Mechanism of Development of Atherosclerosis and Cardiovascular Disease in Diabetes Mellitus

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Diabetic macroangiopathy, atherosclerosis secondary to diabetes mellitus (DM), causes cerebro-cardiovascular diseases, which are major causes of death in patients with DM and significantly reduce their quality of life. The alterations in vascular homeostasis due to endothelial and vascular smooth muscle cell dysfunction are the main features of diabetic macroangiopathy. Although multiple metabolic abnormalities that characterize diabetes are involved in the progression of atherosclerosis in patients with DM, it may be said that prolonged exposure to hyperglycemia and insulin resistance clustering with other risk factors such as obesity, arterial hypertension, and dyslipidemia play crucial roles. Laboratory and clinical researches in the past decades have revealed that major biochemical pathways involved in the development of diabetic macroangiopathy are as follows: overproduction of reactive oxygen species, increased formation of advanced glycation end-products (AGEs) and activation of the AGEs-receptor for AGE axis, polyol and hexosamine flux, protein kinase C activation, and chronic vascular inflammation. Among them, oxidative stress is considered to be a key factor.

Key words: Diabetes, Atherosclerosis, Cardiovascular disease, Oxidative stress, Advanced glycation end-products (AGEs)

Introduction

Diabetes mellitus (DM) causes a lot of complications. Diabetic macroangiopathy, atherosclerosis secondary to DM, especially may cause cerebral vascular disorder, ischemic heart disease, peripheral arterial disease, or other vascular diseases, which are major causes of death in patients with DM and significantly reduce their quality of life.¹-³

Although many factors are involved in the progression of atherosclerosis in patients with DM, it may be said, indeed, that the two most important factors are insulin resistance and hyperglycemia.⁴ A large number of basic studies have demonstrated that insulin resistance in vascular cells plays an important role in the progression of atherosclerosis.⁵-⁷ Insulin resistance in the liver and muscle is not only a major cause of the onset and progression of DM, but also plays a role as a risk factor for the onset and progression of other atherosclerotic conditions such as hypertension and dyslipidemia. Accordingly, patients with insulin resistance often have multiple risk factors that induce the progression of atherosclerosis through various mechanisms.⁸ Patients with insulin resistance may also have obesity or excessive visceral fat, resulting in an abnormal adipocytokine profile.⁹-¹¹ The connection of insulin resistance to DM and atherosclerosis is very complex and involves many factors. As there have been excellent review articles dealing with this subject, the present article does not detail it. The present article describes major mechanisms of how hyperglycemia accelerates the progression of atherosclerosis.

Process of the Development of Atherosclerotic Lesions

Endothelial cells produce and release a variety of bioactive substances to control and maintain the function and structure of intact vessels through balancing between oxidation and anti-oxidation, and inflammation and anti-inflammation in the vascular wall; proliferation and anti-proliferation of vascular smooth
muscle cells; dilation and contraction of vessels; and coagulation and fibrinolysis of blood. According to the study, increased low-density lipoprotein (LDL) cholesterol levels, hyperglycemia, oxidative stress, and smoking may cause vascular endothelial dysfunction that may result in atherosclerosis.

It is believed that the formation of atherosclerotic lesions is triggered by a local inflammation in the vascular wall that is induced by dyslipidemia, specifically high LDL-cholesterol levels, and high remnant lipoprotein levels, as well as other various disease factors. The process is considered as follows: (1) Vascular endothelial cells injured by oxidative stress or other factors express adhesion molecules and release cytokines and chemokines. (2) The chemokines attract monocytes from blood circulation to the injured area, and monocytes attach to the endothelium through interaction with adhesion molecules. (3) Monocytes penetrate the subendothelial space, differentiate, and mature into macrophages that release cytokines. When LDL cholesterol levels are high, LDL cholesterol infiltrates the subendothelial space and is retained in the intima where it is oxidized or otherwise modified. (4) Macrophages take up and accumulate oxidized LDL cholesterol, leading to foam cell formation and atherosclerosis. (5) Oxidized lipids trigger the secretion of various growth factors by the endothelium. Vascular smooth muscle cells of the media transform and migrate into the intima where they proliferate and actively produce extracellular matrix. These transformed vascular smooth muscle cells also take up oxidized LDL cholesterol and transform to form cells that contribute to atherogenesis. (6) On the other hand, the proliferation of vascular smooth muscle cells and an increase in extracellular matrix may cause intimal thickening and sclerosis (Fig. 1).

