

N^ε-(Carboxymethyl)lysine and Coronary Atherosclerosis-Associated Low Density Lipoprotein Abnormalities in Type 2 Diabetes: Current Status

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Summary In comparison to the general population, individuals with diabetes suffer a 3- to 4-fold increased risk for developing complications of atherosclerosis and vascular insufficiency. This fact should be taken into account to develop a suitable determinant for the early detection of these complications and subsequently reduce the adverse effect of type 2 diabetes. *In vitro* experiments have shown that the products of glucose auto-oxidation and Amadori adducts are both potential sources of N^ε-(carboxymethyl)lysine (CML). Excessive formation of CML on low density lipoprotein (LDL) has been proposed to be an important mechanism for the dyslipidemia and accelerated atherogenesis observed in patients with type 2 diabetes. It has been postulated that the uptake of CML-LDL by LDL receptors is impaired, thereby decreasing its clearance from the blood circulation. Alternatively, the uptake of these modified LDL particles by scavenger receptors on macrophages and vascular smooth muscle cells (SMCs) and by AGE receptors on endothelial cells, SMCs, and monocytes is highly enhanced and this, in turn, is centrally positioned to contribute to the pathogenesis of diabetic vascular complications especially coronary artery disease. The present review summarizes the up-to-date information on effects and mechanism of type 2 diabetes-associated coronary atherosclerosis induced by CML-LDL modification.

Key Words: N^ε-(carboxymethyl)lysine, low density lipoprotein, atherosclerosis, type 2 diabetes

Introduction

Type 2 diabetes can lead to cardiovascular damage through a number of mechanisms, each of which in turn may accelerate or worsen the others. Potential mechanisms of how hyperglycemia may induce vascular injury include an increased production of advanced glycation end products (AGEs) and excessive oxidative stress [1]. Glycation, the term adopted by the International Union of Biochemistry, is

given to any reaction that links a carbohydrate to free amino groups of the proteins [2]. The term ‘AGEs’ is now used for a broad range of Maillard reaction products such as N^ε-(carboxymethyl)lysine (CML). Hyperglycemia and hyperlipidemia which are associated with diabetes can lead to irreversible nonenzymatic glycation of proteins and lipids and formation of AGEs [3]. It has been reported that the process of AGEs formation is accelerated by hyperglycemia [4, 5]. Accumulation of AGEs with structural alterations result in altered tissue properties that contribute to the reduced susceptibility to catabolism [6], leading to the gradual development of diabetic complications. It has been reported that AGEs levels are increased in type 2 diabetic patients with CAD [7].

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Several interrelations have been shown between oxidative stress and AGEs. Glycooxidation, a new term proposed by Baynes, refers to AGEs formation through an oxidative pathway [8]. CML modification of proteins is one of the major glycooxidation products formed *in vitro* by the reaction between glucose and protein [9]. Since CML is a major product of oxidative modification of glycated proteins, it has been suggested to represent a general marker of both oxidative stress and long-term proteins damage in aging, atherosclerosis, and diabetes [10]. Mykkanen *et al.* [11] have shown that a dyslipidemic lipoprotein profile characteristic of T2DM precedes the onset of diabetes. Lipoprotein particles are modified by glycation in the presence of hyperglycemia. The clearance of these glycated LDL particles is prolonged, and thus they might be more readily oxidized, leading to their increased uptake by macrophages [12]. In fact, CML has been identified in glucose-modified LDL and found in macrophage-induced foam cells of atherosclerotic plaques [13, 14]. Thus, disturbance of lipid and lipoprotein metabolism which commonly occur in diabetes almost certainly contributes to the pathogenesis of vascular complications.

Type 2 Diabetes and Coronary Artery Disease: General Overview

It has been suggested that type 2 diabetes be considered as: “*a state of premature cardiovascular death which is associated with chronic hyperglycemia and may also be associated with blindness and renal failure*” [15]. Diabetes predisposes its sufferers to cardiovascular disease (CVD) in a number of ways. Subjects with diabetes are at increased risk of atherosclerosis, and, to make matters worse, atherosclerosis in people with diabetes is accelerated in development, more widespread and more severe. The same traditional risk factors for CVD are operative in type 2 diabetic as in non-diabetic individuals. However, the effect of any given risk factor on the incidence of CVD is greater in diabetic than non-diabetic populations [16]. One of the major vascular beds where atherosclerosis clinically manifests is the coronary arteries leading to coronary artery disease (CAD) [17].

The term coronary artery disease refers to the consequences of oxygen deficiency in the myocardium caused by the decrease or complete interruption of the blood supply, generally originating from reduced blood flow from coronary arteries and usually caused by atherosclerotic changes. The process of atherogenesis was previously considered to consist mainly of lipid accumulation within the artery wall. Other processes, such as inflammation, are also involved [18]. CAD, the most important manifestation of CVD, represents a wide spectrum from angina pectoris, myocardial infarction and sudden death to silent myocardial ischemia.

