THE ROLE OF IMMUNE AND INFLAMMATORY PROCESSES IN THE DEVELOPMENT OF MACROVASCULAR DISEASE IN DIABETES

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1. ABSTRACT

Diabetes is associated with a high incidence of cardiovascular disease, which is the major cause of morbidity and mortality in this disease. There is considerable interest in defining factors responsible for the accelerated development of atherosclerosis in diabetes. There is no evidence to suggest that the inflammatory process in diabetic patients is different from those in non-diabetic individuals. The main difference may lie on factors able to trigger the inflammatory process. Diabetes is a major predisposing factor for the generation of modified proteins though advanced glycation and oxidation, two intimately interrelated processes. Advanced glycation end-products modified low density lipoprotein (AGE-LDL) and other AGE-modified proteins as well as oxidized LDL (oxLDL) are able to interact with a variety of cells and induce cell dysfunction and the release of pro-inflammatory mediators. But AGE-LDL and oxLDL are also immunogenic. Activated T lymphocytes reacting with peptides derived from oxidized LDL have been detected in atheromatous lesions. Their pro-inflammatory potential is directly linked to the release of interferon-gamma and other cytokines able to activate macrophages, smooth muscle cells, and endothelial cells. On the other hand, antibodies to oxidized and AGE-modified LDL have been isolated from diabetic patients and shown to belong predominantly to the IgG isotype, subclasses 1 and 3, which have well-defined proinflammatory properties. These autoantibodies to modified lipoproteins have sufficient affinity to form stable antigen-antibody complexes, which have been also shown to have pro-atheromatous and pro-inflammatory properties.

2. INTRODUCTION

Epidemiological studies have shown that diabetes is associated with severe and premature cardiovascular disease (CVD) (1). Recently, in the new National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) guidelines, diabetes was recognized as coronary heart disease equivalent (2). The mechanisms responsible for the accelerated development of CVD in diabetes are not well defined, although dyslipidemia and increased generation of modified lipoproteins has been long recognized as possible mechanisms (3-5). Both factors can, in turn, promote the activation of inflammatory responses and contribute to the onset and/or progression of atherosclerosis.

The re-evaluation of atherosclerosis as a chronic inflammatory disease represents a major change in our understanding of the pathogenesis of this disease (6). This change resulted from the accumulation of evidence over the last decade supporting the involvement of inflammatory processes in the pathogenesis of atherosclerosis. Data suggesting that both cell-mediated and antibody-mediated mechanisms may be involved in the amplification of the inflammatory reaction has been published by several independent groups (7-10). Crucial to our understanding of the pathogenesis of atherosclerosis is the definition of the insults likely to trigger the inflammatory reaction. Modified lipoproteins and infectious agents able to infect and/or activate endothelial cells and macrophages appear most likely to play this role on the light of current evidence. Later, the immune response to modified lipoproteins and heat-shock proteins expressed by activated and damaged cells in the arterial wall is probably the most significant factor leading to the chronicity of the inflammatory process, due to the activating effects on macrophages and other cells. Also critical is the understanding of the role played by the different cell types present in atheromatous lesions. Experimental evidence shows that activated cells in atheroma plaques engage in a variety of amplification circuits, some mediated by cytokines, other by cell surface molecules, perpetuating the inflammatory reaction in vessel walls. The degree of cell activation is likely to be a very important factor leading to the formation of vulnerable plaques and increased risk of acute ischemic events. Thus, immune and inflammatory processes are tightly interwoven in a complex network leading to the progression of atherosclerosis and to acute cardiovascular events.
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3. Evidence for the role of immune and inflammatory processes in atherosclerosis

The evidence supporting the inflammatory nature of atheromatous lesions derives from three types of sources: epidemiological studies, studies of arterial plaque tissues, and studies carried out in animal models.

Strong epidemiological associations have been reported between clinical manifestations of atherosclerosis and serum levels of inflammatory markers, such as C-reactive protein (CRP), amyloid A protein, fibrinogen, and interleukin-6 (IL-6) (11-16). Of those markers, CRP has received considerable attention after reports of poor outcome in patients with stable or unstable angina and elevated levels of CRP (11-13), increased coronary heart disease mortality in patients with elevated levels of CRP, particularly in smokers (17), and increased risk of myocardial infarction and stroke in the participants on the Physicians Health Study with higher levels of CRP (18). It was also demonstrated, in the same group of patients, that the association of CRP levels and lipid parameters (e.g., total cholesterol levels) was a better risk predictor that either of the parameters by themselves (19). In later studies the same general conclusions were reached in a prospective study of 27,939 apparently healthy women (20). High levels of CRP also appear to be a predictor of poor outcomes (coronary heart disease, graft failure) in heart transplant patients (21).

IL-6 has also been the object of considerable attention. This ubiquitous cytokine is released primarily by activated macrophages, but it can also be released by proliferating lymphocytes (22,23) as well as by smooth muscle cells activated in an autocrine fashion by IL-6–IL-6 receptor complexes (24). Once released it has in situ effects and systemic effects. Systemically it is an important factor in the induction of systemic manifestations of inflammation, such as fever and increased synthesis of reactive proteins, including CRP (22,23). In situ, it stimulates macrophage proliferation and enhances leukocyte recruitment after forming complexes with its soluble receptor (25). High levels of IL-6 have been shown to be associated with an increased risk of myocardial infarction in a prospective study involving 14,916 apparently healthy men (26). IL-6 has also been found to be elevated in the serum of patients with unstable angina (13) and in the immediate 12 to 60 hours following a myocardial infarction (15). Also significant is the fact that elevated serum levels of IL-6 in patients with unstable angina pectoris predict a higher risk of cardiovascular mortality (27). In women, the levels of IL-6 and CRP are significantly increased in patients with several risk factors and are independently related to several of these risk factors, such as increasing body mass index, systolic and diastolic blood pressure, and smoking (28).

