Inflammatory mediators in atherosclerosis

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Introduction

In 1915 [1], Allbutt described atherosclerosis as 'a very chronic inflammation' and noted the 'round cell growth' in the adventitia. Brief mention of these cellular aggregates, associated with advanced atherosclerotic plaques, have since been made by other workers who have interpreted them variously [2, 3]. Lymphocytes and macrophages in the intima of atherosclerotic arteries have been described by light and electron microscopy. In 1979 [4], Joris and colleagues, in an electron microscopical study in the rat, observed the infiltration of blood-borne mononuclear cells into the intima and proposed that this was in response to a chemical message, perhaps an antigen, originating from the media. In 1962 [5], Schwartz & Mitchell suggested that arterial adventitial infiltrates of small lymphocytes correlated with the severity of the intimal atheromatous lesion and not with the anatomical site of the plaque nor with the patient's age or sex. Furthermore, these workers described the predominance of lymphocytes and plasma cells in the adventitia and media. They pointed out that in other conditions in which adventitial cellular changes occur, including polyarteritis nodosa, giant cell arteritis and disseminated lupus erythematosus, the cellular pattern was different to that found associated with the advanced atherosclerotic plaque. They suggested that these changes were involved in the pathogenesis of atherosclerosis and have since been due to some 'change in immunological tolerance' to a component of the plaque itself.

The inflammatory nature of human atherosclerosis has been largely ignored until recently. Immunological studies are long overdue to evaluate the role of inflammation in the pathogenesis of human atherosclerosis.

Chronic periaortitis: a local complication of advanced human atherosclerosis

A spectrum of chronic inflammation is commonly seen in association with advanced atherosclerosis when the aortic media is thinned [3]. Chronic periaortitis is a term recently suggested for this condition [2]. Chronic periaortitis may be detected only histopathologically, usually at necropsy, or, in its most severe form, may present clinically in the form of 'idiopathic retroperitoneal fibrosis', 'peri-aneurysmal retroperitoneal fibrosis' or 'inflammatory aneurysm' [2, 6]. Chronic periaortitis is thought to have an autoallergic cause. The allergen is believed to be a component of ceroid, likely to be oxidized low-density lipoprotein (LDL), elaborated in the human atheroma. This allergen is sequestered from the immune response unless the media is breached; the idea being that the atheromatous plaque acts as an immunologically 'privileged site' [3].

The role of macrophages in atherosclerosis

A role for blood-borne mononuclear cells in atherogenesis was first suggested by work on diet-induced lesions in rabbits. Electron microscopy of spontaneous human atherosclerosis supported this, as have cell-marker studies and, most recently, using immunohistochemistry with monoclonal antibodies to human macrophages. Thus it has been shown that lipid-laden 'foam cells' in early and advanced atherosclerotic plaques are monocyte-derived macrophages rather than smooth muscle cells [7].

The recognition that foam cells in human atherosclerotic plaques are macrophages lends new importance to studies on lipoprotein uptake. Of particular significance is the finding that LDL which has been chemically modified, for example by acetylation or acetoacetylation, is taken up more readily by macrophages in vitro and cleared from the bloodstream in vivo than 'native' or unmodified LDL [8]. It has been shown that LDL extracted from aortic wall is taken up more avidly by mouse peritoneal macrophages than is native LDL and so is LDL from inflammatory fluid. Acetylation is by no means the only way in which LDL can be modified, leading to increased uptake by macrophages. A variety of artificial treatments have a similar effect [9] and all seem to have in common the ability to make the LDL particle more electronegative, a property which was shown many years ago to encourage uptake by the macrophage. The potential importance of LDL modification in vivo is suggested by the finding that LDL extracted from human aortic intima, especially from atherosclerotic areas, differs from native LDL in its fatty acid content and its increased negative charge. LDL derived from inflammatory fluid also shows major differences from native LDL, including an increase in electrophoretic mobility. It is known that endothelial cells in vitro are capable of altering LDL particles and causing enhanced macrophage uptake and degradation as are smooth muscle cells and phagocytes including macrophages.