CAD is the major cause of mortality and morbidity in the industrialized world [19]. T2DM and CAD share several important characteristics—both conditions become more prevalent with age, both are associated with obesity, an abnormal serum lipid profile and a sedentary lifestyle. Furthermore, it has been described that type 2 diabetes and coronary artery disease conditions are insulin resistant states associated with atherosclerosis [20]. Howard *et al.*, however, concluded that higher levels of insulin sensitivity are associated with less atherosclerosis in Hispanics and non-Hispanic whites but not in blacks, and this effect is partially mediated by traditional cardiovascular risk factors [20]. Therefore, T2DM has been defined as a CAD risk equivalent by the Adult Treatment Panel III of the National Cholesterol Education Program (NCEP) [21].

Epidemiology of Coronary Artery Disease in Type 2 Diabetes

Approximately 17 million people in the United States or 6.2% of the population are diagnosed as having diabetes mellitus [22]. The risks of developing CAD [23] as well as long-term mortality as a result of CAD are higher in individuals with diabetes than in those without diabetes [24]. For example, a recent meta-analysis showed that the rate of fatal CAD is 3 times higher in diabetic patients than in non-diabetic individuals [25]. In fact, CAD is the major cause of mortality in type 2 diabetes. The overall prevalence of CAD, detected by a variety of diagnostic methods, is reported to be as high as 55% in individuals with diabetes compared with 2% to 4% in the general population [26].

CAD is not only more prevalent in diabetic patients compared with the rest of the population but tends to be more extensive, involving multiple vessels and is rapidly progressive [27]. About 70% of deaths among type 2 diabetes patients result from CAD [28]. Compared with CAD in nondiabetic persons, CAD in patients with diabetes is more advanced at diagnosis and is generally characterized by more extensive atherosclerosis with higher rates of left ventricular dysfunction and cardiac events [26, 29]. Since we are facing a dramatic, worldwide increase in the incidence of type 2 diabetes [30], the cost for healthcare is escalating [31]. The complications associated with T2DM account for the majority of these expenditures and the cardiovascular complications make significant contribution to the cost of diabetes care [32].

Low Density Lipoprotein Metabolism, Glycooxidation and Diabetes-Induced Atherosclerosis

Low density lipoprotein

In type 2 diabetes lipid abnormalities are almost the rule. LDL cholesterol is a well-known risk factor for coronary

heart disease and is now recognized as the primary target of lipid lowering therapy. Elevated LDL cholesterol has been associated with CAD in follow-up studies [33, 34]. Increased LDL cholesterol predicts coronary heart disease (CHD) in patients without macroangiopathy at baseline indicating that elevated LDL cholesterol becomes important after exclusion of high-risk patients with CHD at baseline. More than 70% of people with type 2 diabetes have raised LDL cholesterol levels [11]. The risk of a cardiovascular event increases as the level LDL cholesterol level increases. In the United Kingdom Prospective Diabetes Study, the risk of either angina or a myocardial infarction in people with type 2 diabetes increased 1.57 fold for every 1 mmol/l increase in LDL cholesterol. Those with an LDL cholesterol > 3.89 mmol/l were 2.3 times more likely to develop angina or a myocardial infarction than people with an LDL cholesterol < 3 mmol/l [33].

Advanced glycation end products and diabetic macroangiopathy

In diabetic patients increased AGEs levels have been found in many tissues including dermal connective tissue, small blood capillary walls and vessel walls of arterioles and arteries [35, 36]. Vlassara and co-workers [37] have administered AGE-modified albumin to healthy nondiabetic rats and rabbits. After 2-4 weeks of AGE-administration, animals displayed diabetes-like vascular complications: a significant increase in vascular permeability, significant mononuclear cell migration in subendothelial and periarteriolar spaces and a defective endothelium-dependent and -independent vasodilatation. AGEs were identified in skeletal muscle arteries of streptozotocin-treated diabetic rats within 4-6 weeks of induction of diabetes [38] and in their mesenteric vessels within 3 weeks [39]. Moreover, it has been shown in diabetic rats that some AGEs were found in the aortic collagen after 4 and 12 weeks but this was significantly increased by 20 weeks [40].

Advanced glycation end products formation is enhanced in the presence of hyperglycemia in diabetes, by hyperlipidemia in atherosclerosis, and by oxidative stress in chronic diseases, inflammation, neurodegenerative disorders (such as Alzheimer's disease) and renal failure as well as under conditions where the turnover of lipids and proteins is prolonged. AGE products accumulate in body fluids, cells, tissues, and plasma [41]. In the cardiovascular system, AGEs accumulation contributes to arterial stiffening, myocardial relaxation abnormalities, atherosclerotic plaque formation, and endothelial dysfunction. Physiological AGEs in blood plasma have high renal clearances in normal healthy subjects [41].

Immunohistochemical studies of human atherosclerotic lesions have demonstrated intracellular AGEs deposition in SMCs-derived foam cells in fatty streak and atherosclerotic

plaques in human aorta [42]. Significant extracellular AGEs accumulation was also observed in advanced lesions [43]. AGEs formation on the extracellular matrix component of the vessel wall can cause structural damage by decreasing elasticity, increasing thickness, rigidity, and narrowing of the vessel lumen [40, 44]. It has been shown that the diminished arterial elasticity in humans with diabetes was related to enhanced AGEs formation [45]. AGEs increase collagen cross-linking leading to the arterial stiffness which is commonly observed in normal ageing but at an accelerated rate in diabetes [46]. Commonly found epitopes in AGEs include CML, pentosidine and pyralline [47].

