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Method for collecting and identifying markers of coronary disease

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ABSTRACT

The present invention relates to a method of collecting and identifying markers of coronary disease. It further includes providing a means of monitoring intra-arterial pathology.

In one aspect of the present invention, there is provided a method of identifying a marker of coronary disease, said method including the steps of:

perfusing coronary tissue of patients having atherosclerotic or non-atherosclerotic coronary arteries;

collecting perfusate from the coronary tissues; and

identifying the marker from the perfusate in which it is present.

In another aspect of the present invention, there is provided a method of diagnosing coronary disease, said method comprising:

identifying the presence or absence of a coronary marker said marker being identified by a method described above ; and

correlating the coronary marker to a coronary disease.

The markers may be useful in monitoring coronary disease such as atherosclerosis, angina, stroke and heart attack.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method of identifying a marker of coronary disease, said method including the steps of:

perfusing coronary tissue of patients having atherosclerotic or non-atherosclerotic coronary arteries;

collecting perfusate from the coronary tissues; and

identifying the marker from the perfusate in which it is present.

2. A method according to claim 1, wherein the coronary tissue includes the circulatory system of the heart from the aorta to the coronary sinus or a portion of the circulatory system thereof.

3. A method according to claim 1 or 2 further including determining a marker that is either present or absent from a patient having atherosclerotic or non-atherosclerotic coronary arteries.

4. A method according to any one of claims 1 to 3 wherein the perfusing of the coronary tissue is conducted antegradely through the coronary tissue.

5. A method according to any one of claims 1 to 4 wherein the perfusate is collected from the coronary sinus.

6. A method according to any one of claims 1 to 5 wherein the coronary disease is selected from the group including atherosclerosis, angina, stroke or heart attack.

7. A method according to any one of claims 1 to 6 wherein the coronary disease is atherosclerosis.

8. A method of diagnosing coronary disease, said method comprising:

identifying the presence or absence of a coronary marker said marker identified by a method according to any one of claims 1 to 7; and

correlating the coronary marker to a coronary disease.

9. A method according to claim 8 wherein the coronary disease is selected from the group including atherosclerosis, angina, stroke or heart attack.

10. A method of diagnosing arterial pathology in a patient, said method comprising:

collecting coronary perfusate;

identifying the presence of a coronary marker that is indicative of arterial pathology in the perfusate; and

correlating the coronary marker to a level of arterial pathology.

11. A method according to claim 10 wherein the coronary perfusate is collected by perfusing coronary tissue.

12. A method according to claim 11 wherein the perfusing of the coronary tissue is conducted from the coronary aorta to the coronary sinus.

13. A method according to any one of claims 10 to 12 wherein the coronary marker is identified by a method according to any one of claims 1 to 8.

14. A method according to any one of claims 8 to 13 wherein the coronary marker is selected from haptoglobin (Hap), Ig Kappa V-II, or a combination of Hap and Ig Kappa V-II.

15. Haptoglobin when used in a method according to any one of claims 8 to 13.

16. Ig Kappa V-II when used in a method according to any one of claims 8 to 13.

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ORIGINAL
COMPLETE SPECIFICATION
STANDARD PATENT

Invention Title: METHOD FOR COLLECTING AND IDENTIFYING MARKERS OF CORONARY DISEASE

Applicant: THE HEART RESEARCH INSTITUTE

The following statement is a full description of this invention, including the best method of performing it known to me:

METHOD FOR COLLECTING AND IDENTIFYING MARKERS OF CORONARY DISEASE

The present invention relates to a method of collecting and identifying markers of coronary disease. It further includes providing a means of monitoring intra-arterial pathology.

BACKGROUND

Statistics tell us that heart disease costs Australia upwards of $3.5 billion each year. 14.5% of all cardio-vascular deaths are premature because they are often undetected and then it is too late.

Atherosclerosis is the disease of blood vessels which causes heart attacks and strokes and leads to the death of almost 50% of all Australians. This debilitating disease is characterized by the hardening and thickening of arteries being the major cause of the heart disease. Arteries become blocked generally due to the presence of atherosclerotic lesions.

The atherosclerotic lesions may be small but large enough to constrict the artery and restrict blood flow. However, their presence can only be detected by methods such as coronary angiography. If undetected, heart attack may result for which coronary surgery such as a by-pass operation, is the only immediate solution. However, this may not prevent further heart attacks and it would be desirable to monitor the condition of the arteries of a patient having the propensity for further heart attacks. Currently available methods cannot monitor the arterial pathology particularly at the site causing the attack.

