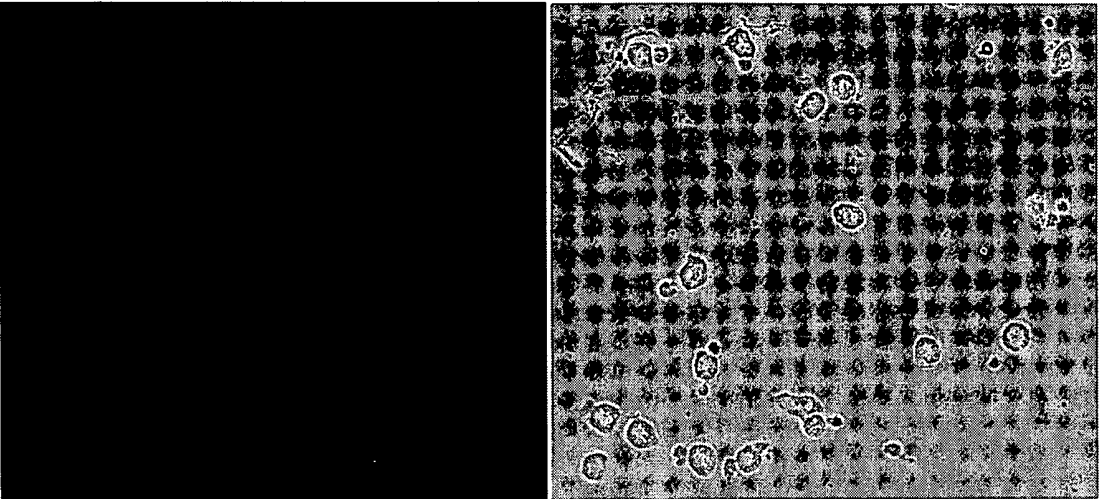


FIG. 7a

FIG. 7b

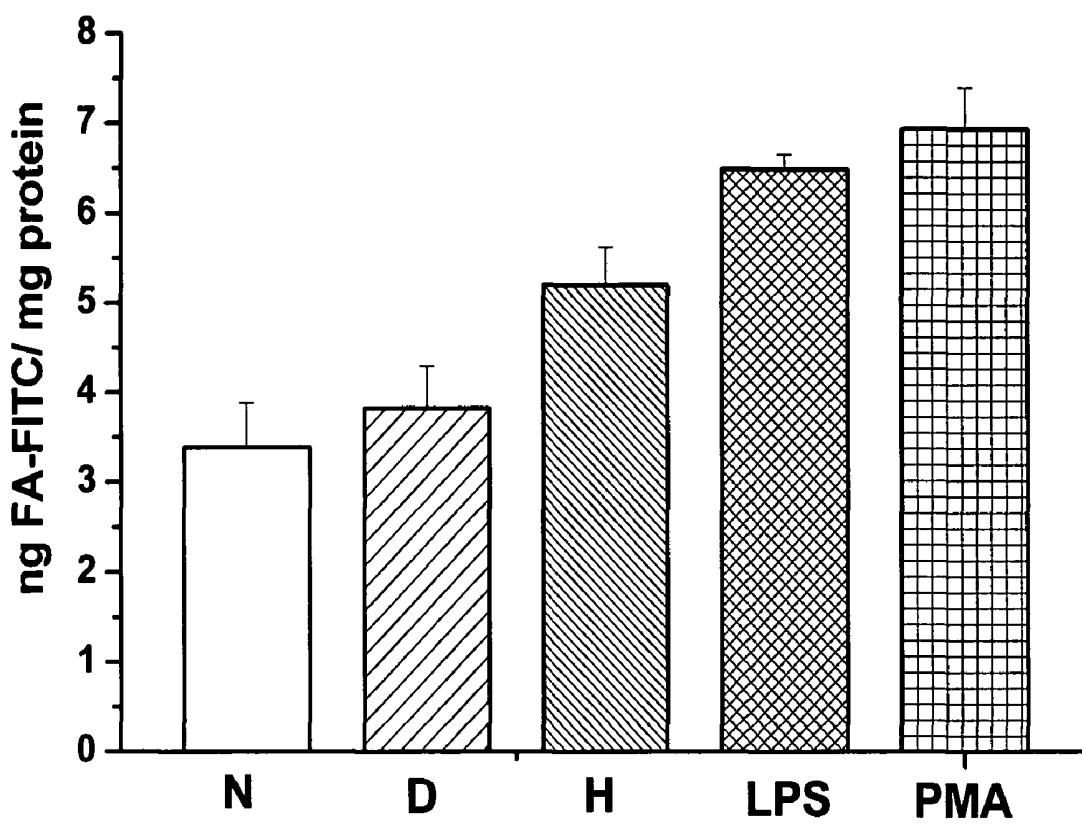