Role of AGEs receptors

The potential pathophysiological significance of AGEs is associated with their accumulation in plasma, cells and tissues and their contribution to the formation of cross-links, generation of reactive oxygen intermediates, and interactions with particular receptors on cellular surfaces [48]. In view of the close association of AGEs with cells in the body and their known adverse effects on cells, many studies have focused on searching for AGE binding proteins. Binding and internalization of AGE-modified proteins is facilitated through several AGE-specific cell surface receptors [49].

Normally, AGE-modified proteins are repaired, replaced, or degraded *in vivo*. The recognition and degradation of such proteins is mediated by cellular receptors for AGEs (RAGEs), which are present on certain critical target cells such as monocytes, macrophages, endothelial cells, mesangial cells and fibroblasts [50, 51]. Interaction of AGE with RAGE on macrophages stimulates these cells to produce and release cytokines, growth factors, proteolytic enzymes, and increase expression of extracellular matrix proteins and vascular adhesion molecules, which are all required for normal tissue remodeling [52]. Excess production of growth factors and cytokines plays an essential role in both micro- and macrovascular alterations. Functional alterations or saturation of the macrophage system would allow anomalous tissue accumulation of AGEs leading to reduced structural protein turnover, increased collagen cross-links, and excessive degeneration and/or proliferation of tissue components as observed in ageing and diabetes [53].

In addition to RAGEs, several other cell surface receptors for AGEs have been identified. These include macrophage scavenger receptor types I and II, oligosaccharyl transferase-48 (AGE-R1), 80K-H phosphoprotein (AGE-R2) and galectin-3 (AGE-R3) [47]. Of these, RAGE has attracted most attention, as it is to date the only AGE receptor reported to have signal-transducing properties. An abundant CML epitope in structurally heterogeneous AGEs is specifically recognized by the RAGE V-domain [54]. In addition, as RAGE is expressed in endothelial cells, vascular SMCs

and monocytes, it is centrally positioned to contribute to the pathogenesis of diabetic vascular complications.

Binding of AGEs to endothelial cells increases expression of adhesion molecules (VCAM-1 and ICAM-1) and tissue factor in a RAGE-dependent manner resulting in vascular hyperpermeability [55, 56]. RAGE is a member of the immunoglobulin superfamily and exists in two forms, 35 kDa and 45 kDa [57]. The 45 kDa form of RAGE has a proteolytic property and is known as soluble RAGE (sRAGE). It is used as a pharmacological agent to prevent the vascular effects of AGEs (acts as an antagonist) in experimental diabetes [58]. For example, vascular hyperpermeability in diabetic rats was inhibited in the presence of sRAGE in a dose-dependent manner [59]. In a murine model of accelerated diabetic atherosclerosis, a six-week treatment with sRAGE suppressed development of atherosclerosis in a glycemia- and lipid-independent manner. Furthermore, vascular lesions that formed in animals receiving sRAGE were arrested at the fatty streak stage and the number of complex atherosclerotic lesions was strikingly reduced [60].

A two-hit model for RAGE-mediated perturbation of vascular function has been suggested [49, 61]. In the first hit, prolonged presence of RAGE ligands changes vascular properties priming the vasculature for a basal level of activation. In the second hit, additional perturbation, such as oxidized lipoproteins, ischemia, physical stress or inflammatory stimuli, results in exaggerated cellular response pro-

moting formation of vascular lesions rather than restitution of vascular homeostasis. Furthermore, Anti-RAGE IgG can also inhibit AGE-RAGE interaction and subsequently the rise of vascular complications seen in diabetes [55].

Oxidative modification of LDL and atherosclerosis

Being the main carrier of cholesterol in blood, LDL is also the principal lipoprotein causing atherosclerosis. Nishi and co-workers have documented that vulnerable carotid plaques from humans are greatly enriched in ox-LDL and that plaque content of ox-LDL was 70 times the plasma concentration [62]. Increased levels of ox-LDL are also associated with increased carotid intima-media thickness [63]. In addition, oxidation of LDL is not recognized by the native LDL receptors and it is preferably taken up via receptors on macrophages of extrahepatic tissues, including artery walls, resulting in cholesteryl esters deposition and foam cells formation [64].