It would also be desirable to identify markers of atherosclerosis which are useful for diagnosis and which are indicative of the intra-arterial pathology *in vivo*. These markers may enable early detection of defects in the heart condition before a serious attack occurs.

SUMMARY OF THE INVENTION

In one aspect of the present invention, there is provided a method of identifying a marker of coronary disease, said method including the steps of:

perfusing coronary tissue of patients having atherosclerotic or non-atherosclerotic coronary arteries;

collecting perfusate from the coronary tissues; and

identifying the marker from the perfusate in which it is present.

In a preferred aspect of the invention there is provided a preliminary step, said method further including:

determining a marker that is either present or absent from a patient having atherosclerotic or non-atherosclerotic coronary arteries.

In a preferred aspect of the invention there is provided a method of identifying a marker of coronary disease, said method including the steps of:

perfusing coronary tissue from the coronary aorta to the coronary sinus or a portion thereof of patients having atherosclerotic or non-atherosclerotic coronary arteries;

collecting perfusate from the coronary sinus; and optionally identifying a marker that is either present or absent from a patient having atherosclerotic or non-atherosclerotic coronary arteries; and

collecting the marker from the perfusate in which it is present.

In another aspect of the present invention, there is provided a coronary marker collected by the methods described above.

In another aspect of the present invention, there is provided a method of diagnosing coronary disease, said method comprising:

identifying the presence or absence of a coronary marker said marker being identified by a method described above ; and

correlating the coronary marker to a coronary disease.

In another aspect of the present invention there is provided a coronary marker molecule useful as an indicator of arterial pathology. Preferably the marker has been collected and identified by the method described above.

In another aspect of the present invention, there is provided a method of diagnosing arterial pathology in a patient, said method comprising:

collecting coronary perfusate;

identifying the presence of a coronary marker that is indicative of arterial pathology in the perfusate; and

correlating the coronary marker to a level of arterial pathology.

The coronary perfusate may be collected as described above by flushing the coronary vessels and collecting the perfusate.

DETAILED DESCRIPTION OF THE INVENTION

In one aspect of the present invention, there is provided a method of identifying a marker of coronary disease, said method including the steps of:

perfusing coronary tissue of patients having atherosclerotic or non-atherosclerotic coronary arteries;

collecting perfusate from the coronary tissues; and

identifying the marker from the perfusate in which it is present.

This method may be useful where the marker may be known.

In a preferred aspect of the invention there is provided a preliminary step, said method further including:

determining a marker that is either present or absent from a patient having atherosclerotic or non-atherosclerotic coronary arteries.

This adaptation of the method is useful where the marker is not known and the difference between the atherosclerotic and non-atherosclerotic coronary artery patients must be determined to identify a potential marker which distinguishes the groups. The marker may be determined to be present or absent from a

patient with atherosclerotic or non-atherosclerotic coronary arteries when one is compared with the other. Hence the marker may be used as a positive or negative marker of coronary disease.

The perfused coronary tissue may include the circulatory system of the heart from the aorta to the coronary sinus (final vein which leaves the heart before the coronary blood mixes with non-coronary blood) or it may include a portion of the circulatory system between the aorta and the coronary sinus. Hence it is preferred that the whole heart is perfused, however a portion of the heart may also be perfused to collect coronary perfusate.

In a preferred aspect of the invention there is provided a method of identifying a marker of coronary disease, said method including the steps of:

perfusing coronary tissue from the coronary aorta to the coronary sinus or a portion thereof of patients having atherosclerotic or non-atherosclerotic coronary arteries;

collecting perfusate from the coronary sinus; and optionally identifying a marker that is either present or absent from a patient having atherosclerotic or non-atherosclerotic coronary arteries; and

collecting the marker from the perfusate in which it is present.

It is preferred that the perfusion starts from the aorta or arteries leading from the aorta. However, the starting point of the perfusion may be any point after the aorta which leads to the coronary sinus. This invention takes advantage of patients under going by-pass surgery and allows collection of *in vivo* perfusate of coronary arteries for identification of coronary markers.

Without being restricted by theory and limited to this description, at the onset of by-pass surgery, a wash solution or solution which paralyses the heart muscle is generally passed antegradely down the coronary arteries via their orifices in the aorta. The solution may be placed in the root of the aorta, and, as the orifices of the arteries are the only openings in the aorta, the solution passes down along the arteries, eventually empting out via the coronary veins (hence antegrade or forward flow). The solution may pass along the arteries, then the

capillaries, and either enters the heart muscle, or comes out into the coronary veins, finally emptying into the right atrium of the heart and the general circulation. The final vein which collects the coronary venous blood from the heart before allowing it to mix with non-coronary blood is the coronary sinus (which is the main vein of the heart).