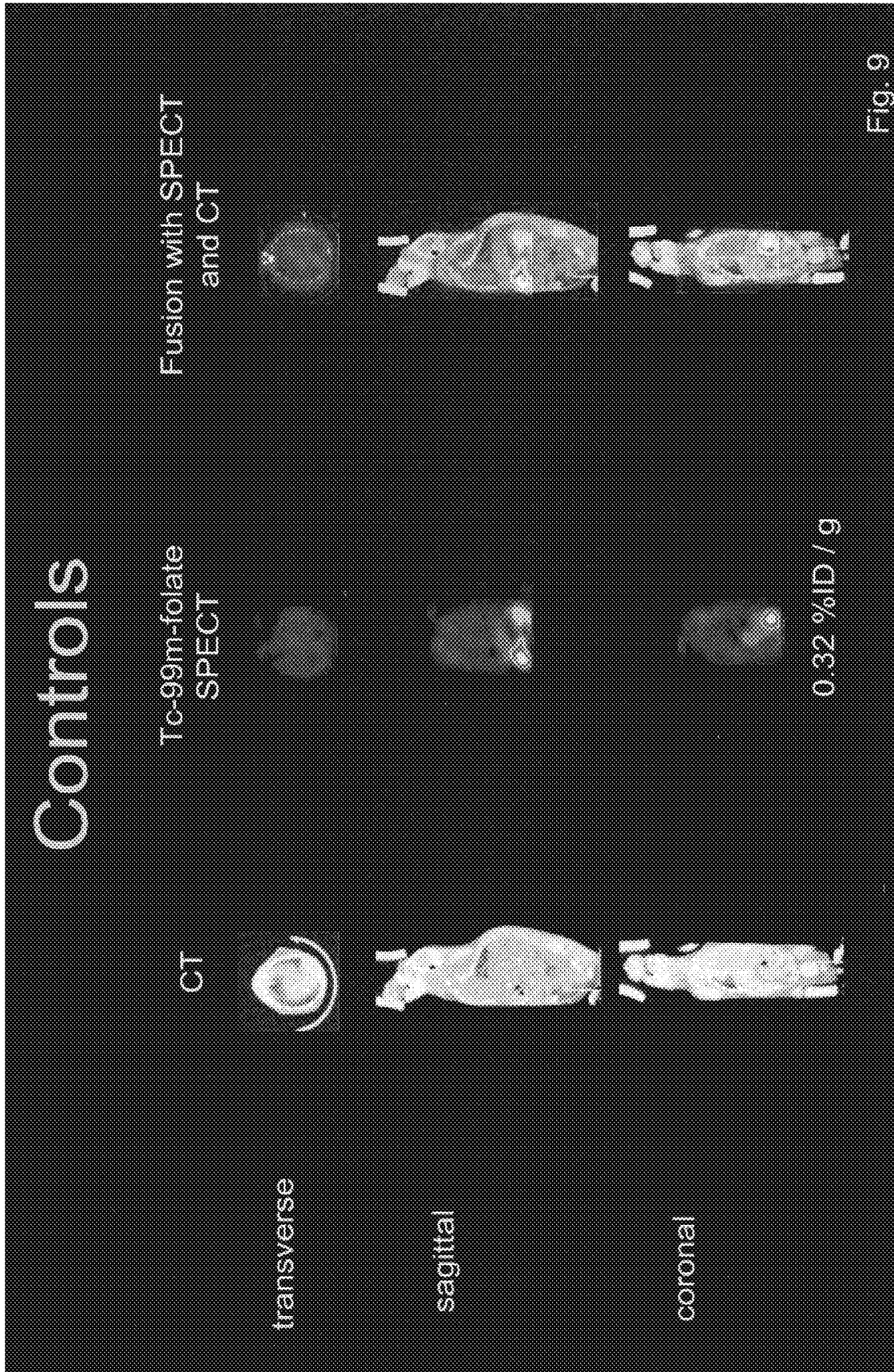
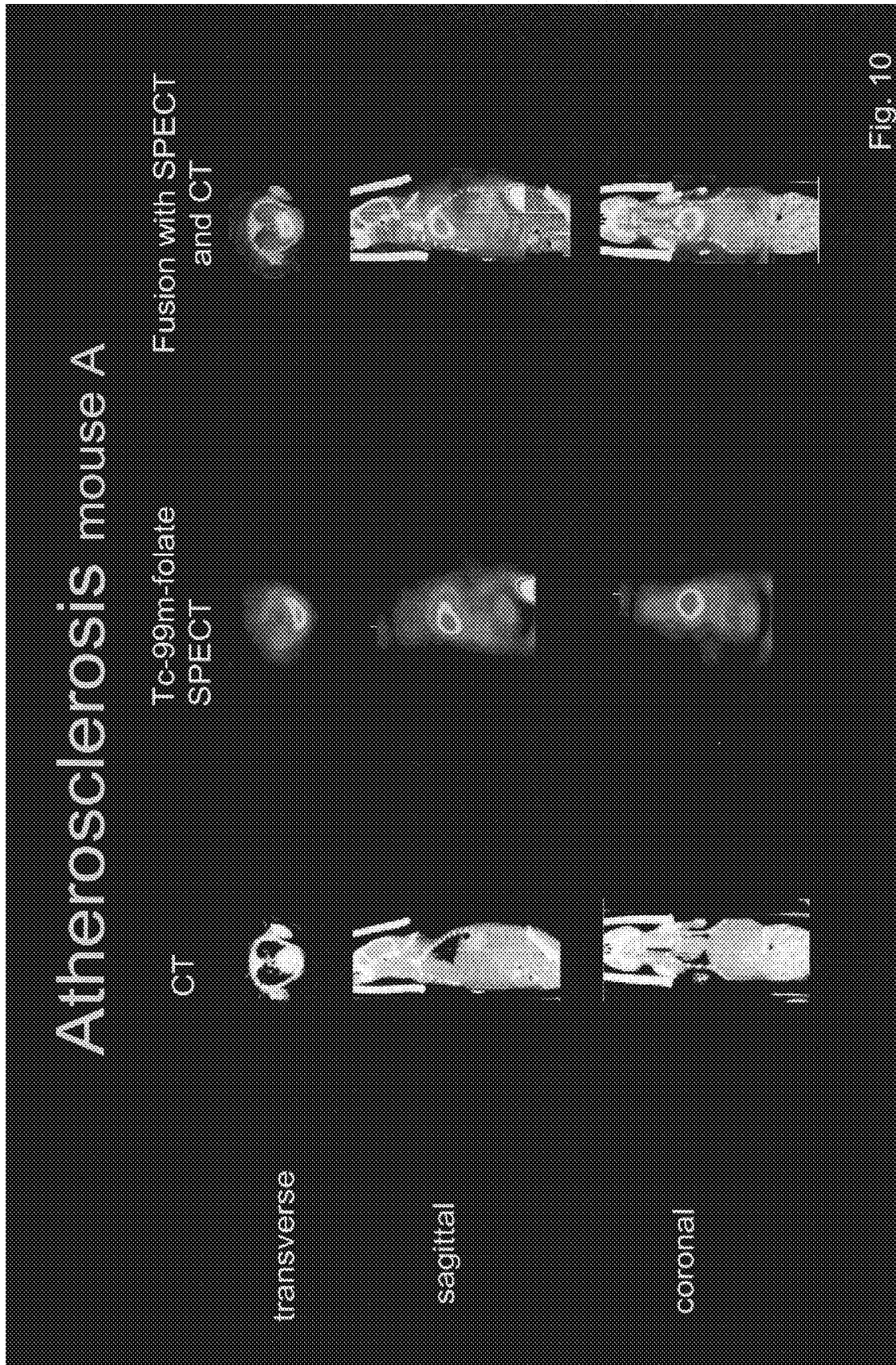
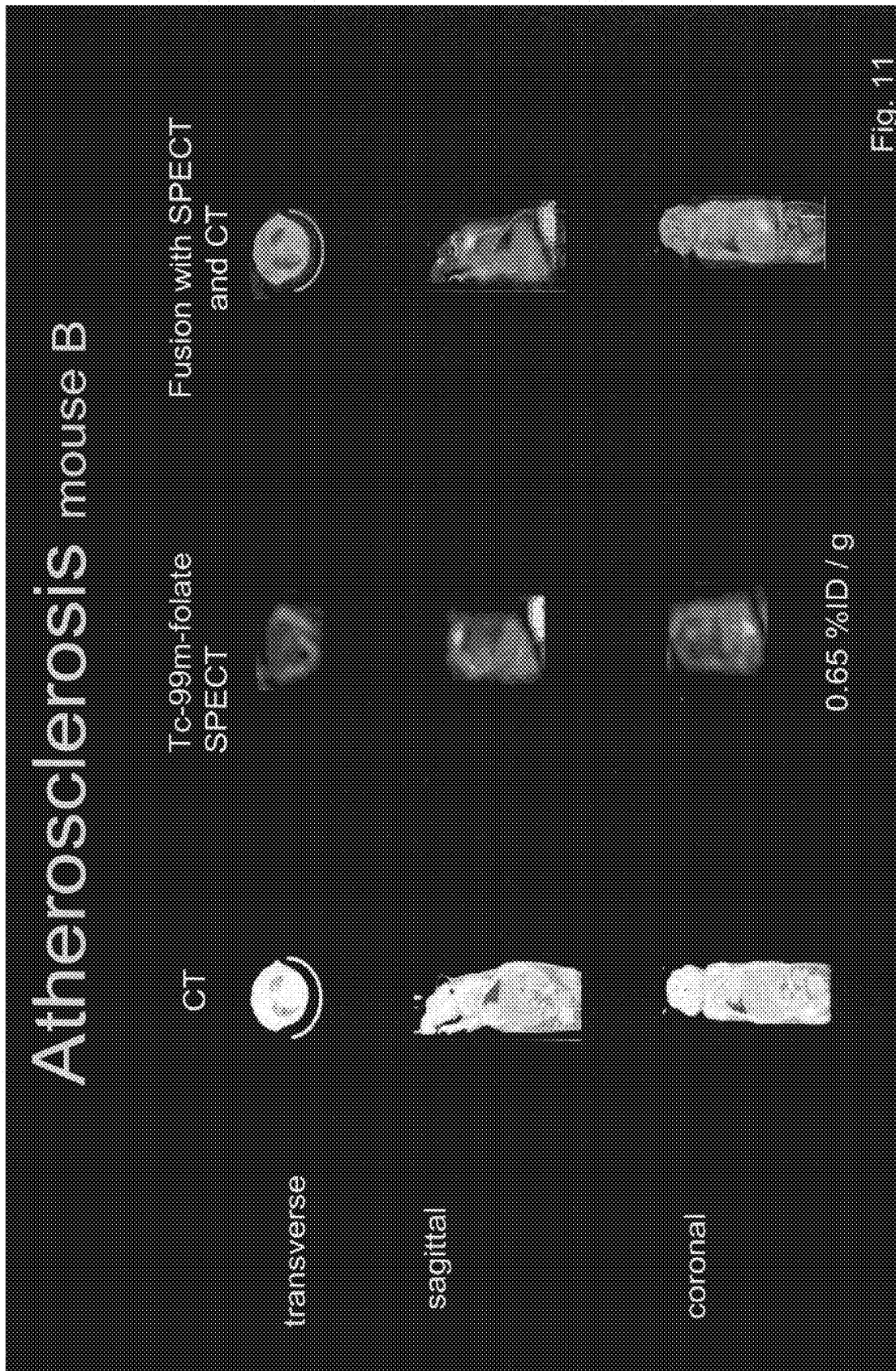


