CURRENT PERSPECTIVES ON LABORATORY MARKERS FOR THE ASSESSMENT OF CARDIOVASCULAR DISEASE AND MYOCARDIAL DAMAGE

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ABSTRACT

The disruption of the endothelium is a prelude to the evolution of arteriosclerosis. Nitric oxide is critical to endothelial function. The oxidation of low density lipoprotein (LDL) mediated by small dense LDL particles contributes to the development of cardiovascular disease. The multiple effects of homocysteine including its potentiating the binding of lipoprotein(a) (LP(a)) to fibrin and its role in the formation of small dense LDL particles highlights its importance in the evolution of cardiovascular disease and justifies its measurement. Problems in measurement of homocysteine should be kept in perspective. The measurement of release of proteins from the cardiac contractile apparatus such as Troponin I has advantages over conventional measurement of cardiac enzymes and isozymes.

KEY WORDS : Cardiovascular disease, nitric oxide, small dense LDL, Homocysteine, LP(a), myocardial damage, Troponin-I.

INTRODUCTION

The evolution of cardiovascular disease is triggered by the dysfunction of the endothelial cell. The invasion into the intima of the vessel wall by inflammatory cells is orchestrated by a variety of pro-inflammatory mediators. The vessel wall progressively occludes with deposition of lipid-laden cells culminating in the formation of the atherosclerotic plaque. The rupture of the plaque promotes thrombus formation leading to tissue ischemia and myocardial damage.

Determinants of Endothelial Cell Function

Nitric oxide (NO) has a key role in maintaining endothelial cell function. It is a soluble gas with a half-life ranging from 6-30 seconds. It is synthesized by the endothelial cell from the amino acid L-arginine

by a calcium calmodulin dependent enzyme called nitric oxide synthase (NOS) (1). The role of NO as a vasodilator is effected by stimulating soluble guanyl cyclase enzyme activity. As a result the level of cyclic guanosyl mono phosphate (C-GMP) is increased. In the vascular smooth muscle cells beneath the endothelium the increase in c-GMP results in the activation of GMP-dependent kinases leading to the relaxation of the vessel wall as the level of intra cellular calcium is reduced(2). NO also modulates the relaxation of the vessel wall by inhibiting a potent constrictor peptide called endothelin-1 and also the release of norepinephrine from sympathetic nerve terminals (3,4).

In addition to maintaining vasorelaxation NO functions as an endogenous inhibitor of vascular smooth muscle cell growth and migration, and the activity of the transcription factor nuclear factor kappa

B (NF- κ B) that regulates the expression of proinflammatory molecules such as vascular cell adhesion molecule-1 (VCAM-1) and chemokines such as monocyte chemoattractant protein-1 (MCP-1) (5).

While NO has a major role in maintaining vasorelaxation, other molecules such as prostacyclin and activators of adenosine triphosphate (ATP) and calcium-sensitive potassium channels also contribute to a lesser extent to the process.

Admittedly a decrease in the bioactivity of NO leads to endothelial dysfunction.

Pro-inflammatory Mediators

The bioactivity of NO is affected by a variety of pro-inflammatory mediators. The capacity of NO to inhibit the activity of the transcription factor NF- κ B can be overwhelmed by excess of hydrogen peroxide (H_2O_2) and superoxide anions (O_2^-) that can inactivate NO (6). H_2O_2 can also activate other pro-inflammatory mediators such as early growth response factor (erg) -1 and activator protein (AP) -1 (7,8). Hydroxyl radicals and peroxynitrite which is formed by the oxidation of NO by superoxide anions and free radicals are pro-inflammatory(6). Indeed the key for the modulation of the activity of pro-inflammatory mediators is the inhibition by NO of the transcription factor NF- κ B. The latter can induce the synthesis of pro-inflammatory cytokines and growth factors (9). In addition to NO other antioxidants such as glutathione, catalase, superoxide dismutase protect the endothelium from the assault of pro-inflammatory oxidants. Antioxidant therapy with vitamins C and α -tocopherol the biologically active form of vitamin E, and the cholesterol lowering drug Probucol have been shown to protect low density lipoprotein (LDL) from oxidation and in turn the progression of atherosclerosis (10-13).