This collection, because it allows a maximal concentration of marker (straight out of the coronary arteries and thus undiluted from other blood streams) and because the perfusate is a clean medium which reduces the contamination of marker molecules with the myriad components in blood, will allow the initial characterisation and identification of a marker. However, this marker, once identified, may subsequently be quantified in complex fluids such as blood with the development and refinement of appropriate assays.

Given the biochemical complexity of an atherosclerotic plaque, and for a diagnostic marker to leave the vessel wall, Applicants have found that by using perfusate of atherosclerotic arteries, markers directly indicative of the condition of an atherosclerotic artery may be collected and identified. Measurement of blood and plasma or other bodily fluids may be too far from the point of heart disease and hence it is believed that samples taken directly from the coronary arteries would not only provide a more accurate picture but provide a stronger source of the marker.

Markers which are identified in coronary artery perfusate may subsequently be sought and quantified in plasma or other complex bodily fluids in order to facilitate the utilisation of markers to diagnose or otherwise characterise coronary disease preferably coronary artery disease.

Coronary disease may be any disease of the heart such as, atherosclerosis, angina, stroke or heart attack. Preferably the disease is atherosclerosis. Atherosclerosis is characterized by the atherosclerotic lesions and it is believed that the lesions would provide a valuable source of markers of atherosclerosis.

A suitable time to perform the coronary perfusion is when the patient is undergoing coronary surgery such as by-pass surgery.

Samples (perfusate) may be collected from the coronary sinus in consecutive fractions or as one perfusate. Samples may be generated by the administration of physiologically acceptable fluid or buffer such as 0.9% saline or phosphate buffered saline into the aorta or a region between the aorta and coronary sinus which depending on where the perfusion starts may then antegradely flush the coronary arteries, the coronary veins, and eventually into the coronary sinus. The coronary perfusate may then be collected via an indwelling catheter placed in the coronary sinus. Preferably, to prevent contamination of the perfusate with blood which may wash around the opening of the coronary sinus, the perfusate is collected via a tube (catheter) which has a balloon cuff, to partially isolate the material coming down the veins from that in the right atrium. There are greater concentrations of coronary sinus blood in initial sample fractions and decreasing blood concentrations in later fractions. However these blood contaminants may be taken into account providing a control sample is compared or by relating quantities of marker molecules to the quantities of the contaminant which are present.

Patients having atherosclerotic coronary arteries and coronary atherosclerosis may be identified according to the characteristics of their preoperative coronary angiograms. Angiographic data are used to establish those patients suitable and defined as having minimal or extensive coronary atherosclerosis. These are described according to the severity (% stenosis) and extent (coronary surface are involved, or number of arteries affected) of coronary atherosclerosis. Patients who have angiographically normal arteries or have angiographically minor arterial irregularities (<20% stenosis) are the control patients (minimal coronary atherosclerosis). Patients with severe and extensive coronary disease affecting two or three major coronary arteries with >50% stenosis are those characterised as being affected with atherosclerosis (extensive atherosclerosis). For the purposes of this invention, patients with intermediate degrees of atherosclerosis may be excluded to ensure marker molecules can distinguish between minimal and extensive atherosclerosis.

The level of stenosis may also be used to separate those having atherogenic or non-atherogenic coronary arteries. Preferably, control or non-atherogenic coronary patients will have no more than 30% stenosis, more preferably 20% or less. Others will be classified as atherogenic coronary arteries.

The presence or absence of a potential marker may be determined by comparing the perfusate of an atherogenic or non-atherogenic coronary artery. Methods of comparison include gel electrophoresis, antibody or chemical detection. The method of detection will depend on the type of molecule destined to be the marker. For instance, if a protein is destined to be a marker, SDS-PAGE gel electrophoresis, 2D-electrophoresis, spectrophotometry techniques, chemiluminescence detection, Western blotting with antibodies, FPLC, affinity chromatography, HPLC, Mass Spectrometry and protein sequencing may be used. Nuclear molecules such as mRNA and DNA may also be detected with appropriate quantitative PCR and blotting techniques and microchip arrays. Fats and carbohydrates may be detected by spectrophotometric methods, HPLC, and mass spectrometry.

Once the difference in marker or molecule is established, the marker may be isolated by methods known to the skilled addressee.

Isolation of the coronary marker will depend on the type of molecule. If the molecule is a protein, standard protein isolation techniques and proteins sequencing techniques are available. For carbohydrates, fats or lipids chromatography may be adopted. This may include standard chromatographic methods such as, HPLC, FPLC, electrophoresis, affinity chromatography, mass spectometry or GC-MS. Where the marker is a newly identified molecule, these may be chemically synthesized or isolated by standard techniques dependent upon the chemical structure.