As described above, atherosclerotic lesions are formed through complex interactions of various factors, and DM accelerates all these interactions. It is known that levels of small dense LDL cholesterol are high in patients with DM. Small dense LDL cholesterol particles are very atherogenic as they circulate in the blood at higher levels since they have lower affinity to LDL receptors, have greater propensity for transport into the subendothelial space due to their small size, and are more likely to be oxidized or otherwise degraded due to their low anti-oxidant content.

Increased Formation of Advanced Glycation End-Products (AGEs) and Activation of the AGEs-RAGE Axis

Reducing sugars such as glucose bind nonenzymatically to proteins in the body. This reaction, called protein glycation, is accelerated when hyperglycemia, oxidative stress, and inflammatory reactions are present. In the early stage of protein glycation, the aldehyde group of sugar reacts with amino acids to produce Schiff’s base, which undergoes a series of modifications to form Amadori rearrangement products. Hemoglobin A1c (HbA1c) and glycoalbumin are known as major products in the early stage of protein glycation. The early-stage protein glycation products undergo complex reactions, such as oxidation, dehydration, and condensation to form advanced glycation end products (AGEs) that have a dark color, fluorescence, and a molecular cross-linking potential and other features characteristic of aged tissues. AGEs are not a single chemical entity, but a collective term of different substances that share the above-mentioned characteristics.

The findings of the Diabetes Control and Complications Trial in patients with type 1 DM and its follow-up study called the Epidemiology of Diabetes Interventions and Complications Study, as well as the United Kingdom Prospective Diabetes Study in patients with type 2 DM, have indicated that the risk of diabetic vascular complications remained higher in patients receiving conventional treatment in whom blood glucose control was inadequate than those receiving intensive treatment to ensure strict blood glucose control even when the level of blood glucose control no longer differed between the two patient populations after the study. This persistent increase in the risk of diabetic cardiovascular complications after an exposure to high glucose levels for a certain period of time is called “metabolic memory” or “legacy effect.” As AGEs are formed more frequently at high glucose levels and are not easily metabolized, they are accumulated more in those with a longer history of inadequate blood glucose control and accelerate the progression of vascular disorders. The “metabolic memory” phenomenon is best explained by the formation of AGEs.

The authors determined skin autofluorescence (AF), a measure of skin AGEs levels, in Japanese patients with type 1 DM and their gender- and age-matched healthy individuals to investigate the relationship between skin AF and the risk of diabetic complications. The findings indicated that skin AF values were significantly higher in patients with type 1 DM than healthy controls, and mean HbA1c over the past 10 years was an independent determinant of skin AF values. It was also found that skin AF was an independent risk factor for carotid atherosclerosis.

AGEs are involved in each step of atherosclerosis. AGEs accelerate the migration of monocytes to the subendothelial space and its transformation to macro-
As AGEs promote autocrine production of vascular endothelial growth factor (VEGF) and thereby induce pathological neovascularization, AGEs may promote neovascularization in plaques and be involved in the bleeding and instability of plaques. Moreover, AGEs promote clot formation by activating the coagulation system through accelerating the production of tissue factors and by suppressing fibrinolysis through promoting the synthesis of plasminogen activator inhibitor 1 (PAI-1). These findings indicate that AGEs are closely involved in the pathophysiology of atherothrombotic diseases.

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AGEs are believed to be involved in the onset and progression of atherosclerosis through multiple mechanisms. One is a direct cytotoxic mechanism...
through modifications and structural changes of proteins and extracellular matrix by glycation and cross-linking. Another is a mechanism controlling cell response to cell-surface receptors that recognize AGEs as specific ligands. Oxidative stress that occurs during the formation of AGEs is also involved in cell and tissue damage \(^{21}\) (Fig. 2).