Relationship between Chronic Infectious Processes and Atherosclerosis

Infectious agents such as cytomegalovirus, chlamydia pneumoniae and herpes virus have been suggested as playing a role in the atherogenic process (14, 15).

In a study of patients who developed narrowing of blood vessel lumen or restenosis after undergoing coronary angioplasty procedure, the presence of increased amounts of tumor suppressor protein p53 and human cytomegalovirus (HCMV) was reported in 23 of the 60 restenosis lesions (38%) that were examined (14). Cultured smooth muscle cells from these restenosis lesions also expressed HCMV protein IE84 and increased amounts of p53. It has been postulated that HCMV through the binding of its protein IE84 to p53 inhibits the function of p53, thus leading to restenosis (14).

Chlamydia pneumoniae has been detected by both immunohistochemical staining and the polymerase chain reaction (PCR) in tissue specimens taken from patients with coronary artery lesions(15).

In a study conducted on 23 fresh tissue specimens taken from patients with primary coronary artery lesions and 15 specimens from patients who had restenosis, chlamydia pneumoniae was detected in 20 of the 38 specimens(15). The organism was confined to macrophages and was seen more frequently in tissue specimens from patients who had restenosis than those who had primary lesions.

Antibodies to chlamydia species TWAR, and to chlamydial lipopolysaccharide group antigen have been detected in patients with acute myocardial infarction and chronic coronary heart disease thus implicating chlamydial infection as a factor in the evolution of

cardiovascular disease and eventual damage to the myocardial tissue (16).

Relationship Between Low Density Lipoprotein (LDL) Size and Heart Disease

Small dense LDL particles are produced when triglycerides from the very low density lipoprotein (VLDL) core are exchanged with the cholesterol esters from the LDL core, with the subsequent hydrolysis of the exchanged triglycerides (17). While the reduced size of these particles are due to the hydrolysis of the more buoyant triglycerides, the increased density is due to the retention of the same amount of apo B protein that was originally present. In plasma of patients with hypertriglyceridemia accompanied by the increased synthesis of VLDL by the liver, significant amounts of small dense LDL particles are in circulation(17).

There have been reports of the association of small dense LDL particles with the incidence of coronary artery disease and the risk of myocardial infarction (18,19).

By nondenaturing polyacrylamide gel electrophoresis of plasma, individuals can be classified according to their LDL diameter into one of the three patterns (19). Large LDL particles with a peak particle diameter usually larger than 26.3 nm are mostly seen in pattern A subjects. Individuals with pattern B, in contrast, have increased amounts of smaller LDL particles which are usually smaller than 25.8 nm. Subjects with characteristics of both patterns A and B are classified as having an intermediate (INT) pattern.

Patients with coronary artery disease generally have LDL subclass pattern B with a predominance of small dense LDL particles.

In one study it was demonstrated that LDL size was smaller in subjects with coronary artery disease (CAD) with a mean diameter $\pm$ S.D. of 26.17 ± 1.00 nm when compared to control subjects (26.68 ± 0.90 nm) (18).

Increased triglyceride levels with the consequent increase in small dense LDL particles, and a decrease in high-density lipoprotein cholesterol (HDL-C) taken together increase the risk for myocardial infarction (19).

The association of small dense LDL particles with the incidence of CAD stems from its increased susceptibility to oxidation (17). Furthermore, these small dense LDL particles are cleared slowly from the plasma as a consequence of its poor binding to the LDL receptor.

The increased risk of small dense LDL for CAD is reported to be twice as high towards the clinical onset of CAD, in contrast to the earlier stages of atherosclerosis where LDL-C level is the most important risk factor (17, 20). Incidentally, the increased tendency to thrombosis due to the increase in the activity of both the coagulation factor VII and plasminogen activator inhibitor-1 (PAI) the fibrinolytic inhibitor, is associated with increased triglyceride levels in plasma (17). Thus the apo B component of small dense LDL, HDL-C and triglyceride levels are important in the later stages of atherosclerosis (17).

Role of Oxidized LDL in Atherogenesis

As noted earlier, small dense LDL particles are more susceptible to oxidation. LDL sequestered within the subendothelial space of the arterial intima undergoes mild peroxidation of its polyunsaturated fatty acids (PUFAs) which is free radical mediated, to become minimally modified. This minimally modified LDL (MM-LDL) is susceptible to uptake by the LDL receptor (21).