FIG. 8







# Authoradiography mouse B

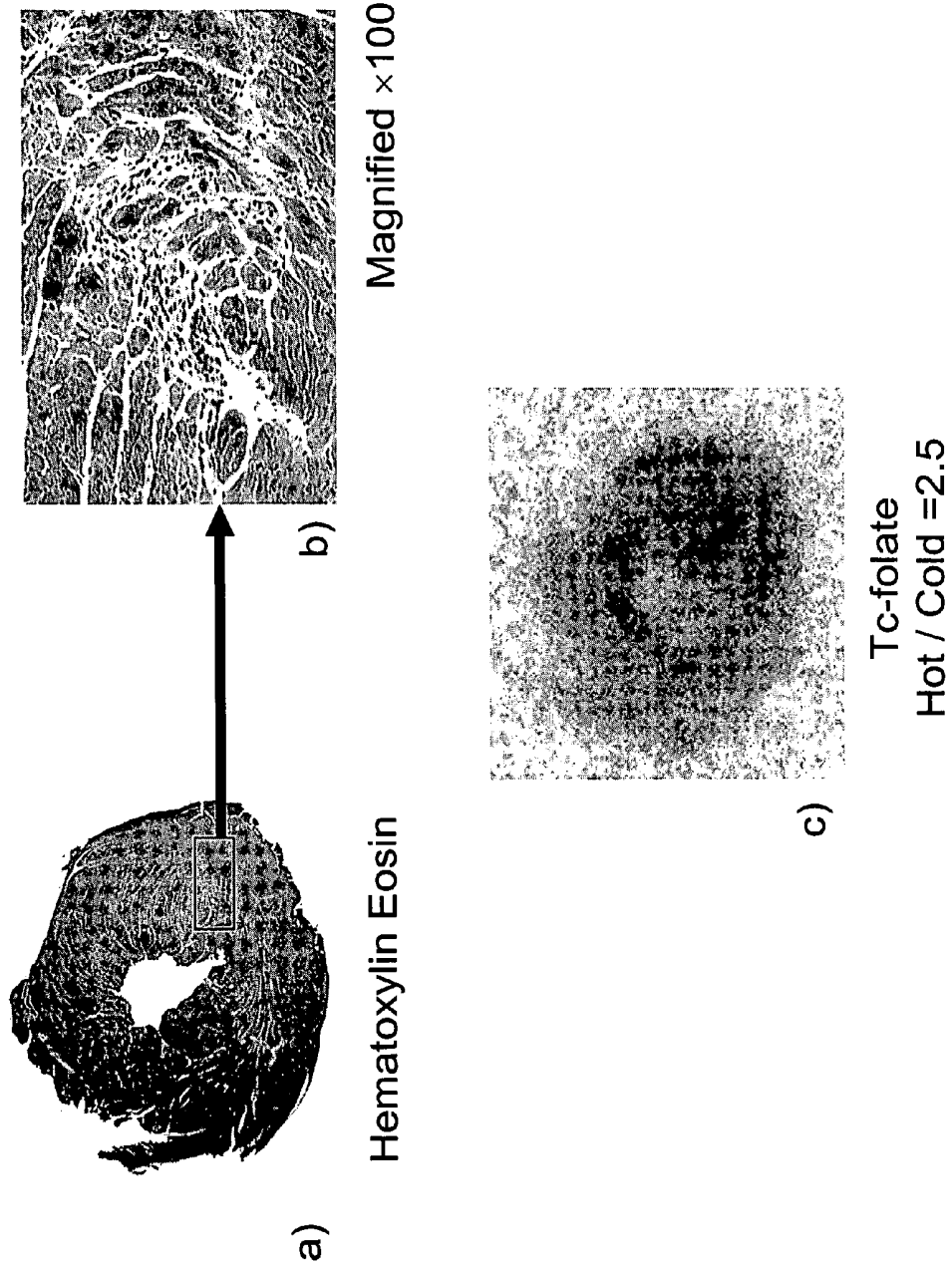


Fig. 12



FIG. 13

## DIAGNOSTIC METHOD FOR ATHEROSCLEROSIS

### CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part application of PCT/US 2004/016667, filed on May 27, 2004, which claims priority under 35 U.S.C. §119(e) to U.S. Provisional Application Ser. No. 60/474,731, filed on May 30, 2003, each of which is incorporated herein by reference.

### FIELD OF THE INVENTION

This invention relates to a method for identifying/monitoring active atherosclerotic plaques. More particularly, ligands that bind to activated macrophages are conjugated to a chromophore or to a chemical moiety capable of emitting radiation for administration to a diseased host for identifying/monitoring active atherosclerotic plaques.

### BACKGROUND AND SUMMARY OF THE INVENTION

Activated macrophages can participate in the immune response by nonspecifically engulfing and killing foreign pathogens within the macrophage, by displaying degraded peptides from foreign proteins on the macrophage cell surface where they can be recognized by other immune cells, and by secreting cytokines and other factors that modulate the function of T and B lymphocytes, resulting in further stimulation of immune responses. Activated macrophages can also contribute to the pathophysiology of disease in some instances. For example, activated macrophages can contribute to atherosclerosis, rheumatoid arthritis, autoimmune disease states, and graft versus host disease.

Atherosclerosis is initiated when a fatty streak forms within a blood vessel wall. Formation of fatty streaks is believed to result from accumulation of lipoprotein particles in the intima layer of the blood vessel wall, the layer of the vessel wall underlying the luminal endothelial cell layer. Lipoprotein particles can associate with extracellular matrix components in the intima layer and can become inaccessible to plasma antioxidants, resulting in oxidative modification of the lipoprotein particles. Such oxidative modification may trigger a local inflammatory response resulting in adhesion of activated macrophages and T lymphocytes to the luminal endothelium followed by migration into the intima layer. The oxidized lipoprotein particles themselves can act as chemoattractants for cells of the immune system, such as macrophages and T cells, or can induce cells in the vascular wall to produce chemoattractants. The atherosclerotic lesion then forms a fibrous cap with a lipid-rich core filled with activated macrophages. Atherosclerotic lesions that are unstable are characterized by local inflammation, and lesions that have ruptured and have caused fatal myocardial infarction are characterized by an infiltration of activated macrophages and T lymphocytes.

The present invention relates to a method of identifying/monitoring active atherosclerotic plaques in blood vessel walls. In accordance with the invention a ligand, that binds to a receptor which is preferentially expressed/presented on the surface of activated macrophages relative to resting macrophages, is conjugated to a chromophore or a chemical moiety capable of emitting radiation and the ligand conjugates are administered to a patient being evaluated for atherosclerosis. The ligand conjugates bind to activated macrophages associ-

ated with active atherosclerotic plaques and emit light (i.e., ligand-chromophore conjugates) or radiation (i.e., ligand-chemical moiety conjugates) and can be detected using a catheter-based device or by external imaging, such as by using X-ray detection. Accordingly, the ligand conjugates can be used to distinguish active atherosclerotic plaques containing activated macrophages from inactive plaques.

Methods are not presently available for distinguishing active and inactive atherosclerotic plaques. Because many unstable (i.e., active) atherosclerotic plaques, capable of rupturing and causing acute atherosclerotic syndromes do not produce luminal narrowing of blood vessels, particularly in the coronary circulation, the method of the present invention represents a significant advance in diagnosing the risk of myocardial infarction, and in evaluating the need for clinical intervention, in patients suffering from atherosclerosis.

In one embodiment, a method is provided of identifying/monitoring active atherosclerotic plaques associated with blood vessel walls wherein the plaques comprise activated macrophages having accessible binding sites for a ligand. The method comprises the steps of administering to a patient being evaluated for atherosclerosis an effective amount of a composition comprising a conjugate of the general formula

L-X

wherein the group L comprises the ligand and the group X comprises a chromophore capable of emitting light under predetermined conditions, allowing sufficient time for the ligand conjugate to bind to activated macrophages associated with the active plaques, subjecting the blood vessel walls to the predetermined conditions using a catheter-based device, and identifying active plaques by detecting light emitted by the chromophore using a catheter-based device.

In another embodiment, a method is provided of identifying/monitoring active atherosclerotic plaques associated with blood vessel walls where the plaques comprise activated macrophages having accessible binding sites for a ligand. The method comprises the steps of administering to a patient suffering from atherosclerosis an effective amount of a composition comprising a conjugate of the general formula

L-X

wherein the group L comprises the ligand and the group X comprises a chemical moiety capable of emitting radiation, allowing sufficient time for the ligand conjugate to bind to the activated macrophages associated with the active plaques, and identifying active plaques by detecting radiation emitted by the chemical moiety using a catheter-based device or by external imaging, such as by X-ray detection.

In these embodiments, the ligand can be any ligand that binds to a receptor which is preferentially expressed/presented on the surface of activated macrophages relative to resting macrophages. Such ligands include vitamins selected from the group consisting of folate, biotin, vitamin B<sub>12</sub>, riboflavin, thiamine, and other vitamin receptor binding ligands.

### BRIEF DESCRIPTION OF THE DRAWINGS

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

FIG. 1 shows the uptake of a folate-targeted <sup>99m</sup>Tc chelating chemical moiety (EC20) in the organs of atherosclerotic Watanabe rabbits.

FIG. 2 shows folate-FITC retention by macrophages harvested from hyperlipemic hamsters (H) compared to animals maintained on either normal (high folate) diet (N) or folic acid (FA) deficient diet (D).

FIG. 3 shows folate-FITC retention by macrophages harvested from hyperlipemic mice (H) compared to animals maintained on either normal (high folate) diet (N) or folic acid (FA) deficient diet (D).

FIG. 4 shows the serum levels of folate-FITC in hyperlipemic hamsters (H) compared to animals maintained on either normal (high folate) diet (N) or folic acid (FA) deficient diet (D).