Oxidized lipids and atherosclerosis

The mechanism of LDL modification which has recently attracted most attention is oxidation, catalysed by certain ions, including Cu+2 [10]. The alteration in LDL produced...
by endothelial cells and smooth muscle cells is possibly the result of oxidation [10]. Oxidized LDL is taken up more avidly by macrophages than is native LDL. Studies in vitro suggest that LDL modification, perhaps especially oxidation, may occur in vivo and that macrophages take up this modified LDL preferentially, much as they phagocytose foreign material in their role as scavenger cells. In the arterial intima, this role of the macrophage may be crucial to the development of the atherosclerotic plaque. Macrophages are known to release harmful enzymes when they die and they also secrete substances which cause cell proliferation. In addition, oxidized lipids have been shown to damage enzymes and membranes, to cause necrosis and to decrease prostacyclin production. One other result of LDL modification is that it may be rendered antigenic [10]. It is therefore possible that autoallergy to this modified LDL is a factor in the development of the pathogenesis of atherosclerosis.

Ceroid, a similar substance to lipofuscin, is present in all advanced atherosclerotic plaques. The nature of this group of the inflammatory cells in atherosclerosis factor in the development or the pathogenesis of athero-sclerosis.

Ceroid may be regarded as the insoluble, end-product of oxidation of LDL in the macrophage. It may also be produced in vitro by oxidizing LDL artificially with Cu++ [11].

Serum antibodies to oxidized LDL and ceroid in chronic periaortitis Immunoglobulin-secreting plasma cells in the aortic adventitial infiltrate occur in chronic periaortitis; this has become evidence that chronic periaortitis is due to an autoallergic reaction to a component of the atherosclerotic plaque [14].

The possibility that ceroid might be antigenic was first suspected when human immunoglobulin was found to localize to ceroid in atherosclerotic plaques from patients with chronic periaortitis [15]. The serum of a large number of patients produced antibodies to human LDL, to oxidized LDL and to ceroid extracted from human atheroma was assessed in 100 subjects using an adaptation of the enzyme-linked immunosorbent assay technique [16]. Patients with chronic periaortitis, subclinical chronic periaortitis, ischaemic heart disease and 'elderly control' individuals were compared with young, healthy adults. Provided that oxidations were carried out in vitro, antibodies were never found to native human LDL. Antibodies to oxidized LDL or ceroid, usually both, were detected in all 20 patients with clinical chronic periaortitis, 17 out of 20 patients with subclinical chronic periaortitis, 12 out of 20 patients with ischaemic heart disease and 10 out of 20 'elderly control' individuals.

Western blotting after SDS/PAGE showed that in some patients with chronic periaortitis, some of these antibodies were directed against oxidation products of apo B [16].

The findings in ischaemic heart disease may be due to a relatively high incidence of subclinical chronic periaortitis in this group. Prospective studies will be necessary to substantiate this.

Characterization of the inflammatory cells in atherosclerosis

Hansson and colleagues [17, 18], using immunohistochemical techniques, have detected T lymphocytes and macrophages in atherosclerotic plaques from carotid endarterectomy specimens, but noted that B lymphocytes were absent. They noted that T cells, macrophages and smooth muscle cells were capable of expressing HLA-DR antigen. They also noted that T cells expressed interleukin 2 (IL-2) receptor and were associated with γ-interferon (IFNγ) secre-
tion. They suggested, from their findings, that in vivo T-cell-smooth muscle cell interactions occur during atherogenesis.

More recently, Van der Wahl and colleagues [19], using double immunohistochemical staining, showed that lymphocyte populations in various stages of human atherosclerotic plaques consist of HLA-DR positive T-helper and T-suppressor cells which express IL-2 receptor molecules. They postulated that local immune-mediated hypersensitivity reactions are associated with approximately 10% macrophages (EBM11 positive), 55% B cells (CD19/22 positive), 35% T cells (CD3 positive) with T-helper lymphocytes (Th) (CD4 positive) and T-suppressor lymphocytes (Tc/s) (CD8 positive) in ratio of beween 3 and 4 to 1. These cells were organized into secondary follicles with germinal centres. The predominance of CD4-over CD8-positive cells was as would be expected in a B-cell response to local extracellular antigen, requiring T-cell help. No polymophonuclear cells or natural killer cells were observed.

The expression of major histocompatibility complex (MHC) Class II antigen was also observed using monoclonal antibodies to the HLA-DR antigen. Of the cells in the inflamed tissue 60–80% expressed this antigen. The antigen was expressed by macrophages and smooth muscle cells and by many of the T cells. This abundant expression indicates a highly immunologically activated site. The MHC class II molecule is important in antigen presentation and is required for recognition of antigen by T-helper cells and for the subsequent initiation of an immune response. It is, however, unclear if all of these cells are acting as antigen presenting cells. This abundant, or perhaps aberrant, expression is also seen in other chronic inflammation reactions, as well as in most autoimmune diseases; it may indicate a loss of immunological control.