In another aspect of the present invention, there is provided a coronary marker collected and identified by the methods described above.

In another aspect of the present invention there is provided a coronary marker molecule useful as an indicator of arterial pathology or coronary disease. Preferably the marker has been collected and identified by the methods described above.

A marker may serve to differentiate between those patients who have minor or extensive atherosclerosis. Alternatively a marker may qualitatively differentiate between patients with known atherosclerosis according to their risk of future disease progression and/or coronary thrombosis.

In a preferred embodiment the coronary marker molecule is haptoglobin (Hap) or Ig Kappa V-II. These molecules have been identified by the applicants to be present in perfusates of coronary tissue from atherosclerotic patients.

In another aspect of the present invention, there is provided a method of diagnosing coronary disease, said method comprising:

identifying the presence or absence of a coronary marker said marker being identified by a method described above ; and

correlating the coronary marker to a coronary disease.

In another aspect of the present invention, there is provided a method of diagnosing arterial pathology in a patient, said method comprising:

collecting coronary perfusate;

identifying the presence of a coronary marker that is indicative of arterial pathology in the perfusate; and

correlating the coronary marker to a level of arterial pathology.

The coronary perfusate may be collected as described above by flushing preferably antegradely, the coronary vessels preferably from the aorta, and collecting the perfusate.

Identification of the coronary marker will depend on the type of molecule. As described above, the identification will depend on whether it is a protein, carbohydrate, fat, lipid or chemical compound. Methods available to the skilled

addressee can be employed to determine levels of the markers in various samples.

The level of arterial pathology is a measure of the arterial pathology. For instance, if a marker is indicative of atherosclerotic lesions, the level of the marker will be high or low in a atherosclerotic coronary artery compared with a non-atherosclerotic coronary arterial. Hence the level of the artery pathology may be quantitated or determined qualitatively.

Preferably, in the described method, haptoglobin (Hap) or Ig Kappa V-II is used as the coronary marker. Hap is an acute phase plasma glycoprotein, of which the beta- subunit is absent in normal intima, but is abundant in atherosclerotic lesions. Hap has been found by the applicants to elute from coronary arteries in patients undergoing coronary artery by-pass surgery. Identification of its presence is an indication of intra-arterial pathology and the presence of atherosclerotic lesions.

Similarly, this molecule is surprisingly found in the coronary perfusates and correlates with patients having coronary atherosclerosis. It is not expected to be present in normal plasma and hence its presence in coronary perfusates of atherosclerotic patients make it a useful indication of atherosclerotic coronary disease.

Hence the present method finds use for those patients undergoing bypass surgery as an indicator of their intra-arterial pathology, and for those patients with hitherto undisclosed undiagnosed coronary pathology, preferably atherosclerosis.

Throughout the description and claims of this specification, the word "comprise" and variations of the word, such as "comprising" and "comprises", is not intended to exclude other additives, components, integers or steps.

The present invention will now be more fully described with reference to the following examples. It should be understood, however, that the description

following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

EXAMPLES

Example 1 - Haptoglobin Elutes From Atherosclerotic Human Coronary Arteries *In Vivo*

The egress of proteins from atherosclerotic arteries *in vivo* may potentially provide an indicator of inra-arterial pathology. Haptoglobin (Hap) is an acute phase plasma glycoprotein, of which the beta subunit is absent in the normal intima, but is abundant in atherosclerotic lesions. (Stastny JJ. and Fosslein, E (1992) Exp Mol pathology 57, 205-214, 1992). The present study investigated whether Hap eluted from coronary arteries in patients undergoing coronary artery by-pass graft surgery to establish the feasibility of detecting arterial protein in coronary bypass surgery.

The study recruited a total of 38 consecutive (24 male and 14 female) subjects, of whom 31 had angiographic evidence of 2 or 3 vessel disease and 7 had angiographically minor disease (no more than 30% stenosis, controls). Antegradely flushed coronary perfusate was collected in sequential portions (total of 155 fractions) from the coronary sinus by retrograde catheter. Blood contamination was quantitated by haemoglobin (Hb) assay on non centrifuged aliquots of each fraction and the erythrocyte free portion was analysed for total protein. Proteins were separated by SDS-PAGE gel electrophoresis and Hap beta chain quantified by Western blotting and probing with mouse anti-human Hap antibody and chemiluminescene detection. To account for blood contamination in perfusates, Hap beta was quantitated as ug per ug Hb in all fractions.