Cell surface receptors that recognize AGEs include the receptor for AGE (RAGE), galectin-3, scavenger receptor class A, CD36, scavenger receptor class B type I, and lectin-like oxidized LDL receptor-1 recognize AGEs \(^{22}\). Among them, the activation of RAGE is an important step of atherogenesis. RAGE is found on the cell surface of endothelial cells, monocytes, macrophages, vascular smooth muscle cells, pericytes, mesangial cells, and other cells that play important roles in the onset and progression of diabetic complications. RAGE expression is especially high on atherosclerotic plaques in patients with DM \(^{23-26}\).

When AGEs bind to RAGE, several intracellular signaling pathways such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38, and phosphoinositide 3-kinase (PI3K) are activated \(^{27}\) to promote the activity of nuclear factor-κB (NF-κB) and cAMP response element binding protein \(^{28}\). This process is mediated by reactive oxygen species (ROS) \(^{29}\) that are produced in endothelial cells and macrophages through the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase \(^{30}\). AGEs binding to RAGE on endothelial cells induce the expression of adhesion molecules and the secretion of cytokines and growth factors through activating the above-mentioned pathways \(^{31, 32}\). AGEs also stimulate monocytes to induce the production and secretion of various cytokines such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 \(^{33}\). The AGE-RAGE axis also induces the migration and proliferation of vascular smooth muscle cells and the production of extracellular matrix through activating transforming growth factor-β (TGF-β) \(^{34, 35}\).

In a RAGE knockout mouse model, atherosclerosis progresses slowly, and the size of myocardial infarction after experimental coronary artery ligation is small. In studies where soluble RAGE was administered to animals as a ligand decoy, it inhibited the AGE-induced ERK phosphorylation and the expression of

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**Fig. 2.** Role of AGEs in the vascular damage.

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overexpression of human AR (hAR) and reduced when the aorta of ApoE knockout diabetes mice with DM37), and inhibited the formation of neointimal after femoral arterial injury in mice38). These findings indicate an important role of AGEs-RAGE axis in the progression of atherosclerosis and the onset of cardiovascular diseases.

Activation of the Polyol Pathway

The polyol pathway consists of just two steps. The rate-limiting enzyme of the first step is aldose reductase (AR) that requires nicotinamide adenine dinucleotide phosphate reduced form as a coenzyme to reduce glucose to sorbitol. Then sorbitol is oxidized to fructose via sorbitol dehydrogenase and a coenzyme, nicotinamide adenine dinucleotide (NAD).

In tissues where glucose uptake is mediated by insulin-independent glucose transporters such as glucose transporter 1, hyperglycemia is more likely to cause metabolic disorders. Glucose taken into the cell has a high affinity to glucokinase and undergoes extensive phosphorylation, but about 5% of glucose is not phosphorylated, but metabolized directly through the polyol pathway into sorbitol and fructose. However, when blood glucose levels are high, the percentage of glucose metabolized through the polyol pathway increase four- to five-fold.

It is believed that the activation of the polyol pathway induces, directly and indirectly, vascular damage for the following reasons: (1) Fructose and its metabolites, e.g., triose phosphate, methylglyoxal, fructose 3-phosphate, and 3-deoxyglucosone, are potent glycating agents. When their production is accelerated, more AGEs are produced39). (2) In the polyol pathway, the polyol pathway and poly (ADP-ribose) polymerase are activated and the NADH/NAD4 ratio increases, the NAD-dependent glycolysis from glyceraldehyde 3-phosphate (GAP), an aldose, to pyruvic acid is inhibited, while levels of dihydroxyacetone phosphate (DHAP), a ketose, are elevated, which results in an increased production of DAG44). DAG activates cPKC and nPKC. Experiments in diabetic animal models have demonstrated that an increase in DAG levels in the heart, aorta, and renal glomeruli correlate with the activity of cPKC (α, β1, β2) and nPKC (δ, ε)45). Elevated oxidative stress associated with DM is also an important activator for PKCs46).

Findings of studies using diabetic animal models have indicated that some PKC isoforms are activated in different organs such as the aorta, heart, retina, and renal glomeruli, and the type of PKC isoforms activated by oxidative stress differs by cell type. The activation of PKCβ is important in vascular disorders.