In response to MM-LDL, vascular endothelial and smooth muscle cells secrete monocyte chemotactic protein-1 and macrophage colony-stimulating factor which promote monocyte chemotaxis or migration into and adhesion to the subendothelial space, where macrophage colony stimulating factor promotes its differentiation into macrophages (22). Macrophages modify MM-LDL to a more oxidized form. The oxidized LDL is taken up by the scavenger receptor on monocyte macrophages leading to foam cell formation (23).

Oxidized LDL has multiple effects. It causes endothelial dysfunction by decreasing the expression of endothelial nitric oxide synthase and in turn inhibiting NO mediated vasorelaxation (24). It can stimulate an immune response leading to secretion of cytokines with atherogenic properties such as interleukin-1 (IL-1), tumor necrosis factor- α (TNF- α), and interferon- γ (IFN- γ) (25). These cytokines can activate smooth muscle cells, monocytes, macrophages and endothelial cells leading to leukocyte recruitment and promotion of its adhesion to the endothelial cell. Furthermore, endothelial cell procoagulant activity is increased due to increases in tissue factor and the down regulation of fibrinolysis through the synthesis of plasminogen activator inhibitor-1 (PAI-1) (23). This oxidized LDL is cytotoxic to the endothelial cell.

Substitution of lysine residues in the apolipoprotein B-100 moiety of LDL by aldehydes such as malondialdehyde (MDA) results in the formation of an oxidatively modified LDL called MDA-modified LDL (26). This marker may result from ischemic injury to the endothelium which may lead to prostaglandin synthesis by endothelial cells through the activation of the cyclooxygenase-dependent pathway, and also due to the increased synthesis of non-cyclooxygenase-derived prostaglandin-like

substances such as $F_{2\alpha}$ isoprostanes which promote platelet adhesion and aggregation.

Thus oxidized-LDL unleashes a chain of events beginning with foam cell formation with its progression to a fatty streak, further lipid accumulation and finally the development of a fibrous cap due to collagen synthesis. Disruption of the fibrous cap by either physical forces or through the activity of enzymes (metalloproteinases) can lead to rupture of the plaque, activation of coagulation, and in turn, occlusion of the vessel lumen by thrombus formation with the attendant risk of developing an acute coronary syndrome (27, 28).

Homocysteine

Increased level of homocysteine, a sulfur-containing amino acid has been associated with cardiovascular risk.

Homocysteine is formed from methionine by a series of enzymatic steps. Homocysteine lacking a methyl group can be remethylated back to methionine. The reconversion of homocysteine to methionine is mediated by two enzymes: 5,10-methylenetetrahydrofolate reductase (MTHFR) and 5-methyltetrahydrofolate homocysteine methyltransferase. Homocysteine is also metabolized in an irreversible step when it combines with the amino acid serine in presence of an enzyme called cystathionine β -synthase to form cystathionine (29).

Defects in enzymes involved in homocysteine metabolism lead to increases in circulating levels of homocysteine causing pathological effects.

Thus the deficiency of cystathionine β -synthase, which is a pyridoxal phosphate-dependent enzyme leads to increased excretion of homocysteine in urine

(homocystinuria), a condition which is characterized by vascular abnormalities with patients at risk for frequent arterial and venous thrombosis (29). Deficiency of the enzyme methylenetetrahydrofolate homocysteine-methyltransferase, which is a cobalamin or vitamin B12-dependant enzyme can lead to rapidly progressive arteriosclerotic plaques (29). Indeed, a mutation in the gene for the enzyme MTHFR in the homozygous state is associated with reduced MTHFR activity leading to an increased level of homocysteine, exposing the patient to increased risk for cardiovascular defects and neural tube defects in the newborn (30).

The effects of increased circulating levels of homocysteine are many fold. For instance, homocysteine has effects on several coagulation factors including platelets, tissue factor, thrombomodulin, activated protein C, thromboxane and factors V, VII and XII (29).