FIG. 5 shows total serum cholesterol and triglyceride levels in hyperlipemic hamsters (H) compared to animals maintained on either normal (high folate) diet (N) or folic acid (FA) deficient diet (D).

FIGS. 6a, 6b, and 6c show Oil Red staining (FIG. 6a) fluorescence analysis (FIGS. 6b and 6c) of tissue fragments from atherosclerotic lesion-prone areas of Golden Syrian hamsters fed the atherosclerotic diet.

FIGS. 7a and 7b show fluorescence analysis of macrophages harvested from the peritoneum of folic acid-Texas Red treated hypercholesterolemic hamsters.

FIG. 8 shows folate-FITC retention by U937 cells grown in either normal medium (N), folic acid deficient medium (D), or hyperlipidemic medium (H) or normal medium supplemented with LPS or PMA.

FIG. 9 shows a CT, a SPECT, and a SPECT-CT image of a control mouse injected with a folate-targeted <sup>99m</sup>Tc chelating chemical moiety (EC20).

FIG. 10 shows a CT, a SPECT, and a SPECT-CT image of a mouse maintained on a high cholesterol and high fat diet and then injected with EC20.

FIG. 11 shows a CT, a SPECT, and a SPECT-CT image of a mouse maintained on a high cholesterol and high fat diet and then injected with EC20.

FIG. 12 shows A) an H & E stain of an atherosclerotic artery, B) an H & E stain, magnified 100x, of an atherosclerotic artery, and C) an autoradiograph of an atherosclerotic artery from a mouse injected with EC20.

FIG. 13 shows electron micrographs of atherosclerotic lesions on the aortic arch and cardiac valves in hyperlipidemic hamsters.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method of identifying/monitoring active atherosclerotic plaques in blood vessel walls. In accordance with the invention a ligand that binds to a receptor which is preferentially expressed/presented on the surface of activated macrophages relative to resting macrophages, is conjugated to a chromophore capable of emitting light or a chemical moiety capable of emitting radiation and the ligand conjugates are administered to a patient being evaluated for atherosclerosis. The ligand conjugates bind to activated macrophages associated with active atherosclerotic plaques. The light or radiation emitted by the ligand-chromophore conjugate or the chemical moiety, respectively, is detected using a catheter-based device or externally using such methods as X-ray detection. Accordingly, the ligand conjugates can be used to distinguish active atherosclerotic plaques, containing activated macrophages, from inactive plaques wherein the plaques are present in the arteries or veins of a patient being evaluated for atherosclerosis.

In accordance with the invention, the word "catheter" means any catheter, guidewire, or other device capable of transluminal delivery (i.e., delivery into the lumen of blood

vessels) of optical energy or of radiation, and/or any catheter, guidewire, or other device capable of detecting, in the lumen of blood vessels, light or radioactivity emitted from the ligand conjugates used in accordance with the method of the present invention, and/or any catheter, guidewire, or other device capable of delivering a therapeutic drug to the lumen of blood vessels.

In accordance with the present invention, the ligand conjugates can be formed from a wide variety of ligands, including any ligand that binds to a receptor expressed or presented on the surface of activated macrophages that is not expressed/presented or is not present in significant amounts on the surface of resting macrophages. Such ligands include N-formyl peptides (e.g., f-Met-Leu-Phe), high mobility group factor 1 protein (HMGB1), hyaluronan fragments, HSP-70, toll-like receptor ligands, scavenger receptor ligands, co-receptors for antigen presentation, ligands that bind to the CD68, BER-MAC3, RFD7, CD4, CD14, and HLA-D markers on activated macrophages, ligands that bind to urokinase plasminogen activator receptors (e.g., the WX-360 peptide), antibodies, or fragments thereof, that bind preferentially to activated macrophages, and vitamins or receptor-binding vitamin analogs/derivatives. The ligand conjugates are capable of preferentially binding to activated macrophages compared to resting macrophages due to preferential expression of the receptor for the ligand on activated macrophages.

Acceptable vitamin moieties that can be used as ligands in accordance with the invention include niacin, pantothenic acid, folic acid, riboflavin, thiamine, biotin, vitamin B<sub>12</sub>, and the lipid soluble vitamins A, D, E and K. These vitamins, and their receptor-binding analogs and derivatives, constitute the targeting entity that can be coupled with a chromophore or a chemical moiety, capable of emitting radiation, to form the ligand conjugates for use in accordance with the invention. Preferred vitamin moieties include folic acid, biotin, riboflavin, thiamine, vitamin B<sub>12</sub>, and receptor-binding analogs and derivatives of these vitamin molecules, and other related vitamin receptor-binding molecules (see U.S. Pat. No. 5,688,488, incorporated herein by reference). Exemplary of a vitamin analog is a folate analog containing a glutamic acid residue in the D configuration (folic acid normally contains one glutamic acid in the L configuration linked to pteric acid).

In the ligand conjugates of the general formula L-X in accordance with the present invention, the group L is a ligand capable of binding to activated macrophages as compared to resting macrophages as described above. In one embodiment the activated macrophage binding ligand is folic acid, a folic acid analog/derivative or other folate receptor binding molecules. In another embodiment the activated macrophage binding ligand is a specific monoclonal or polyclonal antibody or Fab or scFv (i.e., a single chain variable region) fragments of an antibody capable of preferential binding to activated macrophages as compared to resting macrophages.

Activated macrophages express a 38 kD GPI-anchored folate receptor that binds folate and folate-derivatized compounds with subnanomolar affinity (i.e., <1 nM). Importantly, covalent conjugation of small molecules, proteins, and even liposomes to folic acid does not alter the vitamin's ability to bind the folate receptor. Because most cells use an unrelated reduced folate carrier to acquire the necessary folic acid, expression of the folate receptor is restricted to a few cell types. With the exception of kidney, choroid plexus, and placenta, normal tissues express low or nondetectable levels of the folate receptor. However, many malignant tissues, including ovarian, breast, bronchial, and brain cancers express significantly elevated levels of the receptor. Also, it

has recently been reported that the folate receptor  $\beta$ , the nonepithelial isoform of the folate receptor, is expressed in active form on activated, but not resting synovial macrophages.

The binding site for the ligand can include receptors for any ligand molecule, or a derivative or analog thereof, capable of preferentially binding to a receptor uniquely expressed or preferentially expressed/presented on the surface of activated macrophages. A surface-presented protein uniquely expressed or preferentially expressed by activated macrophages is a receptor that is either not present or is present at insignificant concentrations on resting macrophages providing a means for preferential detection of activated macrophages. Accordingly, any receptor that is upregulated on activated macrophages compared to resting macrophages, or which is not expressed/presented on the surface of resting macrophages, or any receptor that is not expressed/presented on the surface of resting macrophages in significant amounts could be used for targeting. In one embodiment the site that binds the ligand conjugates used in accordance with the present invention is a vitamin receptor, for example, the folate receptor, which binds folate or an analog or derivative thereof.

In accordance with the invention the ligand conjugates can bind with high affinity to receptors on activated macrophages. The high affinity binding can be inherent to the ligand or the binding affinity can be enhanced by the use of a chemically modified ligand (i.e., an analog or a derivative) or by the particular chemical linkage, in the ligand conjugate, between the ligand and the chromophore or between the ligand and the chemical moiety capable of emitting radiation.

The chemical linkage in the ligand conjugate between the ligand and the chromophore or between the ligand and the chemical moiety can be a direct linkage or can be through an intermediary linker. If present, an intermediary linker can be any biocompatible linker known in the art. Typically, the linker comprises about 1 to about 30 carbon atoms, more typically about 2 to about 20 carbon atoms. Lower molecular weight linkers (i.e., those having an approximate molecular weight of about 30 to about 300) are typically employed. The linkers can be any of those described in U.S. patent application Ser. No. 10/765,336, incorporated herein by reference.

Generally, any manner of forming a complex between the ligand and the chromophore, between the ligand and the chemical moiety capable of emitting radiation, between a linker and the ligand, or between a linker and the chromophore or chemical moiety capable of emitting radiation can be utilized in accordance with the present invention. With or without a linker, the complex can be formed by conjugation of the components of the conjugate, for example, through hydrogen, ionic, or covalent bonds. Covalent bonding of the components of the conjugate can occur, for example, through the formation of amide, ester, disulfide, or imino bonds between acid, aldehyde, hydroxy, amino, sulfhydryl, or hydrazo groups. Also, in accordance with this invention a linker can comprise an indirect means for associating the ligand with the chromophore/chemical moiety, such as by connection through spacer arms or bridging molecules. Both direct and indirect means for association should not prevent the binding of the ligand to the receptor on the activated macrophages for operation of the method of the present invention. Alternatively, the ligand conjugate can be one comprising a liposome wherein the chemical moiety capable of emitting radiation, for example, is contained within a liposome which is itself covalently linked to the activated macrophage-binding ligand.