The significance of elevated IL-6 levels may extend beyond what is expected of a non-specific marker of inflammation. A specific polymorphism of the IL-6 promoter region (174 G/C) has received considerable attention. According to some groups GG homozygosity of the IL-6 promoter region (174 G/C) locus is associated with higher acute phase levels of IL-6 in inflammatory diseases (29) and after coronary revascularization (30), higher levels of CRP, higher systolic blood pressure (31), and higher levels of heat shock protein-60 (hsp60)-reactive antibodies (32). Healthy individuals with G at position 174 have been reported to have higher levels of fasting triglycerides, VLDL-triglycerides, and post-load free fatty acids, while their levels of HDL-cholesterol are lower (33). The increased levels of IL-6 and CRP in patients with this polymorphism could reflect a genetic tendency to develop intense inflammatory reactions, which in turn would increase the risk for complications. Effects on lipoprotein lipase activity, adipose tissue metabolism, and hepatic triglyceride secretion could explain the lipid abnormalities (33). Hyperactivity of antigen-presenting cells could explain the higher levels of antibodies to hsp60, and possibly of other antibodies, such as those reacting with modified lipoproteins. It must be stressed, however, that not all data is concordant. For example, a recent cross-sectional study of 2559 patients with angiographically documented coronary artery disease (CAD) and 729 controls without angiographic evidence of CAD failed to reveal significant differences between circulating IL-6 levels or -174 G/C polymorphism between the two groups.

The involvement of inflammation in the pathogenesis of atherosclerosis is supported by a considerable wealth of pathological and experimental data. The importance of this data stems from the fact that the epidemiological evidence discussed above is based on correlations with measurements of non-specific inflammation markers that can be increased as a consequence of multiple disease processes besides atherosclerosis.

One major area of investigation has been the detection of pro-inflammatory mediators in the atheromatous lesions. This has been achieved in three types of studies: those in which the mediators have been measured directly in the local blood supply of a diseased vessel, those in which mediators have been demonstrated in tissue samples, and studies in animal models.

The local production of IL-6 in ischemic cardiac tissues has been demonstrated in patients undergoing coronary artery bypass grafting. In these patients there was a significant increase in the levels of IL-6 in coronary venous blood after reperfusion (34). Delyargiris et al. measured IL-6 during cardiac catheterization and found an elevated IL-6 transcardiac gradient (levels on the coronary sinus/levels on the left main coronary artery) in patients with acute coronary syndrome (35). Studies in a canine model of myocardial ischemia and reperfusion have also shown increased expression of IL-6 mRNA on mononuclear cells and myocytes at the viable border of a myocardial infarct (36). Immunohistochemical studies in necrotic tissue have revealed IL-6 overexpression in ischemic myocardium as well in atherosclerotic coronary arteries (37) and atheromatous lesions (38-41).

Besides the localization of IL-6, a plethora of evidence supports the inflammatory nature of atheromatous lesions. Plaque lesions have been shown to contain immunoglobulins and various complement components (42-44) as well as mononuclear cell infiltrates, predominantly constituted by T lymphocytes and monocytes/macrophages (45-48). High levels of IL-2 expression in T lymphocytes
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Figure 1. Macrophages play a central role in inflammatory processes. Macrophages can be activated by a variety of stimuli, including infection by facultative or obligatory intracellular organisms, cytokines (particularly IFN-gamma), chemotactic factors and chemokines, growth factors, modified lipoproteins, and LDL-IC. The uptake of LDL-IC seems particularly significant because their FcgR-mediated uptake not only results in macrophage activation and mediator release, but also in intracellular accumulation of cholesterol esters (foam cell transformation). Activated T lymphocytes, present on atheromatous plaques, contribute to the inflammatory process through the release of IFN-gamma and expression of CD40 L, which can then interact and activate CD40+ cells, including macrophages.

and of MHC-II molecules on T lymphocytes, macrophages and smooth muscle cells indicates that these cell populations are activated. The increased expression of MHC-II molecules on plaque macrophages also suggests an enhanced capacity of these cells to function as antigen-presenting cells (49). T lymphocytes cloned from atherosclerotic lesions are CD4 T cells that do not fit into the defined subtypes of T-helper (Th) cells. The majority of the CD4 cells cloned produce IL-4 and interferon gamma (IFN-gamma), followed by clones that produce predominantly IFN-gamma (Th1 helper cells). Only a very small number of clones produced predominantly IL4 (Th2 helper cells) (50). The predominance of IFN-gamma—producing cells is very significant, because the local synthesis of this cytokine is likely to contribute significantly to macrophage activation (49). While CD4+ T cells predominate in early lesions, the T lymphocytes detected in advanced atheromatous lesions appear to contain equal proportions of CD4+ and CD8+ cells, many of them expressing the CD45RO marker, indicating that memory T cells may predominate in late lesions (51). Another interesting characteristic of the atheromatous lesions is the abundance of cells overexpressing CD40 and the corresponding CD40-ligand (CD40L), enabling activation of a variety of CD40+ cell populations, including macrophages, fibroblasts and endothelial cells (52).