Oxidation of LDL enhances atherogenesis by a number of different mechanisms, in particular by attracting the monocytes into the vascular intima and transforming them into foam cells and subsequent endothelial dysfunction as illustrated in Figure 1. Oxidative modification of LDL has also been shown to increase the ability of LDL to bind to the extracellular matrix [65]. Therefore, oxidation of LDL plays an important role not only in allowing LDL to be taken up by macrophages leading to the formation of foam cells,

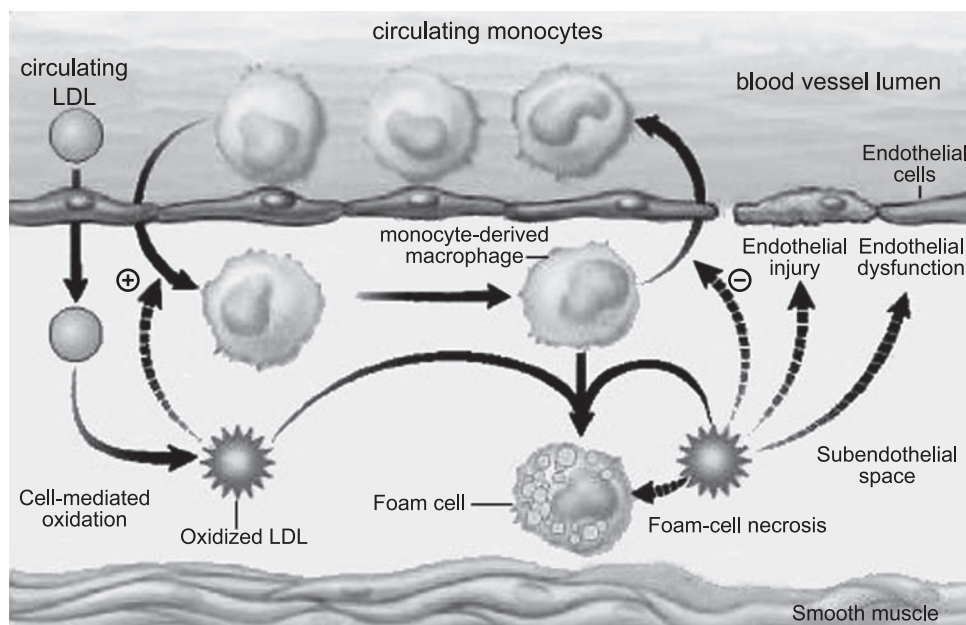


Fig. 1. A schematic illustration of foam cell formation in atherosclerosis. Circulating LDL cholesterol moves into the subendothelial space. Trapped LDL is subjected to oxidative modification by vascular cells such as smooth muscle cells, endothelial cells, and macrophages. Macrophages uptake modified LDL (ox-LDL) by scavenger receptors. In LDL overload, macrophages transform themselves into foam cells which become necrotic due to the accumulation of oxidized LDL. Oxidized LDL also results in endothelial dysfunction and injury. Local inflammation increases the entry of LDL and monocytes. (Adapted from Diaz *et al.*, *N. Engl. J. Med.*, 1997. [68]).

but also in promoting entry of monocytes/macrophages to the sub-intimal space where the process of LDL uptake occurs. However, oxidation of LDL apparently leads to possibly a very large array of consequences other than the generation of foam cells thought to be important in atherogenesis. For example, the oxidative modification of LDL has also been shown to be a chemoattractant for monocytes and to be cytotoxic to endothelial cells, as well as to inhibit nitric oxide-induced vasodilation [66]. Protection of LDL from oxidation could increase nitric oxide bioactivity and bioavailability and improve endothelium-dependent vasomotor, anti-inflammatory, and anticoagulant properties of the endothelium [67].

A prototypical protein target for oxidants in cardiovascular disease is the LDL and oxidation of LDL has been considered as an important mechanism for the development of atherosclerosis [69, 70]. It is noteworthy that oxidized lipoproteins have been implicated in the development of many diseases ranging from diabetes to arthritis. Consequently, there has been a vast amount of interest in evaluating factors that influence the LDL oxidation, as well as development of pharmacological agents and antioxidants that could reduce the oxidative modification of LDL.

LDL peroxidation is probably the most extensively investigated free radical-induced chain reaction [71, 72]. The earliest step in the generation of oxidative modified LDL is peroxidation of polyunsaturated fatty acids (PUFAs) in LDL phospholipids. Thus, PUFAs are particularly susceptible to peroxidation and once the process is initiated, it proceeds as a free radical-mediated chain reaction involving initiation, propagation and termination [73].

Each single substrate radical may result in conversion of multiple fatty acid side chains into lipid hydroperoxides. The length of the propagation chain before termination depends on several factors such as the oxygen concentration and the amount of chain-breaking antioxidants present. Hydroperoxides are fairly stable molecules, but their decomposition can be stimulated by high temperatures or by exposure to transition metal ions (iron and copper ions). Decomposition of hydroperoxides generates a complex mixture of secondary lipid peroxidation products such as malondialdehyde (MDA) and 4-hydroxynonenal.

LDL glycation and glycoxidation

A multitude of secondary complications of T2DM have been attributed to nonenzymatic glycation. Exposure of LDL to advanced glycation end products can prolong its half-life and then it will be trapped in the vascular wall where it is more susceptible to oxidation [74]. LDL has a special place in studies of glycation and oxidation in diabetes because it is a strong risk factor for atherosclerotic vascular diseases. LDL of diabetic patients with poor glycemic control is more susceptible to oxidation when

compared to LDL of normal subjects [75].