In the embodiment where the ligand is folic acid, an analog/derivative of folic acid, or any other folate receptor bind-

ing molecule, the folate ligand can be conjugated to the chromophore/chemical moiety by an art-recognized procedure that utilizes trifluoroacetic anhydride to prepare  $\gamma$ -esters of folic acid via a pteroyl azide intermediate. This procedure results in the synthesis of a folate ligand, conjugated to the chromophore/chemical moiety only through the  $\gamma$ -carboxy group of the glutamic acid groups of folate. Alternatively, folic acid analogs can be coupled by art-recognized procedures through the  $\alpha$ -carboxy moiety of the glutamic acid group or both the  $\alpha$  and  $\gamma$  carboxylic acid entities.

The amount of the conjugate effective for use in accordance with the method of the invention depends on many parameters, including the molecular weight of the conjugate, its route of administration, and its tissue distribution. In accordance with the invention an "effective amount" of the ligand conjugate is an amount sufficient to bind to activated macrophages and to be useful in the identification/monitoring of active atherosclerotic plaques. The effective amount of the ligand conjugate to be administered to a patient being evaluated for atherosclerosis can range from about 1 ng/kg to about 10 mg/kg, or from about 10  $\mu$ g/kg to about 1 mg/kg, or from about 100  $\mu$ g/kg to about 500  $\mu$ g/kg.

The ligand conjugate can be administered in one or more doses (e.g., about 1 to about 3 doses) prior to the catheterization or external imaging procedure. The number of doses depends on the molecular weight of the conjugate, its route of administration, and its tissue distribution, among other factors. When used for identification/monitoring of active atherosclerotic plaques, the catheterization or external imaging procedure is typically performed about 1 to about 6 hours post-administration of the ligand conjugate targeted to activated macrophages, but the catheterization or external imaging procedure can be performed at any time post-administration of the ligand conjugate as long as binding of the ligand conjugate to activated macrophages is detectable.

The ligand conjugates administered in accordance with the method of this invention are preferably administered parenterally to the patient being evaluated for atherosclerosis, for example, intravenously, intradermally, subcutaneously, intramuscularly, or intraperitoneally, in combination with a pharmaceutically acceptable carrier. Alternatively, the conjugates can be administered to the patient being evaluated for atherosclerosis by other medically useful procedures such as in an orally available formulation. In accordance with the invention, a "patient being evaluated for atherosclerosis" means any patient suspected of having atherosclerosis, whether symptomatic or not, who would benefit from an evaluation using the method of the present invention.

The conjugates used in accordance with this invention of the formula L-X are used in one aspect of this invention to formulate diagnostic compositions comprising effective amounts of the conjugate and an acceptable carrier therefor. Examples of parenteral dosage forms include aqueous solutions of the conjugate, for example, a solution in isotonic saline, 5% glucose or other well-known pharmaceutically acceptable liquid carriers such as alcohols, glycols, esters and amides. The parenteral compositions for use in accordance with this invention can be in the form of a reconstitutable lyophilizate comprising the one or more doses of the ligand conjugate. Any orally available dosage forms known in the art can also be used.

The activated macrophage-targeted conjugates used for identifying/monitoring disease states mediated by activated macrophages in accordance with this invention are formed to target and, thus, to concentrate the ligand conjugate at the site of activated macrophage populations (i.e., activated macrophages adhering to the luminal endothelial layer of the

plaque or activated macrophages present in the lipid-rich core of the plaque) in the patient being evaluated for atherosclerosis.

In one embodiment of the invention active atherosclerotic plaques comprising activated macrophages are identified/monitored in a patient being evaluated for atherosclerosis by administering a conjugate of the formula L-X wherein L comprises a ligand capable of preferentially binding to activated macrophages, compared to resting macrophages, and X comprises a chromophore or a chemical moiety capable of emitting radiation. The inner lining of a patient's blood vessels is thereafter examined with a catheter-based device capable of detecting a localized concentration of the chromophore/chemical moiety conjugated to the ligand bound to activated macrophages, or by an external imaging technique. Any external imaging technique known in the art can be used.

The ligand conjugates are typically administered as a diagnostic composition comprising a ligand conjugate and a pharmaceutically acceptable carrier. The composition is typically formulated for parenteral administration and is administered to the patient in an amount effective to enable detection of the locale of activated macrophages. The nature of the chromophore/chemical moiety component of the ligand conjugate is dictated by the methodology used for catheter-based detection of the active atherosclerotic plaques. Thus, for example, the chromophore can comprise a fluorophore, such as fluorescein, (see PCT publication number WO 01/074382, incorporated herein by reference, for a description of a ligand-fluorophore conjugate) or another chromophore such as an hematoporphyrin, or a derivative thereof, or a Raman enhancing dye or agent, or a long wavelength fluorescent dye with optical properties that allow detection through many layers of tissue. The component of the ligand conjugate used for detection can also be a chemical moiety, such as a chelating moiety and a metal cation, for example, a radionuclide. It should be noted that the method of the present invention can be used for detecting light or radioactivity emitted from ligand conjugates bound both at the surface of atherosclerotic plaques and below the surface.

Such conjugates wherein the group L is folic acid, a folic acid analog/derivative, or another folic acid receptor binding ligand are described in detail in U.S. Pat. No. 5,688,488, incorporated herein by reference. That patent, as well as related U.S. Pat. Nos. 5,416,016 and 5,108,921, each incorporated herein by reference, describe methods and examples for preparing conjugates useful in accordance with the present invention. The present macrophage-targeted ligand conjugates can be prepared and used following general protocols described in those earlier patents.

In the embodiment where the ligand conjugate comprises a chromophore for use in identifying/monitoring active atherosclerotic plaques, the blood vessel walls can be subjected to predetermined conditions to detect locations on the inner linings of blood vessels where the ligand-chromophore conjugates are concentrated (i.e., active atherosclerotic plaques). Such predetermined conditions include any conditions known in the art to be useful for the detection of a chromophore, such as a fluorophore, using a catheter-based device or external imaging technique. For example, the blood vessel walls can be subjected to radiation, in the ultraviolet, visible, or infrared region of the spectrum, from a laser. Catheter-based techniques employing optical fibers for the pulsed or steady state illumination of atherosclerotic plaques with laser radiation of a given wavelength can be used. A signal generated by the fluorescent light emitted by the ligand conjugates is then conveyed by one or more of the optical fibers to the end of the catheter where it can be analyzed to yield information

about the atherosclerotic plaque being evaluated. The light emitted can be analyzed using art-recognized techniques as described below to identify/monitor the atherosclerotic plaque being evaluated.

In view of the increase in folate receptor levels during macrophage activation, a ligand conjugate comprising a  $^{99m}\text{Tc}$  chelating chemical moiety targeted to activated macrophages using a vitamin, such as folate, can be used to detect active plaques in vivo. Such a ligand conjugate is described in U.S. Patent Application No. 60/378,571, incorporated herein by reference. Typically the activated macrophage-targeted ligand conjugate is administered to a patient, and following a period of time sufficient (e.g., from about 1 to about 24 hours) for the ligand conjugate to bind to activated macrophages associated with the active plaques, the patient is subjected to the catheterization procedure or an external imaging technique and identification/monitoring of active plaques is enabled by the targeted ligand conjugate.

Active atherosclerotic plaques can be identified/monitored in accordance with the method of the invention by, for example, spectral analysis of fluorescence emitted by the chromophore where the fluorescence emission is stimulated by radiation from, for example, a laser (e.g., laser-induced fluorescence spectroscopy), or by analysis of radioactivity emitted by the chemical moiety. Exemplary analytical techniques are described in U.S. Pats. Nos. 4,718,417 and 4,785,806, and in U.S. Patent Application Publication No. U.S. 2003-0162234 A1, each incorporated herein by reference, but any technique useful for analyzing light or radioactivity emitted from an atherosclerotic plaque to identify/monitor the atherosclerotic plaque in accordance with the invention can be used. In one embodiment, the fluorescence or radioactivity analysis is used to control an ablation laser, and accordingly, the ablation laser is activated, automatically or manually, after the diagnostic laser.

A variety of lasers known in the art can be used in the method of the invention. Exemplary lasers include holmium-doped yttrium aluminum garnet (YAG), holmium-doped yttrium lithium fluoride (YLF), and thulium-doped YAG and thulium-doped YLF. Further details regarding these and other suitable lasers are disclosed in U.S. Pats. Nos. 4,917,084 and 4,950,266, which are hereby incorporated by reference.