The presence of activated T lymphocytes in atheromatous lesions implies that a specific immune reaction against antigens related to the atheromatous place must have taken place. It needs to be noted that the population of T lymphocytes detected in the atheromatous plaque is polyclonal; thus the infiltrating T cells are most likely recognizing more than one antigen (51). Published data supports the involvement of at least two unrelated antigenic systems. Stemme et al. (53) isolated CD4 T lymphocyte clones that respond to oxLDL, strongly suggesting that modified lipoproteins can activate cell-mediated responses. The presence of oxLDL in atheromatous lesions is a strong supporting argument for this hypothesis (54,55). On the other hand, Wick et al. accumulated a considerable body of evidence suggesting that autoimmune responses to heat shock proteins are associated with atherosclerosis. Over expression of hsp60, a major stress protein, can be detected in atherosclerosis-prone sites and in atheromatous lesions (56-58). Of interest is the fact that Chlamydia pneumoniae infection, whose possible role in atherosclerosis is described later on in this review, has been found to be associated with antibodies to hsp60 (59,60), suggesting that it can induce the expression of this protein by infected cells. Whether the infiltrating T cells detected in the same sites and lesions react with hsp60 is not known, but antibodies to hsp60 have been detected in patients with documented coronary atherosclerosis (56,61). Thus, it seems possible that T lymphocytes infiltrating atheromatous lesions may be involved in an autoimmune reaction against hsp60-derived peptides. The pathogenic role of an autoimmune response to hsp60/65 in atherosclerosis has been supported by experimental studies showing that immunization of normocholesterolemic rabbits with mycobacterial hsp65 (which cross-reacts with human hsp60) is followed by the development of atherosclerosis. The atheromatous lesions overexpress hsp60/65 and hsp65-sensitized T lymphocytes were isolated from the lesions. Antibodies to hsp65 are also detectable in these animals (61).

Macrophages and foam cells in the atherosclerotic lesion are believed to play a pivotal role in the progression of atheromatous lesions as a consequence of their ability to be activated by numerous stimuli and to release a wide variety of pro-inflammatory proteins and enzymes (Figure 1). The long list of pro-inflammatory soluble factors released by activated macrophages and foam cells includes cytokines [TNF, IL-1, IL-6, IL-8, IL-12, IL-18], chemokines [such as macrophage chemotactic protein-1 (MCP-1) and IL-8], growth factors [such as platelet-derived growth factor (PDGF)], and metalloproteinases (MMPs) (39,46,47,50,51,62-65). The release of tumor necrosis factor (TNF) and IL-1 is believed to cause the overexpression of cell adhesion molecules (CAM), such as ICAM-1 and VCAM-1, on overlaying endothelial cells (48,66-68). These two cytokines are also able to increase vascular permeability (69), thus facilitating the diffusion of lipoproteins into the extravascular space where either in native form or after oxidation may possibly contribute to macrophage, smooth muscle cell, and endothelial cell activation (70-74). The increased expression of cell adhesion molecules, as well as the release of MCP-1 and IL-8, are likely to ensure the continuing recruitment of monocytes and T lymphocytes to the lesions (48,62,66,75). It is worth noting that CXCR2, the specific receptor for IL-8 (expressed by T lymphocytes and phagocytic cells), is overexpressed in atheromatous lesions (62,75). IL-12 and IL-18 are also expressed in atheromatous lesions (63,65). In the case of IL-18, higher levels of expression in carotid endarterectomy samples were associated with plaque instability, as reflected either by recent clinical symptoms of cerebral ischemia or by visible ulceration on the removed plaque tissue (65). These effects are not observed in double transgenic Apolipoprotein E

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(ApoE) -/- and IFN-gamma -/- mice, suggesting that the effects of IL-18 are mediated through release of IFN-gamma (76).

Several growth factors also appear to be released in atheromatous lesions. Two of them, PDGF and basic fibroblast growth factor-2 (bFGF-2) are able to induce smooth muscle cell proliferation (41,77-79). Once activated by a variety of factors, including PDGF, lysophosphatidylcholine contained in oxLDL, or infection by Chlamydia pneumoniae, smooth muscle cells are able to initiate autocrine activation circuits mediated by PDGF-AA and bFGF-2 (41,80,81). However, the pro-angiogenic properties of bFGF-2 have led to a reevaluation of its role and to attempts to use this factor to promote revascularization in areas of coronary stenosis (82). A second growth factor, monocytes/macrophage colony stimulating factor (M-CSF), is released by macrophages activated by engagement of Fc-gamma receptors (FcγR) (83). This growth factor allows activated monocytes and macrophages to survive and proliferate in atheromatous lesions (62,84) and may also contribute to the proliferation of smooth muscle cells in the plaque (85). Support for the significance of M-CSF as a pathogenic factor in atherosclerosis has been obtained in studies carried out with M-CSF deficient mice. Those animals show significantly reduced atherogenesis when placed on a high fat, high cholesterol diet (84).

Not all cytokines and soluble factors released by activated macrophages and lymphocytes have pro-inflammatory properties. IL-10, tumor growth factor beta (TGF-beta) and IL-1 receptor (IL-1r) agonist (IL-1ra) have anti-inflammatory properties either secondary to the inhibition of TNFα cells (49,86) or to the blocking of IL-1 receptors (87). IL-10 mRNA is found in human atherosclerotic plaques, in regions of decreased apoptosis where both macrophages and smooth muscle cells are present (88). In vitro, the addition of IL-10 to monocyte cultures inhibits the stimulation of IL-12 synthesis by oxLDL (89). IL-12 plays a critical role in the differentiation of pro-inflammatory TNFα cells (90) that can be, therefore, neutralized by IL-10. IL-1ra levels have been reported to be elevated in patients with acute myocardial infarction and stable angina pectoris (91), and IL-1ra is rapidly induced in carotid artery after balloon angioplasty and remains elevated for up to 2 weeks (92). High levels of IL-1ra have also been reported after treadmill exercise in patients with peripheral vascular disease (93). Although it has not been proven, it is logical to assume that patients with strong and sustained elevations of IL-1ra may have a more favorable evolution than those who fail to show this response. The same reasoning could apply to IL-10 and TGF-beta, because of their ability to down-regulate activated lymphocytes in the inflammatory lesions.