Glycation of LDL particles can alter their structure, function, and their susceptibility to oxidation and hence affect their atherogenic potential. One of the most compelling lines of evidence, which allows us to consider glycated LDL atherogenic, is the *in vitro* and *in vivo* studies which suggested the altered biological activity of glycated lipoproteins. Incubation of LDL with glucose leads to AGE formation on both the lipid and apoprotein components. Klein group has shown that recognition of LDL from diabetic patients with poor glycemic control by human fibroblasts was also impaired, supporting the role of glycation in altering recognition of LDL by the classical LDL receptor [76]. Subsequently, incubation of macrophages with glycated LDL results in transformation of these macrophages into cholesterol loaded foam cells. Glycated LDL uptake resulted in increased cholesteryl esters synthesis in macrophages leading to increased intracellular cholesteryl esters accumulation. Lipid peroxidation is known to induce cross-linking of collagen with a high rate in the presence of high glucose [77]. Glycated collagen was shown to be capable of covalently trapping LDL (e.g. in the arterial wall) causing it to be oxidatively modified by free radicals [78].

The increase of plasma levels of LDL and/or changes in their subfractions are associated with an increase of atherogenic risk. In the diabetic patients, although plasma LDL level often stays within the normal range, alterations take place in the lipoproteins components such as diameter, density, and lipid composition that make it more atherogenic [79]. To these, the nonenzymatic glycation that modifies the LDL and affects its metabolism resulting in increase in its potential atherogenicity must be added. In this sense, the glycation is directly linked to the oxidation of the LDL in the arterial wall [80]. Modifications to LDL such as oxidation and glycation are strongly implicated in the pathogenesis and progression of atherosclerosis [81]. Accumulated evidences in the last years suggest the combination of processes of nonenzymatic glycation and oxidation by free radicals acting on the lipoproteins, would contribute to generate a greater atherogenic risk in the diabetic patients, as proposed by Bayne [8]. The combinations of these two reactions, collectively known as glycoxidation, generate products that can be especially atherogenic [80, 82]. The interaction between glycation and oxidation provides a probable explanation for the increase of atherosclerosis frequently associated with diabetes.

The development of atherosclerosis is accelerated in patients with diabetes mellitus, and LDL in a diabetic state is susceptible not only to oxidation [83] but also to glycation [84]. In the diabetic patients several types of modified LDL molecules have been detected, including glycated LDL, oxidized LDL, and glycoxidized LDL. Immunohistochemical studies on human atherosclerotic lesions have

shown that AGEs and oxidized LDL are colocalized in the cytoplasm of macrophage-derived foam cells [13].

The contribution of advanced glycation to the oxidative modification of LDL was first observed in an *in vitro* study [85]. Isolated human LDL was incubated with glucose (in presence of metal ions) and analyzed for both advanced glycation and oxidative modification. Incubation of LDL with 200 mM glucose for 3 days resulted in the formation of readily measurable levels of AGEs on both apoprotein (Apo-B) and lipid. This study indicated also that lipid-linked AGEs formed more rapidly than ApoB-AGEs and measurements of oxidative modification further showed that LDL was oxidized concomitantly with the formation of AGEs. Moreover, increased levels of peroxidation products are detected in glycated or cell-modified LDL particles, which have been known as minimally modified LDL [86]. For better explanation the relationship between advanced glycation and LDL oxidation *in vivo*, Bucala *et al.* analyzed LDL from both diabetic and nondiabetic individuals by an AGE-specific ELISA [85]. Their investigations revealed increased levels of both phospholipids-AGEs and apoB-AGEs of the LDL molecules from diabetic patients compared to healthy controls.

Role of scavenger receptors

Atherosclerosis is an inflammatory disease [87] and macrophages are present at all stages of the disease [88]. Animal studies, using several different models of atherosclerosis, support a key role for macrophages in the development and progression of atherosclerosis. In addition to their contribution in the modification of LDL [81, 87], macrophages, in their role of scavenging tissue and cellular debris, can take up the modified LDL via scavenger receptors [89]. Scavenger receptors recognize chemically and biologically modified lipoproteins, typically acetylated LDL and oxidized LDL. Unlike LDL receptor, the expression of scavenger receptor is not down regulated by cellular cholesterol content and, therefore, results in the intracellular lipid accumulation and consequently induces foam cell formation. Recent studies showed that, at least in macrophages, macropinocytosis has been shown to operate in the uptake of native LDL too, in the process leading to foam cell formation [90]. However, important in this respect is that modified LDL is taken up faster by macrophages than native LDL.

Monocyte-derived macrophages endocytose modified LDL via multiple receptors, including both class A (SR-A) and class B (SR-BI and CD36) scavenger receptors [91, 92], class E receptor (lectin-like oxidized LDL receptor-1), and lipoprotein lipase [93, 94]. Other scavenger receptors include class D receptor (CD68) and scavenger receptors for phosphatidylserine and oxidized lipoprotein (SR-PSOX), which mediate binding and uptake of ox-LDL [95–97].