It is toxic to the endothelial cell. It increases the binding of lipoprotein (a) to fibrin thus providing an opportunity for plaque formation. Homocysteine thiolactone can modify low density lipoprotein (LDL) to form small dense LDL particles that can self-aggregate and be consumed by macrophages to form foam cells. Ultimately the blood vessel is damaged. Cholesterol and lipids accumulate to generate arteriosclerotic plaques, thereby narrowing the arteries and promoting clot formation(29). Damage to the endothelial cell by homocysteine may also be related to its inactivation of nitric oxide and the inhibition of both the activity and synthesis of glutathione peroxidase (29).

Vitamin B6, B12 and folate supplementation can lower plasma homocysteine levels and protect the patient from cardiovascular disease. Indeed in a large

prospective cohort study of 80,082 female nurses over a 14 year period it was concluded that a daily intake of 400 µg folate and 3 mg vitamin B6 (Pyridoxal) was required to minimize cardiovascular mortality and morbidity (31). In this study it was noted that among women with the highest folate intakes, moderate consumption of alcohol considerably decreased cardiovascular risk.

Measurement of Homocysteine

70% of circulating homocysteine is protein-bound. The remainder is in the form of free oxidized and reduced species and the mixed disulfides of cysteine-homocysteine. The reduced or sulfahydryl form is referred to as homocysteine while the oxidized or disulfide form is called homocystine. Total homocysteine levels in plasma is a sum of both protein-bound and other forms mentioned above(32, 33). In healthy subjects total homocysteine (tHcy) levels are usually in the range of 5 to 15µmol/L(33).

Methods for measurement of homocysteine range from high performance liquid chromatography (HPLC)) with fluorescence detection which is widely used, to procedures based on gas chromatography-mass spectrometry (GC-MS) and enzyme immunoassay.

To the laboratorian the measurement of homocysteine presents challenges since this amino acid continues to be formed in the cell even after collection of blood, making the prompt separation of cells from plasma or serum mandatory for obtaining measurements of true homocysteine levels.

Even in samples collected in tubes containing ethylenediamine tetra acetic acid (EDTA) and stored in refrigerator (4°C to 5°C) within 15 to 30 minutes of

collection and centrifuged within 1 hour, an increase in mean plasma tHcy level of 0.5 $\mu\text{mol/L}$ has been reported(32,34).

Alternative approaches would involve lysing of whole blood to inhibit homocysteine generating and converting enzymes. However, lysing the sample would limit its usefulness for other laboratory tests.

Perhaps, the best approach is to centrifuge anticoagulated blood within 15 to 30 minutes of collection to separate plasma from cells. Separated plasma should be refrigerated as soon as possible or stored frozen at -20°C in the event of delay in analysis.

Laboratories using serum should centrifuge the blood as soon as the specimen has clotted which is usually thirty minutes, and separate serum from cells. Thus prompt separation of plasma or serum from cells can minimize the invitro generation of homocysteine after blood collection.

Other pre-analytical variables are age dependent variations with levels increasing with age, gender dependent variations with plasma tHcy values being higher in men than in women, and variations caused by smoking(32).

The increased emphasis on homocysteine as a risk factor for cardiovascular disease, and attempts to lower homocysteine levels by folate and vitamin B12 therapy, has turned the focus on the measurement of homocysteine for detection of elevated homocysteine levels in individuals and for monitoring the efficacy of therapy. However, a recent statement by the American Heart Association notes that not all prospective studies have supported a relationship between homocysteine and cardiovascular disease (35) It does not recommend population-wide screening

of subjects for homocysteine, until data from controlled clinical trials become available. Screening should thus be restricted to high-risk subjects with a plasma homocysteine value equal to or greater than 10 $\mu\text{mol/L}$, in patients with a history of premature cardiovascular disease, malnutrition, malabsorption, those on selected medications such as nicotinic acid, theophylline, methotrexate, bile acid binding resins, L-dopa and those who have had recent exposure to nitrous oxide (35). These high risk patients should be on an optimum daily intake of 400 μg folic acid, 2 mg vitamin B6 and 6 μg of vitamin B12 and should be monitored for effect of therapy by following homocysteine levels in plasma (35).

Lipoprotein (a) or LP(a)

Increased plasma levels of LP(a) have been regarded as an independent risk factor for coronary heart disease (36). LP(a) is an LDL-like particle containing apo B-100 and apo (a) covalently linked to each other by at least one disulfide bond. apo (a) has partial structural similarity to plasminogen. The molecular masses of apo(a) isoforms vary in size between 280 to 800 kd (36). Normally plasminogen upon binding to fibrin is activated to plasmin by tissue plasminogen activator (tPA). Fibrin is solubilized by plasmin. (37).