The contribution of activated T cells secreting IFN-gamma to plaque destabilization is two fold. In addition to reducing the synthesis of collagen, IFN-gamma has the potential of activating macrophages. Activated macrophages in the atheromatous lesion release both MMPs (63,94,95) and oxygen active radicals (67). The release of products of the respiratory burst may contribute to LDL oxidation, which has significant pathogenic potential as another factor able to cause cell activation or dysfunction, as described later. The release of MMPs has been the object of considerable attention because many investigators believe they are critical for plaque destabilization and rupture (63,96,97). The triple helical structure of fibrilar collagens strongly resists degradation by most proteolytic enzymes, except MMPs (98). Three members of the metalloproteinase family (interstitial collagenase or MMP-1, stromelysin or MMP-3, and 92 kD gelatinase or MMP-9) have been found to be expressed in atheroma lesions, in plaques' shoulders and regions of foam cell accumulation, but not in normal arteries (63). In contrast, the 72 kD gelatinase (MMP-2) is present in normal arteries. MMP-1 can also be expressed by dysfunctional endothelial cells (63).

The pathological significance of the local overproduction of metalloproteinasises is directly related to the modern concepts about the mechanisms underlying acute arterial ischemic events. Until recently the central dogma of clinical cardiology was that the greater the degree of vessel stenosis, the greater the risk of an acute coronary event. However, angiographic studies on patients with acute myocardial infarction (AMI) led to the surprising finding that the atherosclerotic lesions that gave rise to the occlusive thrombus in more than half of the cases did not have high-grade stenosis (99-102). Second, state-of-the-art pathology studies provided evidence that rupture of atherosclerotic plaques precipitates the formation of occlusive thrombus that causes AMI, regardless of the size of the plaques (103). These observations have led to the current understanding that the characteristics of the plaque are the main factor determining plaque stability (104). An atherosclerotic plaque consists of an atheromatous core and a fibrous cap. A positive correlation between the size of the atheromatous core and plaque disruption was reported. A plaque containing a core occupying >40% of the plaque is considered particularly vulnerable and at high risk for disruption and thrombosis (105). The thickness and collagen content of a fibrous cap are also very important for the stability of the plaque. In vulnerable plaques, the fibrous cap is often thinner at the shoulder regions where rupture is most frequently observed (106). Pathology studies found that disrupted caps contain less collagen and fewer smooth muscle cells than undisrupted caps (107). Most of the collagen (50-75%) in a normal artery or in a diseased intima is type I (98,108), synthesized and assembled by vascular smooth muscle cells. It has been shown that cytokines and growth factors regulate the expression of collagen in smooth muscle cells (109-111). On one hand, IFN-gamma released from activated T cells inhibits both collagen biosynthesis in smooth muscle cells and smooth muscle cell proliferation (109-111). IFN-gamma also promotes apoptosis of smooth muscle cells (99). On the other hand, TGF-beta inhibits cytokine-induced metalloproteinase synthesis, thus contributing to plaque stabilization (112). Thus, the balance between pro-inflammatory and inhibitory cytokines will determine the degree of stability of atheromatous plaques.

Finally, studies in animal models also support the significance of T cell activation, cytokine-mediated inflammatory processes, and the associated regulatory processes. The effects of the interruption of intracellular signaling mediated by CD40-CD40L (CD154) interactions, in which activated T cells overexpressing CD40L are likely to play a critical role has been studied in detail. In LDL receptor (LDLr) -/- mice administration of CD40L antibodies reduces
the size, lipid content, and inflammatory cell content of aortic atherosclerotic lesions (113), changes that are likely to promote plaque stability (114,115). Similar results were obtained in CD40L -/- ApoE -/- mice (116). Other experimental approaches have focused on cytokine-mediated steps in the inflammatory reaction. Huber et al (117) showed that administration of recombinant IL-6 to Apo-E deficient mice weekly for a total of 6 to 21 weeks led to larger atheromatous lesions than those observed in Apo E deficient controls that received weekly injections of saline. Boisvert et al. (62) reconstituted irradiated LDLr-/- mice with bone marrow expressing or lacking CXCR2 genes (coding for CXCR-2, the IL-8 receptor) and placed all animals in an atherogenic diet. The development of atheromatous lesions was slowed down in animals reconstituted with CXCR-2 -/- bone marrow. The administration of recombinant IFN-gamma to apoE -/- mice also exacerbated the development of atheromatous lesions in these animals, which showed increased numbers of activated mononuclear cells within the lesions (118). To further prove the pathogenic role of IFN-gamma Whitman et al. crossed ApoE-/- mice IFN-gamma-/- mice (119). The male F1 offspring showed decreased atherogenesis, with reduce numbers of activated T lymphocytes in the lesions. No effect was observed in F1 females, suggesting that hormonal factors may have a protective effect in female mice.

The protective effects of TGF-beta have been demonstrated in apoE-deficient mice using two different approaches: the administration of TGF-beta antibodies (120) and the administration of soluble TGF-beta receptor II (121). Both resulted in the development of highly inflammatory cells with decreased fibrosis. The protective effect of IL-10, in turn, is supported by experiments in which LDL-receptor -/- mice were given bone marrow grafts from IL-10 transgenic mice or wild-type bone marrow grafts and fed an atherogenic diet. The mice receiving the IL-10 transgenic bone marrow showed decreased formation of early fatty streaks in comparison with controls.

4. WHAT ARE THE TRIGGERS OF THE INFLAMMATORY PROCESS?

It is generally accepted that endothelial damage or dysfunction is likely to be the initiating insult leading to atherosclerosis. Many different insults have been proposed, including modified LDL, free radicals generated by smoking, hypertension, elevated plasma homocysteine concentrations, and infection (6). In this review we will concentrate our attention on infectious agents and modified LDL.

The role of infectious agents was first suggested by studies performed with Marek’s virus (122). Human viruses of the herpes group and Chlamydothila pneumoniae have been suggested as involved in human atherosclerosis (123-129). Several pathogenic mechanisms by which C. pneumoniae may cause the onset or progression of atherosclerosis have been postulated. Most of them are based on the ability of C. pneumoniae to infect endothelial cells, as well as macrophages and smooth muscle cells in the arterial intima (128,130). The direct consequences of such infection may include cell damage caused by endotoxin, increased expression of cell-adhesion molecules that may then attract inflammatory cells to the local of infection, (128), pro-inflammatory effects of overexpressed hsp60 (131,132), increased expression of tissue factor on infected endothelial cells (133) and increased uptake of LDL leading to foam cell transformation of infected macrophages (134,135). Indirect effects may be related to the immune response to this organism, directed both against bacterial components such as endotoxin (136) and hsp60, with the definite potential of affecting C. pneumoniae-infected cells (59,60). The immune response to C. pneumoniae may also contribute to the inflammatory process associated with atheroma development, either through the activation of Chlamydothila-reactive T lymphocytes (50,137), or through the activation of phagocytic cells as a consequence of the uptake of antigen-antibody complexes (138).