Uptake of lipoproteins in excess of its metabolism results in the formation of cholesteryl esters in macrophage derived-foam cells. Similarly, foam cells can develop from vascular SMCs by the uptake of oxidized LDL via class A and class B scavenger receptors [98, 99]. Lipid-laden macrophages have been shown to exhibit strong immunoreactivity to CD36, but only low or moderate levels of immunoreactivity to SR-A [100], suggesting that CD36 could be the predominant macrophage receptor for ox-LDL in human atherosclerotic lesions.

Diabetes exacerbates the uptake of modified LDL by foam cells via several mechanisms. Firstly, diabetes causes a proatherogenic dyslipidemia resulting in high serum triglyceride levels, reduced levels of HDL, and increased number of small dense LDL particles [101]. Secondly, diabetes increases the expression of macrophage class B scavenger receptor CD36 [102], which enhances oxidized LDL endocytosis. Thirdly, hyperglycemia increases glycoxylation, resulting in increased levels of oxidized and glycated LDL, which increases ligand availability for scavenger receptors and lipoprotein lipase [85]. Modification of proteins by oxidation, glycation, or glycoxylation has been proposed to impair binding of LDL to the LDL receptor, and increase binding and endocytosis by unregulated scavenger receptor pathways.

N^ε-(Carboxymethyl)lysine Accumulations in Diabetes-Associated Complications

N^ε-(Carboxymethyl)lysine: formation and chemical properties

AGEs form a large group of heterogenous compounds of which only a few have been identified. N^ε-(Carboxymethyl)lysine, a product of glycation and oxidation *in vivo*, is generated by oxidative cleavage of the Amadori product threulosyl-lysine and is also a product of metal-catalyzed oxidation of LDL or peroxidation of polyunsaturated fatty acids in the presence of fructose-lysine. Thus, CML is known to be a glycoxylation product which means that it requires oxidation reactions for their formation from glucose [8]. Although CML is the smallest one of the AGE modifications, it might be functionally relevant. It leads to a change in charge, since, after modification, the former positively charged lysine residues carry a negatively charged carboxylic group.

CML is an irreversible protein modification which is stable to acid hydrolysis and can be quantified from protein hydrolysates. It has been reported that CML binds to RAGE and activates cell-signaling pathways [54]. Moreover, colocalization of CML with adducts derived from products of lipid peroxidation, such as 4-hydroxynonenal and MDA, supports the concept that lipid peroxidation itself, in addition to and apart from advanced glycation, triggers the formation of CML [103]. CML contents of proteins exist in

several tissues such as lung, skin, bone, and blood vessels. However, CML epitopes are not uniformly distributed in these tissues, with more intensely staining of the elastic fibres of blood vessels and skin than surrounding tissues [35].

N^ε-(Carboxymethyl)lysine and vascular complications of diabetes

Inefficient clearance of degraded low molecular weight AGE-rich peptides and recirculation of these 'toxic' molecules might be responsible for vascular damage in diabetic patients. It has been described that highly modified AGE proteins are able to bind to macrophage scavenger receptors [104] and receptor for AGE (RAGE) [105]. Studies have identified CML of AGE proteins as a ligand for RAGE [106, 54]. Although other studies have revealed that CML proteins are not recognized by scavenger receptors

[107] and were unable to interact with RAGE or activate an inflammatory response in RAGE-expressing cells [108], these observations have not been confirmed in other laboratories. Further studies are required to clarify this issue of CML interaction with RAGE and macrophage scavenger receptors.

Since specific CML antibodies have become available, several studies have demonstrated the accumulation and distribution of CML in atherosclerotic lesions [109, 110] and observed within arteriosclerotic plaques, in foam cells [35, 111], in a variety of chronic degenerative, chronic inflammatory diseases [112], and recently in human heart valves [113] as well as in the heart tissue of diabetic patients [114]. CML has become a key marker of protein modification in response to glyoxidative, lipoxidative and carbonyl stress *in vitro*. This has led to suggestion that it could also represent a biomarker for systemic or local oxidative stress in tissue