PKC activation causes many abnormal changes related to atherosclerosis, such as an increase in vascular permeability47), activation of NADPH oxidase48), endothelial dysfunction, and impaired vasodilation due to decreased NO production49); activation of intracellular signaling pathways, such as Akt, ERK, and p38 MAPK; modified expression of transcription factors, such as early growth response protein 1 (Egr-1), NFκB, and specificity protein 1 (SP1); increased expression of cytokines and growth factors such as VEGF, ICAM-1, ET-1, and PAI-1, among others; apoptosis and increased production of extracellular matrix.

It has been reported that in ApoE knockout mice, the inhibition of PKCβ by LY333531, a PKCβ inhibitor, or knockout of PKCβ gene inhibits the formation of atherosclerotic lesions50).

Elevation of O-GlcNAc Protein Modifications

Glycosylation is a reaction where a carbohydrate
Glucosamine-6-phosphate is converted to N-acetylglucosamine-6-phosphate (GlcNAc-6-P) and finally to UDP-N-acetylglucosamine (UDP-GlcNAc). O-GlcNAc transferase (OGT) uses UDP-GlcNAc as the substrate for the attachment of N-acetylglucosamine to proteins. Reversely, N-acetylglucosaminidase (O-GlcNAcase or OGA) removes N-acetylglucosamine from O-GlcNAcylated proteins. These indicate that the progression of diabetic macrovasculopathy relates to O-GlcNAcylation levels. In a study of diabetic ApoE knockout mice, the progression of O-GlcNAcylation in coronary endothelial cells and vascular smooth muscle cells causes decreased levels of NF-κB inhibitory protein A20 to accelerate atherosclerosis. In another study, coronary endothelial cells isolated from type 1 diabetic mice have low levels of OGA expression and high levels of OGT expression with impaired endothelium-dependent vasodilatation, but OGA overexpression reversed coronary dysfunction.

Fig. 3. Role of the polyol pathway in the vascular damage.

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Abbreviations: SDH, sorbitol dehydrogenase; AGEs, advanced glycation end-products; PKC, protein kinase C.

molecule is added to a protein or fat. O-linked N-acetylgalactosamine (O-GlcNAc) is a form of protein glycosylation where a single sugar, N-acetylgalactosamine (GlcNAc), is added to serine and threonine residues on nuclear and cytoplasmic proteins. More than 1,000 proteins are known to be O-GlcNAcylated. As O-GlcNAcylation is known to compete with phosphorylation for the same serine/threonine residues, it is believed to inhibit phosphorylation temporarily. It is thus considered that O-GlcNAcylation contributes to protein–protein associations and the stability of protein complexes through affecting multiple signaling pathways, and that abnormal O-GlcNAcylation may be involved in some diseases, including DM and cancer.

High blood glucose levels lead to high intracellular glucose levels, leading to increased levels of fructose-6-phosphate and its metabolite glucosamine-6-phosphate that is converted by glutamine:fructose-6-phosphate amidotransferase, the rate-limiting enzyme. Glucosamine-6-phosphate is converted to N-acetylgalactosamine-6-phosphate (GlcNAc-6-P) and finally to UDP-N-acetylgalactosamine (UDP-GlcNAc). O-GlcNAc transferase (OGT) uses UDP-GlcNAc as the substrate for the attachment of N-acetylgalactosamine to proteins. Reversely, N-acetylgalactosaminidase (O-GlcNAcase or OGA) removes N-acetylgalactosamine from O-GlcNAcylated proteins.

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The production of ROS in mitochondria is also increased in patients with DM. Even in normal physiological situations, superoxides are generated as byproducts of oxidative phosphorylation in the mitochondrial electron transport chain, but when blood glucose levels are high, glycolysis is enhanced, which consequently increases the flow of electrons to the mitochondrial electron transport chain and the production of superoxides in cells. As normalizing mitochondrial superoxide may reverse the increased production of AGEs, activated hexosamine pathway, and activated PKCs, it is considered that the abnormal intracellular metabolism is closely associated with oxidative stress57).