Antigen-antibody complexes (immune complexes, IC) may also contribute to atherosclerosis by binding to the endothelium and thus mediating the anchoring and activation of proinflammatory cells. Although normal endothelial cells do not express Fc receptors or complement receptor 1 (CR1) (139), both of which can mediate the binding of IC, these two receptors may be expressed when endothelial cells are damaged (139). Infectious agents, such as cytomegalovirus and herpes viruses induce endothelial damage and they were among the first infectious agents shown to induce the expression of Fc and CR1 receptors (140,141) in endothelial cells. Bacteria also appear able to induce the expression of Fc receptors. This was first demonstrated by Bengaulid et al. in human umbilical vein endothelial cells infected with Staphylococcus aureus (142). We have also shown that endothelial cells infected with C. pneumoniae express FgR, particularly FgRII (CD32) (143). The FgR induced by human cytomegalovirus to seems to be distinct from human cellular FgR (144), but irrespectively of the exact structure of the Fc receptors expressed in infected endothelial cells, the binding of IC may contribute to tissue injury or to the initial lesion involved in the development of atherosclerosis (144), as exemplified in an in vitro model of leukocytic vasculitis developed by Moser et al. (145) and by other "in vitro" models using IgG-containing IC adsorbed to red blood cells, which have shown that PMN are strongly activated by cell-bound IC but not by soluble IC (146,147). As a consequence of the recognition of RBC-bound IC, polymorphonuclear leukocytes release large quantities of platelet activating factor (PAF) (146,147), and this could certainly initiate changes associated with a vascular inflammatory response (148). On the other hand, the co-expression of FgR and ICAM-1 could certainly create favorable conditions for the recruitment and subsequent activation of monocytes into the subendothelial regions subjacent to infected endothelium, one of the hallmarks of a vulnerable atheromatous plaque (6).

The pathogenic role of modified lipoproteins in atherosclerosis is well established. As illustrated in Figure 2, there is evidence supporting a direct pro-atherogenic effect of modified forms of LDL (70,72,73,149-151), but probably even more significant are the consequences of the immune response directed against neoepitopes resulting from the modification of lipoproteins (9,152,153). Both types of effects have been extensively characterized in the case of oxLDL. Oxidized LDL is taken up by macrophages via receptor-mediated pathways other than the classic LDL receptor (154-160) and it induces
TH1 lymphocytes is a starting point for macrophage activation recognizing modified LDL-derived peptides. The activation of autoantibodies and the activation of T lymphocytes LDL are immunogenic and trigger both the synthesis of inflammation. At the same time, these modified forms of initiating the self-perpetuating activation circuits characteristic and in the release of pro-inflammatory mediators, thus molecules (e.g., cell adhesion molecules in endothelial cells) activation of these cells results in overexpression of membrane macrophages, T lymphocytes, and smooth muscle cells. The can activate or potentiate the activation of endothelial cells, amounts in diabetic patients. These modified LDL molecules particularly AGE-LDL and oxLDL, are generated in greater of vascular inflammatory reactions. Modified forms of LDL, oxidation products have been identified as cytotoxic (165,166) both endothelial and smooth muscle cells (163,164). Several experimental data suggests that it can injure vascular cells, foam cells (161,162). In addition, oxLDL is cytotoxic and CE accumulation and the transformation of macrophages into foam cells (161,162). In addition, oxLDL is cytotoxic and experimental data suggests that it can injure vascular cells, both endothelial and smooth muscle cells (163,164). Several oxidation products have been identified as cytotoxic (165,166) but the mechanisms responsible for cell injury are not yet known.

Other pro-atherogenic properties of oxLDL include enhanced synthesis of growth factors including PDGF-AA and PDGF receptor in smooth muscle cells, granulocyte-macrophage colony stimulating factor, macrophage colony stimulating factor and granulocyte-colony stimulating factor in aortic endothelial cells from humans and rabbits (167). Human endothelial cells exposed to oxLDL show increased expression of MMP-1, while the corresponding inhibitory protein is downregulated (168). Thus, the end result is a state of augmented proteinase activity. OxLDL has also been found to have chemotactic effects on monocytes (150) and to enhance monocyte adhesion to endothelial cells in culture (169-171). It has been reported that minimally modified LDL can induce binding of monocytes to endothelial cells (169) and stimulate the synthesis of MCP-1, a monocyte chemotactic protein (172) and that the expression of VCAM 1 and ICAM 1 by human aortic endothelial cells induced by TNF is enhanced by the addition of oxLDL (ICAM 1) and glycated or oxidized LDL (VCAM-1) (70). In addition, oxLDL may affect fibrolysis, by inhibiting the secretion of tissue plasminogen activator (tPA) by human endothelial cells (173,174) and stimulating the secretion of plasminogen activator inhibitor-1 (PAI-1) (174). Recent work performed in our laboratory (175) using mildly modified LDL demonstrated that the release of tPA by human umbilical vein endothelial cells exposed to oxLDL is lower than the release observed when the same cells are incubated with unmodified LDL (p<0.01). In contrast, oxLDL is a more efficient inducer of the release of PAI-1 than unmodified LDL (p<0.05). Thus, oxidized LDL is unable to stimulate the endothelium-dependent activation of fibrolysis and may promote a chronic prothrombotic state.