Because of the partial homology of LP(a) with plasminogen it may compete with the latter for binding to fibrin and to plasminogen receptors on the surface of the endothelial cell. As a result LP(a) can inhibit the activation of plasminogen to plasmin by tissue plasminogen activator thus preventing lysis of the fibrin clot (36). The fibrin-LP (a) complex can deposit within the arterial intima and initiate atherosclerotic plaque formation. Homocysteine can increase the affinity of LP(a) to fibrin (29).

Just like LDL, LP(a) is susceptible to oxidation. The oxidatively modified LP(a) can be taken up by the scavenger receptor on macrophages resulting in foam cell formation thus narrowing the arterial intima (36).

The interindividual variation in plasma LP(a) concentration can be as high as a thousand-fold. The size of apo(a) isoforms is inversely related to LP(a) concentration in plasma (36).

Although several studies have demonstrated a relationship between elevated plasma LP(a) levels and the increased risk of coronary heart disease, a few studies have failed to show any association between increased plasma LP(a) levels and coronary heart disease (36).

Immunological methods for the measurement of LP(a) rely upon antibodies raised to the apo(a) portion of LP(a). The standardization of these assays has been problematic due to differences in assay design and calibration. 20 out of 40 assay systems evaluated in a standardization study were found to be non optimized (38).

Clearly along with the standardization efforts, more longitudinal studies using a standardized assay are needed to establish definitively the relationship between increased plasma LP(a) levels and the risk of coronary heart disease.

Markers of Myocardial Damage

Laboratory diagnosis of myocardial infarction (MI) has traditionally been based on the measurement of enzymes and proteins released into the blood stream by the injured myocardium.

Myoglobin because of its low molecular mass

(17.8 Kd) is released within 2-4 hours of MI. As such it has value as a early indicator of MI. The fact that myoglobin is also present in skeletal muscle presents a limitation for its use in the diagnosis of MI, unless the involvement of skeletal muscle is ruled out by the simultaneous measurement of a skeletal muscle marker such as carbonic anhydrase III (39).

The combined measurements of myoglobin and creatine kinase - MB isozyme (CK-MB) by mass assay have been widely used especially in the emergency setting to diagnose MI. It is common knowledge that CK-MB with a molecular mass of 82 Kd, is released into the circulation 3 to 6 hours after infarction, with peak levels reached within 12 to 24 hours, and returning to normal in 24-72 hours.

The recent focus on the measurement of cardiac contractile proteins such as Troponin T and I has shifted attention from other markers such as the measurement of CK-MB isoforms which result by a cleavage of the terminal lysine from the M subunit by the enzyme carboxypeptidase (39). Likewise, the traditional method of confirming MI by following the sequentially timed-release of enzymes such as aspartateaminotransferase (AST) and Lactate dehydrogenase (LD), and LD isozymes and LD-1 could well be of historical interest.

The troponins are a part of the cardiac contractile apparatus, with the interaction of the troponin complex (Troponin I, T and C) regulating cardiac muscle contraction and relaxation. The similarity of the amino acid sequence between cardiac and skeletal troponin C precludes its use as a marker of myocardial damage.

Cardiac troponin T (cTnT) is a 37 kd polypeptide subunit compartmentalized in the cardiomyocyte into

a minor cytosolic (5%) and a major myofibrillary bound fraction (95%). Hence its leakage during MI is biphasic with a initial leakage of the minor cytosolic fraction followed by the prolonged release of the major bound fraction (40).

The cross-reactivity of cTnT with skeletal muscle TnT appears to have been significantly overcome in 2nd generation enzyme linked immunosorbent assays (ELISA) using high affinity cardiac specific antibodies (41). Detection limit of such assays are as low as 0.012 µg/L, with no cross-reactivity being demonstrated with 1000 µg/L of added purified skeletal muscle TnT. However, 5 out of 40 patients with renal failure and 4 out of 20 muscular dystrophy patients had cTnT levels greater than 0.2 µg/L (41).