As mitochondrial DNA is not protected by histones and is located near the inner mitochondrial membrane that contains the enzymes of the electron transfer system, it is susceptible to oxidative damage caused by ROS. Mitochondrial DNA injury decreases the production of ATP and thereby suppresses cellular functions. The processes of mitochondrial fusion, fission, biogenesis, and mitophagy that determine mitochondrial dynamics are important in maintaining appropriate cellular bioenergetics and oxidative stress homeostasis58). An increased mitochondrial fission/
fusion ratio causes a decrease in energy production efficiency and an increase in ROS production. In coronary endothelial cells isolated from diabetic mice the expression of mitochondrial fusion promoting genes is decreased while that of mitochondrial fission promoting genes is increased with elevated susceptibility to mitochondrial fragmentation and augmented oxidative stress in cytoplasm and mitochondria. Administration of a superoxide scavenger to diabetic mice led to a decrease in mitochondrial fragmentation through decreasing ROS.

When nuclear factor E2 related factor 2 (Nrf2), a transcription factor, is activated by ROS or reactive nitrogen species, it enters the nucleus of a cell to modulate the expression of oxidative stress resistance genes such as those encoding glutathione synthesis enzymes, glutathione S-transferase, heme oxygenase-1, and thioredoxin reductase. Since Nrf2-deficient mice showed enhanced neointimal hyperplasia in a wire injury model, Nrf2 appears to be related to atherosclerosis.

Nrf2 is regulated by Kelch-like ECH-associated protein 1 (Keap1), a sensor of oxidative stress, and is held inactive by Keap1 under normal cellular conditions. Under oxidative stress conditions, Nrf2 dissociates from Keap1 and promotes the transcription of target genes through anti-oxidant responsive elements (AREs). A heterodimer of Nrf2, a basic-region-leucine zipper (bzip) transcription factor, with small Maf binds to AREs to strongly activate gene expression. Surfforaphane, a substance found in broccoli, blocks the breakdown of Nrf2 through its interaction with Keap1.

In diabetic mice, sulforaphane recovered Nrf2 levels in the aorta and the expression of Nrf2-dependent anti-oxidative genes, and prevented wall hypertrophy, fibrosis, inflammatory reactions, apoptosis, and cell proliferation in the aorta.

ROS react with components of the body, such as fats, proteins, and nucleic acids to degenerate them. ROS induce erroneous expression of many genes through their direct effects or by promoting AGE production or activating PKCs, which leads to the onset and progression of complications. Genes that are known to be affected by ROS include genes coding (1) catalase and other anti-oxidant enzymes, (2) heme oxygenase-1, metallothionein-1 and other stress-response proteins, and (3) VCAM-1 and other cell adhesion molecules, VEGR, monocyte chemoattractant protein-1 (MCP-1), and other cellular growth factors and cytokines. Under oxidative stress, these genes express themselves with the assistance of activated transcription factors, such as NF-κB, AP-1, and serum response factor (SRF) that are regulated by the intracellular redox status.

Activation of pro-oncogene under oxidative stress plays an important role in the proliferation of vascular smooth muscle cells. The authors have indicated a possible involvement of the activation of proto-oncogene Pim-1 by oxidative stress in the pathogenesis of atherosclerosis. PDGF and RAGE/STA3 signaling pathways are upstream of the activation of Pim-1.

**Chronic Inflammation**

Atherosclerosis is a metabolic disorder and also a chronic inflammatory disorder. Studies have gradually revealed how inflammation develops and persists in arteries.

The relationship between infections and atherosclerosis has been pointed out by epidemiological studies. Substances that induce immune responses and inflammation during infection include pathogen-associated molecular patterns (PAMPs), a group of molecules released by microorganisms in the host, and damage-associated molecular patterns (DAMPs), a group of intrinsic molecules released by damaged tissues or necroting cells. DAMPs include AGEs, cholesterol, and uric acid. Toll-like receptors found on the surface of macrophages, endothelial cells, and smooth muscle cells recognize PAMPs and DAMPs, and activate NFκB and thereby promote the production of inactive nucleotide-binding oligomerization domain-like receptors 3 (NLRP3) and pro-IL-β. PAMPs and DAMPs promote the formation of the active NLRP3 inflammasome that consists of NLRP3, an apoptosis-associated speck-like protein containing caspase recruitment domain (ASC), and pro-caspase-1. In the inflammasome, molecules are brought into close proximity, resulting in the activation of pro-caspase-1, which converts pro-IL-β into mature active IL-β to maintain inflammation. Cells that activated the inflammasome die and release DAMPs, which cause further inflammation.