The advanced glycosylation end-products of human low density lipoprotein (as well as those of other proteins) have also been shown to have pro-inflammatory properties (176,177). Endothelial cells, fibroblasts, T lymphocytes, and phagocytic cells have been shown to be affected by modified proteins. Endothelial cells show increased permeability and pro-coagulant activity (178) and overexpress VCAM-1 (179), fibroblasts proliferate, activated T cells release increased amounts of IFN-gamma and mononuclear cells are chemotactically attracted and activated releasing pro-inflammatory cytokines (178). The impact of AGE in the atherosclerotic process associated with diabetes was recently assessed in streptomycin-induced diabetic ApoE -/- mice. Administration of soluble forms of AGE receptors (RAGE) resulted in reduction of vascular permeability and slowed down the progression of atheromatous lesions (180).

The pro-inflammatory properties of modified lipoproteins appear to be considerably enhanced as a consequence of their immunogenicity. The immunogenicity of modified lipoproteins was first reported by Steinbrecher et al., based on the immunization of laboratory animals with modified lipoproteins (181). From all the modified forms of LDL, oxLDL is the most extensively studied. Steinbrecher as well as Palinski et al. characterized its immunogenic epitopes (182,183). Furthermore, human autoantibodies to oxLDL were the first to be purified and characterized (184-186). Initially the attention of the researchers concentrated on finding evidence supporting a pathogenic role for oxLDL antibodies (using them as a surrogate measurement of oxLDL). However, epidemiological studies conducted by several groups produced conflicting data. While some groups reported a positive correlation between the levels of oxLDL antibodies and different endpoints considered as evidence of atherosclerotic vascular disease, progression of carotid atherosclerosis, or risk for the future development of myocardial infarction (183,187-192) others failed to show such correlation or showed an inverse correlation (152,193-202).

These discrepancies in the literature concerning the correlation of autoantibodies against oxLDL and CAD are not surprising. Levels of antibody formed against a specific antigen are highly variable, depending on individual variations in the immune response. Furthermore the avidity of the

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**Figure 2.** The central role of modified lipoproteins as triggers of vascular inflammatory reactions. Modified forms of LDL, particularly AGE-LDL and oxLDL, are generated in greater amounts in diabetic patients. These modified LDL molecules can activate or potentiate the activation of endothelial cells, macrophages, T lymphocytes, and smooth muscle cells. The activation of these cells results in overexpression of membrane molecules (e.g., cell adhesion molecules in endothelial cells) and in the release of pro-inflammatory mediators, thus initiating the self-perpetuating activation circuits characteristic of inflammation. At the same time, these modified forms of LDL are immunogenic and trigger both the synthesis of autoantibodies and the activation of T lymphocytes recognizing modified LDL-derived peptides. The activation of T lymphocytes is a starting point for macrophage activation through the release of interferon-gamma. The synthesis of autoantibodies to AGE-LDL and oxidized LDL is likely to result in immune complex formation in the subendothelial space, and LDL-IC have well-defined pro-inflammatory properties, mainly related to their ability to activate macrophages.
antibody to the respective antigen also shows individual variations. Thus, the measurement of free circulating autoantibodies depends not only on the magnitude of the antibody response but also on antibody avidity and on the amount of antigen present in circulation. If the average avidity of circulating autoantibody is sufficiently high and antigen is present in circulation, soluble IC are formed and in their presence the assays for serum oxLDL antibody concentrations become inaccurate and underestimate the absolute concentration of circulating oxLDL antibody (152,203).

However, some authors have raised questions concerning the pathogenic role of oxLDL antibodies and postulated a protective effect for the humoral response to oxLDL in humans (10) based on animal studies. In some of these studies Apo-E deficient mice and LDLr deficient mice deliberately immunized with homologous malondialdehyde-LDL (MDA-LDL) showed a reduction in the development of atheromatous lesions (204,205). Similar observations were reported in hypercholesterolemic rabbits immunized with autologous oxLDL (206). More recently, it was reported that a human-derived oxLDL monoclonal antibody inhibited the uptake of oxLDL by macrophages (207). This protective effect, however, was a consequence of the fact that the antibody was synthesized as Fab fragments (207); the inhibition of oxLDL uptake was just a consequence of the fact that Fab fragments cannot activate complement nor interact with Fcg receptors (208,209). This lack of interaction prevents the uptake of any type of antigen-antibody complexes, including those formed between oxLDL and corresponding antibodies. The LDL-containing IC (LDL-IC) formed in vivo are obviously formed with complete antibodies, able to fully interact with Fc receptors on phagocytic cells.

The data obtained in active immunization experiments is riddled with contradictions. For example, in Ameli’s study (206), antibodies to oxLDL developed spontaneously in non-immunized animals, as well as in animals immunized with native LDL. The greatest "protective" effect of immunization was actually observed in animals immunized with native LDL. The same "protective" effect of immunization with native LDL was observed in LDLr deficient mice, where the reduction in atherosclerosis development was seen in the absence of antibodies to modified LDL (210). It is also worth remembering that Palinski et al. demonstrated increased oxLDL autoantibody titers in LDLr -/- mice that were significantly correlated with the extent of atherosclerosis (211), an observation hard to reconcile with a protective role of such antibodies.

Another line of evidence has been based on adoptive transfer of splenic B cells to splenectomized ApoE -/- or rag-1 knock-out mice (212,213). The results observed in these experiments can be interpreted as suggesting that the transferred lymphocytes included a population of regulatory lymphocytes that reduced Tg1 activity and thus reduced the pro-inflammatory contribution of these cells. But the identification of the postulated regulatory cells as B lymphocytes cannot be considered as proven when data concerning the proportion of B cells in those spleen cell suspensions is either not given (212) or indicated to be ≥ 90% (213).