Although there are three isoforms of troponin I (TnI) which are products of 3 separate genes: fast skeletal, slow skeletal and cardiac muscle, after birth the slow skeletal form is lost. Towards nine months after birth, the only detectable form of TnI is the cardiac isoform (42). It is this increased cardiac specificity that might provide TnI with the edge over TnT. Both TnT or TnI have similar release characteristics and stay elevated much longer than CK-MB or even LD after MI. Indeed failure of either TnT or TnI to rise above the cut-off limit 10 hours after on-set of chest pain rules out the occurrence of MI. TnI with a molecular mass of 24 kd begins to increase 4-8 hours after MI, reaching peak levels between 12 and 16 hours and returns to normal within 5 to 9 days. Like TnT, TnI has a biphasic release (39). With TnI minor myocardial injuries mostly due to ruptured atherosclerotic plaques can be detected. Identification of such patients allowed treatment with inhibitors to platelet glycoprotein IIb/IIIa receptor, thus reducing the risk of thrombotic episodes. Indeed TnT is a highly sensitive and specific marker of myocardial necrosis detectable in serum 3

to 12 hours after the onset of symptoms.

Bedside qualitative tests for the determination of both cardiac TnT and TnI have undergone evaluation and have been useful to rule out MI with TnI offering slightly higher sensitivity over TnT (43).

Results for TnI obtained with several of the currently available immunoassays may differ by a factor of 10 (45). This is due to the fact that monoclonal antibodies used in the assay may not recognize all of the circulating forms of TnI.

TnI is not released from necrotic myocytes in just the free form. It can be released either as a cardiac TnT/TnI/TnC complex or as a cardiac TnI-TnC binary complex and in phosphorylated form. In one study it was demonstrated that the TnI-TnC binary complex was the predominant circulating form, with the other species when present being in relatively smaller amounts (45). The standardization of TnI assays in terms of its ability to measure accurately the circulating forms of this protein is thus a pre-requisite to realizing the potential cardiospecificity of this marker. Preanalytical issues should also be kept in perspective. A preanalytical artifact may be limited to a particular assay system making it difficult to generalize the effect for all systems. Thus hemolysis, incomplete clotting, effect with a particular anticoagulant such as heparin are examples of some of the variables that have been reported to interfere with assay specific TnI measurements. In this context the extent of the effect of hemolysis on creatine kinase activity measurements due to adenylate kinase released from the ruptured erythrocytes, is dependent on the inclusion in the assay system sufficient amounts of inhibitors of adenylate kinase such as adenosine 5' pentaphosphate and 5' adenosine monophosphate (46).

Until the standardization of cardiospecific markers such as Tnl across assay systems is achieved the laboratory will still be dependent on classical markers such as creatine kinase (CK) activity and CK-MB mass measurements. The calculation of CK-MB index $(\text{CK-MB} + \text{total CK}) \times 100$ can be used to rule out skeletal muscle involvement if the index values exceed the range of 2 to 5%.

Finally, non-specific laboratory markers such as c-reactive protein and serum amyloid A protein may be useful in identifying patients at risk of developing acute coronary events due to unstable coronary plaques (47).

Conclusion

Markers of myocardial disease are useful for predicting the risk and for instituting appropriate therapy to reduce the incidence of cardiovascular damage and mortality. Emerging specific laboratory markers for detection of myocardial damage can be combined with non-specific markers that are released

earlier following MI so that prompt thrombolytic therapy can be instituted to perfuse and salvage the damaged myocardium.

It is to be hoped that advances in molecular genetics will assist in identifying families that are at risk for cardiovascular disease. We already know that a mutation causing replacement of arginine at residue 150 of apolipoprotein E (apo E) by cysteine is implicated in type III hyperlipidemia where there is an accumulation of intermediate density lipoprotein (IDL) (48). The endoglin gene on chromosome 9 that encodes a receptor for transforming growth factor- β and is expressed on vascular endothelial cells is defective in hereditary hemorrhagic telangiectasia which is a microvascular disorder characterized by vascular dilations and recurrent bleeding (49). Another example is the identification of a methionine 149 valine mutation in the cardiac contractile protein called essential light chain of myosin in hypertrophic cardiomyopathy. Since such patients are at risk for atrial and ventricular arrhythmias and sudden death early identification can help in initiating preventive treatment (50).

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