In each subject, successive fractions contained decreasing blood contamination, and individual fractions were stratified (A-D) as containing >10%(A), 1-9%(B), 0.1-0.9%(C) and <0.1%(D) contaminating blood volume/volume. For each individual patient total protein Hap beta increased relative to Hb with successive fractions, indicating that protein and Hap beta

were simply not arising from contaminating blood, but from the coronary circulation. Hap beta/Hb was greatest in fractions with least blood contamination (0.08±0.01 ug/ug, n=65) for fraction D versus 0.04±0.01 ug/ug for fraction C, mean±SEM, n=35, $P<0.05$). In both fractions C and D, atherosclerotic arteries eluted more Hap beta than corresponding controls, (fraction C: 0.089±0.01 ug/ug (n=65) versus 0.023±0.014 ug/ug (n=11) and fraction D, 0.04±0.006 ug/ug (n=35) versus 0.01±0.004 ug/ug (n=7), ($P<0.05$). In fractions A and B similar trends were seen but these were not statistically significant.

This model establishes the principle that proteins such as Hap do elute from the atherosclerotic coronary circulation. Hap may be a future useful indicator of arterial pathology.

Example 2 - Ig Kappa V-II elutes from Atherosclerotic Human Coronary Arteries *in vivo*

The present study investigated the presence of a molecule which has the potential as coronary marker.

Perfusates from patents with coronary atherosclerosis were collected by perfusing coronary tissue from the coronary artery to the coronary sinus using a wash solution comprising a physiologically acceptable fluid or buffer such as 0.9% saline or PBS.

Successive fractions were collected as described in Example 1 for Hap.

Using SDS-PAGE, an approximately 30kDa protein was identified in coronary atherosclerosis patients correlating with those also showing the presence of Hap beta chain.

The 30 kDa chain was analysed by Edman degradation and N-terminal sequencing after electrophoretic transfer PVDF membrane. The N-terminal

sequence to 8 residues identified the protein as Ig KAPPA chain V-II Region GM607.

Thus, this molecule identified in the same perfusates that identified Hap and is thus likely to be similarly useful as an indication of atherosclerotic coronary disease.

Finally, it is understood that various modifications, alterations and/or additions may be made to the example specifically described and illustrated herein without departing from the spirit and scope of the invention.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method of identifying a marker of coronary disease, said method including the steps of:

perfusing coronary tissue of patients having atherosclerotic or non-atherosclerotic coronary arteries;

collecting perfusate from the coronary tissues; and

identifying the marker from the perfusate in which it is present.

2. A method according to claim 1, wherein the coronary tissue includes the circulatory system of the heart from the aorta to the coronary sinus or a portion of the circulatory system thereof.

3. A method according to claim 1 or 2 further including determining a marker that is either present or absent from a patient having atherosclerotic or non-atherosclerotic coronary arteries.

4. A method according to any one of claims 1 to 3 wherein the perfusing of the coronary tissue is conducted antegradely through the coronary tissue.

5. A method according to any one of claims 1 to 4 wherein the perfusate is collected from the coronary sinus.

6. A method according to any one of claims 1 to 5 wherein the coronary disease is selected from the group including atherosclerosis, angina, stroke or heart attack.

7. A method according to any one of claims 1 to 6 wherein the coronary disease is atherosclerosis.

8. A method of diagnosing coronary disease, said method comprising:

identifying the presence or absence of a coronary marker said marker identified by a method according to any one of claims 1 to 7; and

correlating the coronary marker to a coronary disease.

9. A method according to claim 8 wherein the coronary disease is selected from the group including atherosclerosis, angina, stroke or heart attack.

10. A method of diagnosing arterial pathology in a patient, said method comprising:

collecting coronary perfusate;

identifying the presence of a coronary marker that is indicative of arterial pathology in the perfusate; and

correlating the coronary marker to a level of arterial pathology.

11. A method according to claim 10 wherein the coronary perfusate is collected by perfusing coronary tissue.

12. A method according to claim 11 wherein the perfusing of the coronary tissue is conducted from the coronary aorta to the coronary sinus.

13. A method according to any one of claims 10 to 12 wherein the coronary marker is identified by a method according to any one of claims 1 to 8.

14. A method according to any one of claims 8 to 13 wherein the coronary marker is selected from haptoglobin (Hap), Ig Kappa V-II, or a combination of Hap and Ig Kappa V-II.

15. Haptoglobin when used in a method according to any one of claims 8 to 13.

16. Ig Kappa V-II when used in a method according to any one of claims 8 to 13.

17. A method according to claim 1 substantially as hereinbefore described with reference to the examples.

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