The involvement of LDL-IC in the pathogenesis of atherosclerosis was suggested over a decade ago by the elegant studies of Yla-Herttuala and co-workers (55,184). These authors purified oxLDL and the corresponding IgG antibodies from atheromatous lesions of humans and Watanabe hyperlipidemic rabbits, thus demonstrating that the ingredients necessary for the formation of LDL-IC are present in the damaged arterial wall. Orekhov and co-workers reported that the level of cholesterol in isolated circulating IC, which reflects the amount of LDL-IC in circulation, correlated with the severity of coronary atherosclerosis (214). Turk et al. reported that the concentrations of ApoB in isolated serum IC, another parameter reflective of the concentration of circulating LDL-IC, was significantly higher in patients with coronary heart disease (215). A prospective study involving 98 diabetic subjects recruited as part of the Pittsburgh EDIC study showed that LDL-IC and oxoLDL antibodies, correlated with the development of coronary artery disease over a period of seven years, but while the correlation with LDL-IC levels was direct, the correlation with oxoLDL antibody levels was inverse (152,202). Recently we had the opportunity to analyze the correlation between circulating LDL-IC levels and the circulating levels of cell adhesion molecules and inflammation markers in 1068 patients with type 1 diabetes, recruited as part of the DCCT study (unpublished results). We found significant correlations between the levels of these immune complexes and ICAM-1, C-reactive protein, and fibrinogen. We also found that LDL-IC levels had a highly significant correlation with measurements of internal carotid intima-media thickness in that same group of patients. That strong correlation did not change after correction by conventional risk factors (smoking, hypertension, age, HbA1c, duration of diabetes, total and LDL cholesterol, and triglycerides). Therefore, we have accumulated strong epidemiological evidence supporting the pathogenic role of modified LDL-IC in atherosclerosis.

Supporting the postulated pathogenic role of LDL-IC are studies carried out with immune complexes isolated by polyethylene glycol (PEG) precipitation (203,216-219) from the sera of patients with type 1 and type 2 diabetes or with clinical manifestations of atherosclerosis (219,220). These studies demonstrated that LDL-IC can elicit cholesteryl ester (CE) accumulation in macrophages, a property shared by model IC prepared with oxLDL and rabbit antibodies against LDL (216,217,221-224). The incubation of human monocyte-derived macrophages and macrophage-like cells with IC prepared with native human LDL and rabbit polyclonal LDL antibodies has been found to cause CE accumulation and induce the morphological transformation of macrophages into foam cells (221,222). At the same time, the cells ingesting LDL-IC become activated and release TNF alpha, IL-1, oxygen active radicals (225), and MMP-1 (226). The accumulation of cholesteryl esters by macrophages incubated with LDL-IC is primarily dependent on IC uptake through Fcg receptors, primarily the high affinity FcgRII (223) and appears to be a consequence of the delayed degradation of ingested LDL (227). Paradoxically, macrophages incubated with LDL-IC show an upregulated expression of LDL receptors (217,222,225,228,229).

The isolation of oxLDL antibodies from human sera (185,186) provided the opportunity to further evaluate the
pathogenic potential of oxLDL IC, using human oxLDL and purified human oxLDL antibodies for their preparation. The predominance of IgG1 and IgG3 isotypes in the purified antibody preparations led us to hypothesize that these immune complexes would have pro-inflammatory properties, given the ability of IgG1 and IgG3 immunoglobulins to interact with high affinity with the Fcg receptors of human macrophages and induce their activation (148,208). Indeed, human LDL-IC were shown to induce CE accumulation in human macrophages to a much higher degree than of oxLDL, even when oxLDL concentrations about ten times greater than the amount of oxLDL contained in the oxLDL-IC was used as control(230). Besides promoting CE accumulation, human oxLDL-IC activated macrophages leading to the release of TNF (230). Thus, we successfully reproduced the experiments previously performed using LDL-IC prepared with rabbit antibodies (221,222) proving that oxLDL-IC prepared with purified human antibodies have the same atherogenic properties.

5. FACTORS POSSIBLY CONTRIBUTING TO THE DEVELOPMENT OF MACROVASCULAR DISEASE IN DIABETES

The increased incidence of macrovascular complications including coronary heart disease, cerebrovascular and peripheral vascular disease (4,5,231) in diabetes mellitus has been long recognized. However, the mechanisms by which diabetes accelerates atherosclerosis are not fully understood. In recent years it was proposed that an increased level of modified lipoproteins may be a significant factor contributing to the accelerated development of macrovascular complications in diabetes (4). The persistence of high plasma glucose levels in diabetic individuals creates favorable conditions for some of these modifications to occur, including glycation, glycoxidation (4) and advanced glycation (5).

An excessive formation of oxLDL would certainly have a negative impact, given the atherogenic properties of oxLDL by itself, and its immunogenicity, leading to the formation of proatherogenic IC, as discussed above. Advanced glycation is also likely to play a significant role. This modification involves a chain of chemical reactions that starts with the covalent, non-enzymatic addition of reducing sugars to protein amino groups (Schiff base, Amadori adducts). If the half-life of a protein is sufficiently long, additional reactions take place leading to the formation of an heterogeneous family of sugar-amino acid adducts collectively know as 'Advanced Glycosylation End-products (AGE)' (5). LDL, like most plasma proteins, is susceptible to AGE modification (232). Glycation and AGE modification of LDL and other proteins is associated with free radical production (233), resulting in the formation of oxLDL (234). But the pro-inflammatory consequences of AGE modification of LDL and other proteins are not just indirect, due to oxidation of LDL and perhaps other proteins. There is experimental evidence demonstrating that AGE-LDL by itself can induce pro-inflammatory circuits (178,179,232) and cause trapping of protein in atherosclerotic plaques (232). In addition, AGE-modified proteins are immunogenic (235), a property that has been used to great advantage for their detection in serum (4) and localization in tissues (4,236).