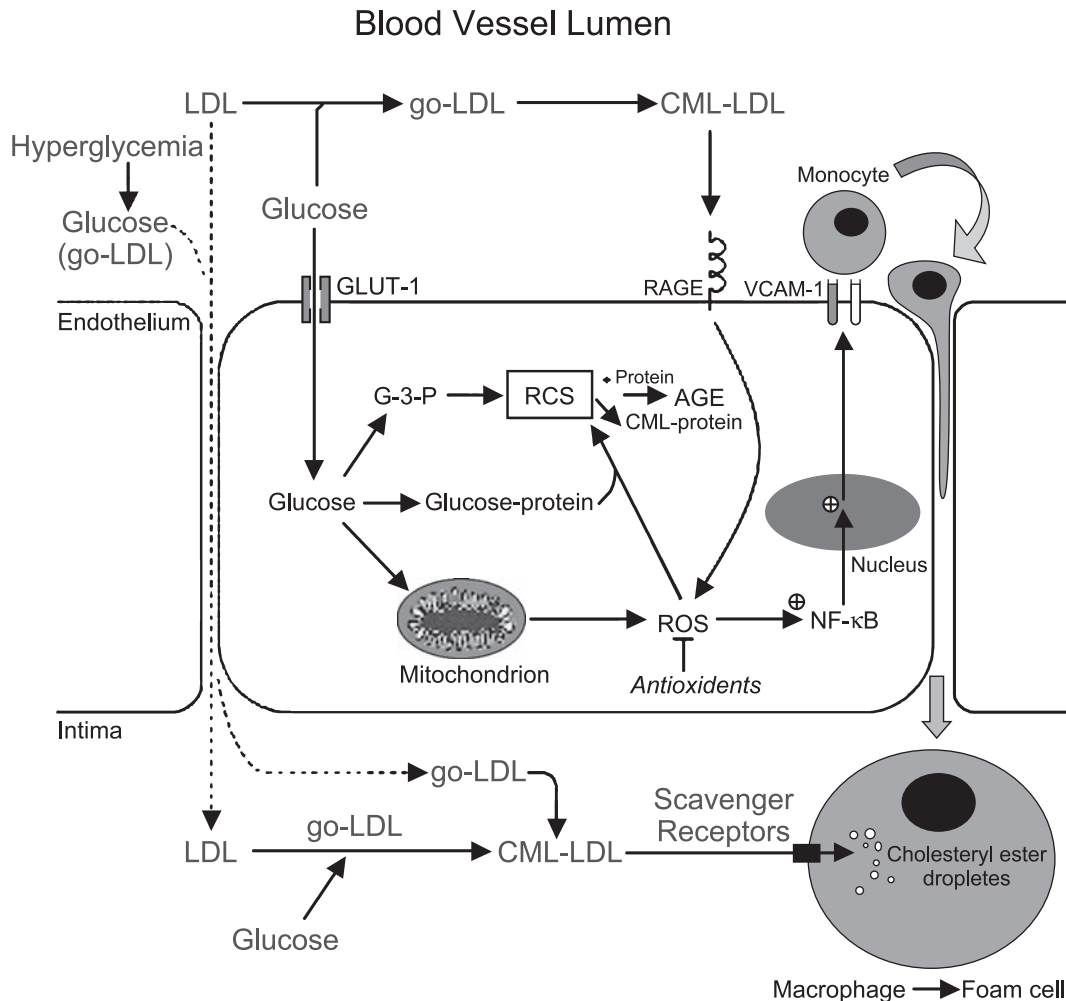


Fig. 2. Proposed model of CML formation on LDL in the blood circulation and in the subendothelial space. CML can be formed on low density lipoproteins in the blood circulation and trapped CML-LDL as well as CML-LDL formed in extracellular matrix will be taken up by macrophages through scavenger receptors followed by macrophages transformation into foam cells. Other processes in this figure are discussed in the text.

lesions *in vivo* [115]. CML content increases with the chronological age of proteins [4, 116] and detected in patients with diabetes mellitus [35]. Figure 2 showed the different pathways of CML formation on LDL and their uptake by macrophages. Hyperglycemia leads to glycation of serum proteins including LDL and an increase in endothelial glucose concentration via GLUT-1 transporters. Increased intracellular glucose leads to the formation of reactive carbonyl species (RCS) such as methylglyoxal, glyoxal, and 3-deoxyglucosone, which may be released into the bloodstream or the subendothelial space and react with LDL to form CML-LDL. In both blood circulation and arterial intima, LDL is subjected to glycooxidation and CML formation. CML-LDL is recognized by endothelial cell through AGE receptor (RAGE) leading to reactive oxygen species (ROS) formation. Monocytes and CML-LDL particles cross the endothelium and can become trapped in the extracellular matrix followed by CML-LDL binding to scavenger receptors on the macrophages, which undergo foam cell formation and, thus, atherosclerosis lesions.

In diabetes, the rate of CML formation is accelerated as compared to that seen in aging and in some studies, CML seems to correlate with the severity of diabetic complications independent of age [117, 118]. CML has been reported to stimulate superoxide radical and H₂O₂ generation via activation of NADPH oxidase in human endothelial cells [119]. The cytoplasmic domains of foam cells also contain the CML epitopes, which may be due to receptor-mediated uptake and degradation of AGE-modified proteins by macrophages within plaques [13].

Since CML can be formed from glycation and lipid peroxidation reactions, the observed increase in CML in diabetes may be accounted for either an increase glycation or increased oxidative stress. However, it has been reported that CML can be formed by a nonoxidative mechanism and its elevations in diabetic serum support the notion that the nonoxidative glycation of proteins may contribute to AGE accumulation in diabetes [116, 120]. The observation of CML accumulation in diabetic muscle is in good accordance with this general notion. The vasculature is the preferred environment for localization of CML accumulation in diabetic macroangiopathy. Although several studies showed CML elevations in diabetic microvascular complications, there is ongoing debate about the effect of CML on macrovascular diseases resulting from T2DM.