It has been reported that patients with type 2 DM have high NLRP3 levels in monocytes, an elevated activity of the inflammasome, and high levels of IL-1β and IL-18 in peripheral blood. Studies have pointed out the progression of atherosclerosis in DM involves inappropriate persistent inflammation induced through excessive inflammasome activation by PAMPs and DAMPs.

Unlike other cell types, neutrophils release nuclear chromatin into the extracellular space. This extracellular chromatin, called neutrophil extracellular traps (NETs), stays local to capture bacteria. These sticky “nets” consisting of nucleic acids, proteins, and proteases released from neutrophils catch pathogenic bacteria and never release them. Bacteria trapped by NETs undergo phagocytosis by neutrophils and macrophages and are also killed by NETs that have bactericidal
thrombus formation, which induces the formation of platelet thrombi. Also, neutrophil elastase and cathepsin G, components of NETs, decompose tissue factor pathway inhibitor to promote blood coagulation and thrombus growth.

Triple-knockout mice that lack ApoE as well as the two major enzymes found in NETs, neutrophil elastase, and proteinase-3, had decreased NETosis, lower IL-1β levels in the blood, and substantially smaller atherosclerotic formation than ApoE-knockout mice had.

Recent studies have reported that hyperglycemia may promote NETosis, and levels of NETosis markers are high in patients with type 2 DM, which suggests the involvement of excessive NETosis in the progression of atherosclerosis in DM.

properties. Neutrophils die immediately after releasing NETs. This process is called NETosis and is attracting attention as a new type of cell death that differs from necrosis and apoptosis.

NETs is an excellent mechanism to eliminate bacteria but may induce excessive inflammation through the release of intracellular components, e.g., nucleic acids, proteins, and proteases, which act as intrinsic ligands affecting natural immunity and tissue damaging enzymes. In fact, it has been revealed that NETs are also involved in noninfectious conditions, such as chronic inflammation, autoimmune disorders, atherosclerosis, and thrombosis.

NETs promote inflammation through stimulating macrophages to release pro-IL-1β, among other mechanisms. Histones, a component of NETs, induce platelets aggregation, and NETs provide a scaffold for thrombus formation, which induces the formation of platelet thrombi. Also, neutrophil elastase and cathepsin G, components of NETs, decompose tissue factor pathway inhibitor to promote blood coagulation and thrombus growth.

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**Fig. 5. Role of oxidative stress in the vascular damage**

The production of ROS is increased in patients with diabetes, hypertension, dyslipidemia, obesity, smoking habit, etc. ROS react with components of the body such as fats, proteins, and nucleic acids to degenerate them. ROS induce erroneous expression of many genes through expression of redox-regulated genes, which leads to the onset and progression of atherosclerosis.

Abbreviation: ROS, reactive oxygen species.
Summary

Since cardiovascular risk burden is not still totally relieved by intensive glycemic control associated with optimal multifactorial treatment, mechanism-based therapeutic strategies are in highly required. In this context, as described above, a number of basic studies using animal models and cell cultures have conducted, and then, revealed that major biochemical pathways involved in the development of diabetic macroangiopathy are as follows: overproduction of ROS, increased formation of advanced glycation end-products (AGEs) and activation of the AGEs-RAGE axis, polyol and hexosamine flux, PKC activation, and chronic vascular inflammation.

However, these findings from laboratory researches have several limitations that should be recognized. Firstly, atherosclerotic lesions created in rodent models differ substantially from complex lesions found in patients with coronary atherosclerosis, and are similar to foam cell accumulation and fatty streaks that are found in the early stage of atherosclerosis in humans. Secondly, findings from cell cultures have also limitations since crosstalk between many cell types plays an important role in the process of atherogenesis. Thirdly, as immortalized cell lines commonly used in laboratory researches may differ from primary cells in regard to substrates, including glucose, being metabolized, findings found in cell lines may reflect those in primary cells. Thus, although experimental work has helped to unravel some of the principles of atherosclerosis pathophysiology, another investigative dimension, where basic scientists and clinicians communicate closely to provide feedback in both directions, may be required before full understanding of mechanism of the development of atherosclerosis in patients with DM.

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