The immunogenicity of AGE-modified lipoproteins is not limited to the induction of heterologous antibodies in experimental animals. Autoantibodies to AGE-modified serum albumin and AGE-modified IgG have been demonstrated in human sera, both from diabetic patients as well as in non-diabetic subjects (237-239). Data suggesting that these antibodies are able to combine with circulating AGE-modified antigens and form soluble IC has also been recently reported (238). AGE-LDL antibodies have also been recently isolated and characterized; they share the same isotypes as oxLDL antibodies but have a higher avidity (240). Because of the higher avidity of AGE-LDL antibodies, IC formed with AGE-LDL and corresponding antibodies are likely to have stronger pro-atherogenic properties than oxLDL-IC.

The nature of the LDL molecules found in IC isolated by PEG precipitation from the serum of diabetic patients has been also investigated. We have detected predominantly carboxymethyl lysine (CML) and MDA-lysine, suggesting that both oxLDL and AGE-LDL are involved in IC formation (240). Antibodies to oxLDL have also been isolated from PEG-precipitated IC and shown not to react with CML-LDL (unpublished data), further supporting the conclusion that the PEG-precipitable IC from diabetic patients contain at least two different forms of modified LDL and their corresponding antibodies.

Another interesting correlation revealed in our studies has been the one that exists between LDL-containing IC and diabetic nephropathy. Initial investigations in this area focused on the correlation between serum oxLDL antibody concentrations and diabetic nephropathy or nephropathy-related macroangiopathy and the results were negative (241). We were also unable to show a significant correlation between circulating oxLDL antibody levels and proteinuria, although trend analysis demonstrated a near significant correlation. However, when we focused our attention on LDL-IC levels we found an association between LDL-IC and abnormal albuminuria (242). Statistically significantly higher levels of cholesterol and apolipoprotein B were detected in the circulating IC of subjects with abnormal AER, particularly those with macroalbuminuria (p<0.03). More significantly, we were able to purify antigen-free IgG from PEG-precipitated IC from diabetic patients with normal and abnormal AER and demonstrate that such IgG contained oxLDL antibodies, and that these antibodies were of higher avidity than free, circulating oxLDL antibodies. Furthermore, the average avidity of antibodies contained in PEG-precipitated IC was significantly higher in subjects with abnormal AER values than in subjects with normal AER (242). It thus appears that LDL-IC may play a pathogenic role not only in macrovascular disease, but also in diabetic nephropathy, which in type 1 diabetes is a well-defined risk factor for macrovascular disease.

The process by which oxLDL-IC may cause increased albuminuria is not clear. OxLDL has been shown to induce proliferation of mesangial cells, a prominent feature of diabetic nephropathy (243), and cause the expansion of the extracellular matrix (149,244). In turn, mesangial cells have been shown to oxidize LDL in vitro (245). This observation has obvious implications, because in situ oxidation of LDL would create the necessary conditions for the formation of.
LDL-IC in the mesangium. In the case of human macrophages, the higher atherogenic potential of ox-LDL IC relative to free oxLDL seems to be related to the engagement of FcgRI by the IC (223). Human mesangial cells have been reported to express this same receptor after activation by IFN-gamma (246). As in the pathogenesis of large vessel disease, it is likely that a variety of immune cells are involved in the process. Activated T cells reacting with oxLDL and hsp60-derived epitopes have been detected in atheromatous lesions (53). A similar involvement at the glomerular level could account for the release of IFN-gamma and mesangial cell activation. Thus, the mechanisms leading to nephropathy and atherosclerosis could be rather similar and that would explain the link between nephropathy and macrovascular complications in type 1 diabetes.

6. PERSPECTIVE

The re-definition of atherosclerosis as a chronic inflammatory process has had significant implications not only in our understanding of disease processes associated with the onset and progression of atherosclerosis, but also has provided a strong impetus to adopt therapeutic interventions that reduce the activity of the inflammatory process and stabilize the endothelium. However, there is a recognized need to develop criteria to stratify patients on the basis of the inflammatory activity in their vessel walls and on the basis of the detection of factors able to induce vascular inflammation. This has resulted in the active search for parameters reflecting the intensity of the vascular inflammatory reaction and the extent of endothelial cell damage/dysfunction, as well as for ways to assay factors that may act as triggers of the inflammatory reaction or of the endothelial damage/dysfunction.

The intensity of the inflammatory reaction has been assessed by measurement in the blood of pro-inflammatory cytokines/chemokines (particularly IL-6) and other circulating molecules considered as inflammation markers (CRP, fibrinogen). However, a problem with those markers is their lack of specificity. Inflammatory processes involving any tissue other than the vessel wall can cause their increase, so their value for the assessment of possible vascular inflammation is limited to patients without clinical evidence of any other inflammatory condition, but this caveat still leaves inflammation is limited to patients without clinical evidence of any other inflammatory condition, but this caveat still leaves inflammation is limited to patients without clinical evidence of any other inflammatory condition, but this caveat still leaves inflammation is limited to patients without clinical evidence of any other inflammatory condition, but this caveat still leaves inflammation.

Among the recently defined triggers of the inflammatory reaction associated with atherosclerosis, antigen-antibody complexes containing modified lipoproteins and antigens or nucleic acids of Chlamydia pneumoniae deserve closer scrutiny. The infection of endothelial cells with C. pneumoniae could play the role of an initiating insult, by inducing the expression of cell adhesion molecules and Fcg receptors. However, serological assays for C. pneumoniae antibodies do not prove persistent active infection. It seems critical that reliable serum assays for C. pneumoniae antigens or DNA should be developed, to allow reassessment of this issue.

Modified LDL-IC have been clearly shown to activate macrophages leading to the release of pro-inflammatory cytokines, growth factors and proteolytic enzymes that have the potential to play significant roles in the initiation and/or perpetuation of the inflammatory process and in the induction of endothelial damage. In diabetes, the significant association between the levels of circulating LDL-IC and different end-points for macrovascular disease, microvascular disease, and inflammation supports the atherogenic and pro-inflammatory role of modified LDL IC and provides a strong rationale for additional studies of the predictive value of LDL-IC levels with regard to the development of clinical atherosclerosis.

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