The methods described in U.S. Pats. Nos. 5,217,456, 5,275,594, 5,562,100, 6,167,297, 6,217,847, 6,246,901, 6,387,350, 6,507,747, incorporated herein by reference, can also be used to stimulate emission of light from ligand-chromophore conjugates in accordance with the present invention and to detect/analyze light or radioactivity emitted from the ligand conjugates.

The method of the present invention can be used alone or in combination with any other method(s) known in the art for the detection/analysis/ablation of atherosclerotic plaques. For example, the invention can be used in combination with methods to ablate atherosclerotic plaques in cases where active plaques cause narrowing of blood vessels. In such cases, the ligand conjugates of the present invention can be used not only to identify active atherosclerotic plaques as compared to inactive plaques, but also to distinguish between atherosclerotic and normal tissue to help in ablation procedures. Thus, the present invention can be used to analyze both the physiological and the morphological state of atherosclerotic plaques. For example, angioplasty involves the nonsurgical widening of a vessel narrowed by plaque deposition, and laser energy, for example, directed through optical fibers in a catheter-based device, can be used to ablate or partially remove the plaque deposits. Catheter-based devices for ablating

plaques using laser energy are described in U.S. Pat. Nos. 4,817,601, 4,850,351, and 4,950,266, incorporated herein by reference.

When laser energy is used to ablate an atherosclerotic plaque, thermal damage to normal tissue is a serious risk because the energy level of radiation emitted from lasers used for ablation of plaque can damage or destroy normal tissue with the possibility of inadvertent perforation of an artery. Accordingly, the ligand conjugates of the present invention can be used to not only identify active atherosclerotic plaques, but to distinguish between atherosclerotic plaques and normal tissue to avert damage to normal tissue during plaque ablation. Pulsed laser emission can also be used whenever continuous laser exposure might damage the tissue.

The method of the present invention can also be used in combination with other techniques for differentiating between atherosclerotic plaques (e.g., fibrous plaque, calcified plaque, and lipid plaque) and normal tissue during plaque ablation. Such techniques include techniques based on analysis of laser-induced calcium photoemission from calcified plaque and laser-induced fluorescence from noncalcified plaque. Other such techniques include the analysis of fluorescence (e.g., laser-induced fluorescence), at selected wavelengths from tissues in an artery, with or without the use of a dye to enhance the contrast between the fluorescence emitted from atherosclerotic plaques and the fluorescence emitted from normal tissue (see U.S. Pat. Nos. 4,641,650, 4,718,417, and 4,785,806, incorporated herein by reference). Other laser-based techniques that can be used in combination with the method of the present invention to differentiate between atherosclerotic plaques and normal tissue include techniques utilizing laser-induced Raman light scattering and laser-induced plasma photoemission. Any other type of technique employing diagnostic and/or ablation lasers known in the art can also be used in combination with the method of the present invention (see U.S. Pat. Nos. 4,817,601 and 4,850,351, incorporated herein by reference).

The method of the present invention can also be used in combination with any other method(s) known in the art for the detection/analysis/ablation of atherosclerotic plaques, including the methods described in U.S. Pat. Nos. 5,217,456, 5,275,594, 5,562,100, 6,167,297, 6,217,847, 6,246,901, 6,387,350, 6,507,747, incorporated herein by reference. Furthermore, the invention can be used to guide the positioning of therapeutic drugs and nucleic acid constructs positioned in the same catheter assembly or a different catheter assembly (see U.S. Patent Application Publication No. U.S. 2002-0192157 A1, incorporated herein by reference).

#### EXAMPLE 1

##### Analysis of EC20 Uptake in the Organs of Atherosclerotic Rabbits

The Watanabe Heritable Hyperlipidemic rabbit model (i.e., a model for atherosclerosis) was used to determine whether binding of a folate-targeted  $^{99m}\text{Tc}$  chelating chemical moiety (EC20; see U.S. Patent Application No. 60/378,571, incorporated herein by reference) could be detected in the arteries of atherosclerotic rabbits. Watanabe rabbits are LDL receptor deficient and, thus, provide a model for hypercholesterolemia and atherosclerosis.

Watanabe rabbits (2 rabbits each 6 months old) were purchased from HRP-Covance (PA, USA). The rabbits were kept on a folate deficient diet for 3 weeks at which time the biodistribution of EC20 was evaluated. For the EC20 biodistribution studies, each rabbit was administered with 3.4 mCi of

$^{99m}\text{Tc}$  and  $7.4 \times 10^{-9}$  moles of EC20 (EC20 in FIG. 1). One rabbit (i.e., control) also received 1000-fold excess of free folic acid (EC20+FF in FIG. 1). The total injection volume for each rabbit was 400  $\mu\text{l}$  via the marginal ear vein. The rabbits were sacrificed 4 hours after injection, and organs were extracted for EC20 biodistribution analysis (i.e., the radioactivity per gram of tissue was measured; see FIG. 1).

The results presented in FIG. 1 show that specific binding of  $^{99m}\text{Tc}$ -EC20, competed by a 1000-fold excess of free folate, was detected in the aortic arch, the inferior vena cava, the superior vena cava, the pulmonary artery, the thoracic aorta, and the abdominal aorta of the Watanabe atherosclerotic rabbits.

#### EXAMPLE 2

##### Materials

Fmoc protected amino acid derivatives, Fmoc-glycine loaded Wang resin, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and N-hydroxybenzotriazole were purchased from Novabiochem (San Diego, Calif.).  $\text{N}^{10}$ -trifluoroacetylpteronic acid was synthesized from folic acid (Sigma Chemical Company, St. Louis, Mo.) according to a previously published report (Godwin, 1972). The synthesis of gamma-carboxy linked folate-fluorescein (folate-FITC) has been described in a previous publication (Kennedy et al., 2003). RPMI Medium 1640 with L-glutamine, but without folic acid was from Gibco-BRL (Grand Island, N.Y., U.S.A.); folic acid and triglyceride reagent GPO-PAP were from DIALAB (Vienna, Austria); OCT compound embedding medium for frozen sections were from Division Miles Laboratories Inc. (Illinois, USA); Hoechst 33342 and Texas Red N-hydroxysuccinimide from Molecular Probes, Inc. (Eugene, Oreg., USA); INFINITY CHOLESTEROL reagent and other chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.).

#### EXAMPLE 3

##### Synthesis of Folate-Texas Red Conjugate

Standard Fmoc peptide chemistry was used to synthesize a folate-derivatized peptide linked to Texas Red via the gamma carboxyl of folic acid. The sequence Gly-Lys( $\gamma$ )Glu-pteronic acid was constructed by Fmoc chemistry using HBTU and N-hydroxybenzotriazole as the activating agents along with diisopropylethylamine as the base and 20% piperidine in DMF for deprotection of the Fmoc groups. Fmoc-protected lysine containing a 4-methyltrityl protecting group on the  $\epsilon$ -amine was linked to Fmoc-protected glycine attached to a Wang resin. An  $\alpha$ -t-Boc protected N- $\alpha$ -Fmoc glutamic acid was then linked to the peptide to provide a  $\gamma$ -linked conjugate on folate after attaching  $\text{N}^{10}$ -trifluoroacetylpteronic acid to the peptide. The methoxytrityl protecting group on the  $\epsilon$ -amine of lysine was removed with 1% trifluoroacetic acid in dichloromethane to allow attachment of Texas Red. Texas Red N-hydroxysuccinimide (Molecular Probes, Eugene, Oreg.) in DMF was reacted overnight with the peptide and then washed thoroughly from the peptide resin beads. The folic acid-Texas Red peptide was then cleaved from the resin with 95% trifluoroacetic acid:2.5% water:2.5% triisopropylsilane solution. Diethyl ether was used to precipitate the product and the precipitant was collected by centrifugation. The product was then washed twice with diethyl ether and dried under vacuum overnight. The product was then analyzed and confirmed by mass spectroscopic analysis ( $[\text{M}^-]$  calculated: 1423, found:

1422). In order to remove the N<sup>10</sup>-trifluoroacetyl protecting group, the product was dissolved in 5 mls of water containing 0.5 mls of 10% ammonium hydroxide with the pH adjusted to 9.5-10.0 and stirred for 30 minutes at room temperature. The product was then precipitated using isopropanol/ether and the precipitant was collected by centrifugation. The product was then added to a G-10 Sephadex gel filtration column (1.5×15 cm) using water as the eluent. The product peaks were collected and lyophilized.