Although Kilhovd and co-workers found that serum AGEs levels increase in patients with CHD and T2DM [121], little data are available on the relationship between serum CML and macrovascular complications in individuals with T2DM. Kiuchi *et al.* showed that serum AGE concentrations were higher in type 2 diabetic patients with obstructive CAD than in patients without obstructive CAD, and higher than in non-diabetic patients with and without

obstructive CAD. Furthermore, serum AGE was associated with the degree of coronary arteriosclerosis in type 2 diabetic patients with obstructive CAD. These results indicate that serum AGE concentrations may be associated with long term poor glycaemic control and reflect the severity of coronary arteriosclerosis in type 2 diabetic patients [7]. Miura *et al.* pointed out that serum non-CML AGE levels were significantly associated with the severity of diabetic nephropathy and retinopathy, suggesting a role of non-CML AGE in the progression of microvascular complications [122]. The finding of the relationship between blood CML levels and the severity of microangiopathy suggested that the blood CML levels are related to the severity of both nephropathy and retinopathy [123]. Wautier *et al.* demonstrated that in type 2 diabetic patients with retinopathy or microalbuminuria, serum CML levels were significantly higher, and even more elevated in patients with both complications [124].

Our work on CML has revealed a significant role of CML levels on the development of ischemic heart disease in patients with type 2 diabetes [125]. Since it has been established that CML levels are increased in the serum with aging [126], our findings exclude the effect of age on CML levels in type 2 diabetic patients with ischemic heart disease. In a more recent work, it has been shown for the first time the significant predictive power of high serum CML concentrations to CAD in type 2 diabetes patients and this association between CML and CAD is independent of other risk factors [127].

We have also studied the effect of increased CML content of LDL (unpublished data), enhanced LDL oxidation, and the high CML levels on the defective uptake of LDL by its receptor in T2DM patients with CAD [127]. Our measurements have revealed that LDL particles obtained from diabetes patients without CAD, AGE-LDL, and oxidized-LDL have similar uptake and distribution in the cytoplasm of Hepatoma (HepG2) cells. These LDL particles did not appear to be defective in terms of their ability to bind to the LDL receptor, rather, their interaction with the LDL receptor was reduced when compared to native LDL. On the other hand, the binding and uptake of LDL from T2DM subjects with CAD were impaired, indicating that LDLs obtained from these patients may have increased atherogenic potential. CML-LDL particles may be more atherogenic than the classic oxidized- and glycated-LDL due to the concomitant effect of both the chronic state of diabetes and oxidative stress in type 2 diabetes patients with artery disease [111, 128]. Nonenzymatic glycation of proteins including LDL may be closely correlated to lipid peroxidation in the arterial intima as explained by localization of CML in the cytoplasm of macrophage-derived foam cells in atherosclerotic lesions [13]. Furthermore, Sakata *et al.* [14] have described the synergistic effect of chronic high blood glucose and oxida-

tive stress on producing CML-LDL. These results also suggest that LDL particles from T2DM patients with CAD have similar glycoxidative behaviors and properties as CML-LDL prepared *in vitro* and these modified LDL molecules were not found in diabetes patients without CAD.

Conclusions

In summary, this review shows the significant association between serum CML levels and type 2 diabetes complications, especially CAD and that raised CML levels may reflect the enhanced oxidative stress in these patients due to prolonged higher blood glucose levels in diabetic CAD patients. As the prevalence of diabetes increases globally, laboratory findings will continue to play a major role in the early diagnosis and subsequently better management of the diabetic vascular complications. Since it has been established that CML is accumulated in atherosclerotic lesions of diabetic subjects, elevation of carboxymethylated-LDL (CML-LDL) might be the rule in this process. From previous investigations and our study on LDL metabolism, it is concluded that carboxymethylation of LDL may be largely responsible for the defective LDL uptake by LDL receptor rather than AGE-LDL or ox-LDL. The impairment in receptor-specific metabolism might be attributed to the findings that LDL molecules of diabetic CAD patients exhibit higher CML content levels than diabetics without complications, indicating that both type 2 diabetes and CAD are superimposed. Moreover, the studies on modified LDL metabolism not only confirmed the previous observations that glycoxidized LDL is present *in vivo* but rather, it shows that glycoxidized LDL may be of CML-epitope that could be found in higher concentrations in patients with T2DM and CAD than diabetes patients without macrovascular complications especially CAD. Thus, CML may play a crucial role in the development of CAD in patients with T2DM and may be considered as an independent endogenous marker of interest for early detection of CAD in type 2 diabetic patients. In view of its cumulative effect on impairment of lipoprotein metabolism, CML may serve as an important target for glycemic control in these patients.

Abbreviations

AGEs, Advanced glycation end products; CAD, Coronary artery disease; CHD, Coronary heart disease; CML, N^ε-(Carboxymethyl)lysine; CVD, Cardiovascular disease; go-LDL, Glycoxidized-low density lipoprotein; LDL, Low density lipoprotein; MDA, Malondialdehyde; PUFAs, Polyunsaturated fatty acids; RCS, Reactive carbonyl species; ROS, Reactive oxygen species; SMCs, Smooth muscle cells; sRAGE, Soluble receptor for advanced glycation end product; T2DM, Type 2 diabetes mellitus.

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