## EXAMPLE 4

## Animal Model

Male healthy RAP mice and Golden Syrian Hamsters were selected for the experiments. Animals were divided in three experimental groups: animals fed with (i) normal diet (N) containing 0.1 g folic acid/100 kg; (ii) folic acid deficient diet (D), and (iii) folic acid deficient diet administered together with hypercholesterolemic food containing 3% cholesterol and 15% butter (H) for 6 months. The folate deficient diet was designed to bring the serum folate levels into the physiologic folate concentration range. Early experiments demonstrated that animals fed with a hyperlipidemic diet and folic acid (0.1 g/100 kg food) developed extensive atherosclerosis; however the specific uptake of folate in these animals could not be measured because of the competition with the high excess of folic acid and the consequent receptor down regulation. Because commercial animal diets are supplemented with supra-physiological concentrations of folic acid, serum folate levels in these "normal" animals will often exceed natural levels by forty-fold (Wang et al.). Such high serum folate contents results in suppression of FR expression.

## EXAMPLE 5

## Folate Receptors on Macrophages in Hyperlipidemic Animals

Folic acid conjugated to either FITC (FA-FITC) or to Texas Red (FA-TR) were injected i.p. (10 µg/100 g body weight), and after 4 hours animals were bled (under light ether anesthesia) and then killed by carotid section. A macrophage-enriched suspension (also containing some mast cells, PMN, and monocytes) was then obtained by peritoneal lavage (Aviram, 1989). Briefly, the peritoneal cavity was injected with 10 ml cold phosphate buffered saline (PBS), pH 7.4, and gently massaged for 2 min. The suspension of peritoneal cells was filtered through nylon cloth and washed two times with PBS by centrifugation at 250×g, 10 minutes at 4° C. The pellet was then solubilized in cold lysis buffer (1% NP-40, 50 mM TRIS-HCl, 2 mM EDTA, 1 mM DTT, PMSF, protease inhibitors (leupeptin, antipain, and pepstatin A, 10 µg/ml each)), cleared by centrifugation, and the fluorescence was quantified with a RF-5001 PC spectrophotofluorimeter using a standard curve. Protein concentration was determined by Amido Black and the results were expressed as a ratio of ng FA/mg protein for each group of animals. The same determinations were performed for the serum sample collected from each animal (ng FA-FITC/serum protein). In control experiments animals were injected with the FA-FITC conjugate in the presence of a 100× excess of free folic acid. The total cholesterol and serum triglyceride concentration after six months of diet were measured using specific reagent kits.

In order to determine whether peritoneal macrophages from Golden Syrian hamsters express functional folate receptors, folate-FITC was injected into the hamsters intraperito-

neally and uptake of the folate conjugate by macrophages extracted 4 hours later was examined by quantitative fluorimetry. The results in FIG. 2 show a significant increase in the folate conjugate's retention by macrophages harvested from hyperlipemic hamsters (H) compared to animals maintained on either normal (high folate) diet (N) or FA deficient diet (D). The folic acid deficient diet induced a significant increase in folate-FITC uptake in both groups D and H, but the folate-FITC levels were 2.4 times higher in macrophages from group H animals (which received hyperlipidemic diet) than controls (N). These data suggest that maintenance of the animals on a folate deficient diet stimulates partial up-regulation of the folate receptor, and that the added exposure of the animals to a high fat/high cholesterol diet further enhances FR expression on peritoneally-derived macrophages. Importantly, the same differences were not detected in macrophages isolated from mice maintained on a similar diet (FIG. 3), a result that may be explained by the fact that mice are resistant to atherosclerosis, in contradistinction to hamsters which develop lesions similar to those of humans (Sima et al. 1990).

In all experimental groups, the serum level of FA-FITC 4 hours post injection was measured. The data indicate a higher concentration of the folate conjugate in serum of hyperlipemic hamsters, suggesting either an acquired deficiency in a clearance mechanism or an increase in folate conjugate binding by serum proteins has occurred in response to the high fat diet (see FIG. 4). The hyperlipidemic condition of the hamsters maintained on the hypercholesterolemic diet was also confirmed by the high level of both total serum cholesterol and triglyceride (FIG. 5). Taken together, the results in FIGS. 2-5 show that the hypercholesterolemic diet induces abnormalities in folate homeostasis, including at least an increase in folate uptake by macrophages and a decrease in clearance of the conjugates from serum.

## EXAMPLE 6

## Preparation of Tissue Sections

Tissue fragments from atherosclerotic lesion-prone areas (aorta and valves) were collected from each experimental group and processed for fluorescence microscopy. The aorta was exposed, the branching arteries were cut off, and loose adventitial tissue was removed in situ. The vessel was then cut open and thoroughly washed with cold sterile PBS. Aorta and cardiac valves were carefully excised and all segments were fixed in 4% p-formaldehyde in PBS for 90 min at room temperature. The tissues were then immersed in OTC medium, flash-frozen in liquid nitrogen, and crysectioned. The frozen sections were stained with oil red O and counterstained with hematoxylin (Mancini, 1995). Similar semithin cryosections were examined both in phase contrast and fluorescent microscopy using appropriate filters. Some tissue fragments were processed for standard electron microscopy (Simionescu et al. 1990) in order to visualize the lesion morphology. Thin sections were mordent with uranyl acetate and lead citrate and examined with a Phillips 400 electron microscope.

To determine whether common sites of atherosclerosis might be affected by altered folate homeostasis, 4 hours after i.p. injection of the folate-TR conjugate, tissue fragments from atherosclerotic lesion-prone areas were collected from Golden Syrian hamsters fed on the atherosclerotic diet and processed for fluorescence microscopy. Although animals maintained on either the normal or low folate diet did not develop atherosclerotic lesions and displayed normal serum concentrations of total cholesterol and triglycerides, within 6

months of transfer to the hyperlipidemic diet hamsters developed extensive lesions on both the aorta and cardiac valves (FIG. 13). The lesions displayed a heterogeneous structure and are heavily laden with lipid, as revealed by Oil Red staining of the semithin cryosections (FIG. 6a). Importantly, adjacent unstained sections examined by fluorescence microscopy reveal significant uptake of the folate-Texas Red conjugate, as indicated by the prominent red fluorescence of the fatty lesions (FIG. 6b). Thus, intense FA-TR uptake is seen in distinct foci throughout the atherosclerotic valve (FIG. 6c), while low level FA-TR retention is observed rather uniformly across the fatty lesions. It is possible that the intense fluorescent spots constitute active microdomains where younger activated macrophages are accumulating, whereas the less intensely fluorescent regions may correspond to areas enriched in lipid laden cells (foam cells) that have gradually become more quiescent. The double staining of the sections with Oil Red O (for lipids) and Hoechst (for nucleic acids) confirms clearly that numerous foam cells are tightly packed in the lesion area (FIG. 6c).

#### EXAMPLE 7

##### Specific Uptake of FA-Texas Red in Cultured Peritoneal Macrophages

Hamster peritoneal macrophages ( $10\text{-}20 \times 10^6$  per hamster) were harvested from peritoneal fluid and washed by centrifugation  $3 \times$  in PBS at  $1000 \times g$  for 10 minutes. Cells were suspended in 10 ml of RPMI Medium 1640 containing L-glutamine but lacking folic acid and supplemented with 10% fetal bovine serum (heat inactivated at  $56^\circ\text{C}$ . for 30 minutes), 100 U/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin. Folic acid was left out of the culture medium to prevent down-regulation of cell surface FR, which usually occurs when cells are cultured in the high folate concentrations present in unmodified RPMI. The peritoneal macrophages were then plated into 50 mm Petri dishes and cultured in a humidified incubator (5%  $\text{CO}_2$ , 95% air). After 2 hours of incubation, the cells were washed to remove non-adherent cells, and further incubated under similar conditions for an additional 18 hours. The cells were briefly washed, mounted with slow fade and examined under a Texas red filter in the fluorescent microscope (Nikon).

Macrophages harvested from the peritoneum of FA-TR treated hypercholesterolemic hamsters were also found to be significantly more fluorescent (FIG. 7a) than macrophages similarly isolated from animals fed a normal lipid diet, demonstrating that the elevated uptake previously observed for folate-FITC (FIG. 2) can also be replicated using an independent folate conjugate, i.e., FA-TR. Since activated, but not resting macrophages have been reported to express a folate receptor (Nakashima-Matsushita, 1999), the data support the hypothesis that atherosclerosis is an inflammatory disease in which activated macrophages contribute to the development of the plaque.

#### EXAMPLE 8

##### Specific Uptake of FA-FITC in the U937 Cell Line

To extend the study of live animal models, cell culture experiments were also conducted using U937 cells (Harris, 1985), a cell line that originated from the pleural fluid of a patient with diffuse histiocytic lymphoma and which exhibits many characteristics of monocytes. To facilitate their expression of folate receptors, U937 cells were grown in folate-

deficient RPMI supplemented with either 5% FCS or with 5% serum from patients with high cholesterol and glucose levels (Chl 295 mg/dl, Glc 315 mg/dl). In parallel experiments aimed at evaluating FR expression following a more classical method of U937 cell activation, U937 cells were incubated in RPMI without folic acid but supplemented with 5% FCS in the presence or absence of 0.5 g/ml bacterial lipopolysaccharide (LPS) or 40 ng/ml phorbol-12 myristate acetate (PMA). After 24 hours of activation either with patient's serum or LPS, cells were incubated with 2.5 g/ml FA-FITC. The cells were then washed with PBS and solubilized in lysis buffer (50 mM Tris-HCl, 1% NP-40, 2 mM EDTA, and 1 mM DTT and protease inhibitors), and FA-FITC uptake was assayed by spectrophotofluorimetry. Protein concentration was determined by BCA methods and the results were expressed as ratio of ng FA-FITC/mg protein for each experimental condition.

U937 cells (Sundstrom, 1976), are cells derived from committed progenitors of the monocyte lineage. To detect the stimuli that induce FR up-regulation during macrophage activation, experiments were performed on U937 cells exposed to hyperlipidemic sera, LPS or PMA. In preliminary experiments, it was verified that uptake of FA-FITC by U937 cells is specifically folate receptor mediated by demonstrating that addition of 100-fold excess free folic acid would block uptake of the FA-FITC conjugates. FA-FITC retention by U937 cells grown in either normal (N) medium (RPMI supplemented with 5% fetal calf serum), hyperlipidemic (H) medium (RPMI supplemented with 5% serum from a hypercholesterolemic patient), or in folate deficient RPMI supplemented with 5% fetal calf serum (D), was then evaluated. For comparison, we also examined folate-FITC internalization by U937 cells grown in the same folate deficient RPMI treated with LPS or PMA. As shown in FIG. 8, by the end of the 4 hour incubation, higher FA-FITC uptake (1.6 $\times$ ) was observed in U937 cells grown in medium supplemented with the hyperlipidemic serum (H) than in cells maintained in normal (N) medium (FIG. 8). Interestingly, an even higher level of folate-FITC retention ( $\times 2$ ) was obtained when cells were exposed to bacterial lipopolysaccharide (LPS), a ligand known to induce secretion of chemokines responsible for differentiation of U937 cells into cells with characteristics of an activated macrophage (Wang, 1998). A similar increase ( $\times 2.1$ ) was observed when the cells were stimulated with the phorbol myristate acetate (PMA), a mimic of diacylglycerol known to activate protein kinase C (Smart et al. 1994). Taken together, these experiments suggest that hypercholesterolemic conditions induce over-expression of folic acid receptors on macrophages, in a manner similar to direct activation of the macrophages by treatment with lipopolysaccharide or PMA.

#### EXAMPLE 9

##### $^{99m}\text{Tc}$ -Folate SPECT-CT

EC20 was prepared as described in U.S. Patent Application No. 60/378,571, incorporated herein by reference. C57BL/6 male mice were prepared for imaging assays by maintaining the mice for more than 6 months on a high cholesterol and high fat diet of coconut butter (see Paigen et al., *Atherosclerosis*, 57:65-73 (1985), incorporated herein by reference). The mice were rendered diabetic by 6 daily intraperitoneal injections of streptozotocin at a dose of 55 mg/kg.

After 6 months on the high cholesterol and high fat diet, the animals were injected with 10 mCi of  $^{99m}\text{Tc}$ -folate intravenously. After about 5 hours, the animals were anesthetized with isoflurane and microSPECT-CT images of the distribu-

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tion of  $^{99m}\text{Tc}$ -folate were recorded (see FIGS. 9-11). SPECT-CT was performed with an instrument from Gamma Medica Instruments, Northridge, Calif. SPECT was performed with a 1 mm pinhole collimator using 64 projections and 60 seconds/projection. CT was performed with a project resolution of 128 and a 360-degree rotation. 5

As shown in FIG. 9 (control mouse) as compared to FIG. 10 (mouse maintained on a high cholesterol and high fat diet),  $^{99m}\text{Tc}$ -folate (EC20) localizes to the heart in atherosclerotic mice and is detectable by microSPECT-CT imaging. In the mouse shown in FIG. 11 localization to the heart was not detectable by microSPECT-CT imaging, likely because the mouse (mouse B) did not develop atherosclerosis that was as severe as mouse A. However, as described in Example 10 below, inflammation and formation of atherosclerotic plaque occurred in mouse B (maintained on a high cholesterol and high fat diet) and the inflammation and plaque is detectable with  $^{99m}\text{Tc}$ -folate (EC20). 10

## EXAMPLE 10

 $^{99m}\text{Tc}$ -Folate Biodistribution and Autoradiography

Mice were maintained on a high cholesterol and high fat diet as described in Example 9 and were injected with 10 mCi of  $^{99m}\text{Tc}$ -folate intravenously. After microSPECT-CT imaging as described in Example 9, the mice were sacrificed by  $\text{CO}_2$  asphyxiation. Autoradiography of the aorta and of individual heart slices was performed. The specimens were placed on the autoradiographic plate and exposed for an additional month to record the distribution of  $^{99m}\text{Tc}$ -folate (see FIG. 12 C). Some samples of the mouse hearts were stained with H & E and examined microscopically to confirm the findings of inflammation in atherosclerotic arteries (see FIGS. 12 A and B). The results presented in FIG. 12 A-C show that inflammation and formation of atherosclerotic plaque has occurred in mice (mouse B in this case) maintained on a high cholesterol and high fat diet and that the inflammation and plaque is detectable with  $^{99m}\text{Tc}$ -folate (EC20). 15

The invention claimed is:

1. A method of identifying/monitoring active atherosclerotic plaques associated with blood vessel walls wherein the

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plaques comprise activated macrophages having accessible binding sites for a ligand said method comprising the steps of: administering to a patient suffering from atherosclerosis an effective amount of a composition comprising a folate receptor binding ligand conjugate of the general formula

L-X

wherein the group L comprises the folate receptor binding ligand wherein the folate receptor-binding ligand exhibits specific binding to the folate receptor, and the group X comprises a chemical moiety capable of emitting radiation;

allowing sufficient time for the folate receptor-binding ligand conjugate to bind to the activated macrophages associated with the active plaques; and

identifying active plaques by detecting radiation emitted by the chemical moiety associated with blood vessel walls using a method selected from the group consisting of the use of a catheter-based device and external imaging. 20

2. The method of claim 1 wherein the chemical moiety comprises a metal chelating moiety.

3. The method of claim 2 wherein the chemical moiety further comprises a metal cation.

4. The method of claim 3 wherein the metal cation is a radionuclide.

5. The method of claim 1 wherein the radiation emitted by the chemical moiety is detected using a catheter-based device.

6. The method of claim 1 wherein the radiation emitted by the chemical moiety is detected by external imaging. 25

7. The method of claim 4 wherein the radiation emitted by the chemical moiety is detected using a catheter-based device.

8. The method of claim 4 wherein the radiation emitted by the chemical moiety is detected by external imaging. 30

9. The method of claim 7 wherein the ligand is folate or a folate analog.

10. The method of claim 8 wherein the ligand is folate or a folate analog. 35

11. The method of claim 9 wherein the ligand is folate. 40

12. The method of claim 10 wherein the ligand is folate.

\* \* \* \* \*

|                |   |         |            |
|----------------|---|---------|------------|
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摘要(译)

本发明涉及鉴定/监测与血管壁相关的活动性动脉粥样硬化斑块的方法，其中所述斑块包含具有配体的可接近结合位点的活化巨噬细胞。该方法包括给予正在评估动脉粥样硬化的患者施用有效量的组合物的步骤，所述组合物包含配体和能够在预定条件下发光的发色团的缀合物，允许配体缀合物有足够的时间与活化的巨噬细胞结合。使用基于导管的装置使血管经受预定条件，并通过使用基于导管的装置或通过使用外部成像技术检测由发色团发射的光来识别活性斑块。本发明还涉及类似的方法，其中能够发射辐射的化学部分与配体缀合。

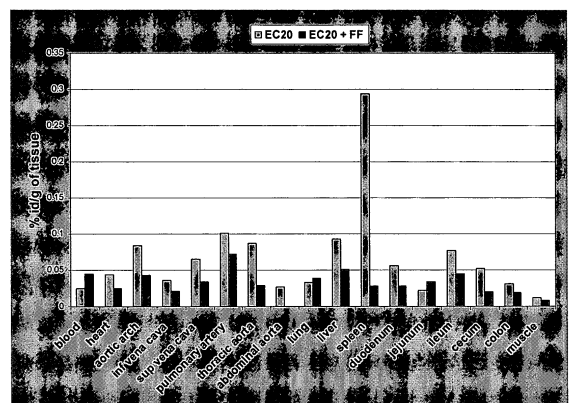


FIG. 1