Proliferating cells have been investigated using the monoclonal antibody Ki67, which binds a proliferation-associated nuclear antigen [22]. The number of proliferating cells appears to correlate with the degree of inflammation. These proliferating cells are found predominantly around the germinal centre within the secondary follicles. They can comprise up to 10% of the lymphocytes. Monoclonal antibodies to the IL-2 receptor revealed a small proportion of the cells, again mostly around germinal centres, to be activated [20].
The role of cytokines in atherosclerosis

Studies have begun to look at the role of cytokines in this chronic inflammatory reaction, since these molecules regulate inflammation and all immune responses [23, 24]. Of special interest are tumour necrosis factor-α (TNFα) and IFNγ, which synergize and regulate the expression of the MHC class II molecule, which is so abundant in this tissue. Preliminary immunohistochemical studies have demonstrated IFNγ in and around some of the mononuclear cells. This method, however, has met with limited success in detecting the presence of cytokines, as these are often produced and secreted in picomolar quantities which are below the threshold for immunohistochemical detection. To observe the cytokines further, tissue culture supernatants have been collected for c.l.i.s.a. assays, and tissue and peripheral blood RNA has been extracted for Northern blot and polymerase chain reaction (PCR) analysis.

Cell culture of inflammatory cells in vitro associated with atherosclerosis: clinical implications

The atherosclerotic plaque may represent a site of relative immunological privilege until the media is breached or new vessels form in advanced or complicated plaques. Chronic periaortitis may be the end-stage of the immune response, directed at antigens elaborated in the atherosclerotic plaque during atherogenesis. Functional studies of the lymphocyte populations in atherosclerotic plaques in various stages of development in vitro, including those with associated chronic periaortitis, would establish whether or not this was the case.

Inflammatory cells have been cultured from the fresh surgical samples. These cells migrate out of the tissue into IL-2-conditioned medium [25], and within 2-3 weeks there is an expanded and workable population of cells. The distribution of these cells has been examined by immunocytochemistry on cytosin preparations over 6-week periods (after which the cells start to die without the exogenous addition of feeder cells or other factors). After 4 weeks, there are still live B cells in culture (confirmed by electron microscopy); this implies that factors required for their sustenance (possibly oxidized lipids) are still present. This could be very important for future work in trying to develop a human mononuclear antibody directly from the tissue's B cells.

It is interesting to note that fibroblast overgrowth is not seen in culture. In fact, fibroblasts are only seen in the wells without lymphocyte outgrowth or when there is stress put on the system. This has implications for observing factors involved in fibrosis and fibroblast proliferation.

Chronic periaortitis is characterized by the presence of adventitial mononuclear cells together with a varied amount of periaortic fibrosis. What determines the degree of fibrosis is unknown, but in its severest form, it is this fibrotic reaction which leads to the clinical manifestations of the disease, such as ureteric obstruction. Cells of the immune system are known to be sources of mediators regulating fibroblast proliferation [26]. The macrophage is a source of fibroblast growth factor; the T lymphocyte is known to produce fibroblast growth factor and collagen synthesis stimulatory factors [26]. The mechanisms of fibrosis in chronic periaortitis require further examination using techniques in vitro.

Future research

Chronic periaortitis may not represent a classic 'classical' autoimmune disease as there is no change in self-tolerance. Rather, there is a change in a self-protein. It is likely that anyone with oxidized LDL present in their serum could perhaps mount an immune response to this antigen, but that in the atheroma the modified LDL is both persistent and abundant and the immune system is unable to clear it. This antibody response may be a normal, physiological mechanism to clear harmful oxidized lipid from the serum. Patients with severe, chronic clinical periaortitis may be demonstrating an immunological derangement of a phenomenon commonly seen in those with advanced atherosclerosis.

Lymphocyte proliferation assays are presently under way. If there are T cells specific for oxidized LDL one would expect them to proliferate in vitro in the presence of antigen and antigen-presenting cells.

If this inflammatory reaction is specific, as proliferating cells are present in situ, there may be clonal dominance in the B- or T-cell populations. There may be some preferentially expanded clones in a 'sea' of polyclonal cells. It may be useful to look at T-cell receptor (TCR) gene rearrangement with probes for example, to the TCRβ constant gene using Southern blot analysis on T cells in vitro. PCR may also be carried out using a family of TCR Vβ-region primers on blood and tissue RNA [27]. Southern blot data from two patients whose T cells were expanded in culture have so far revealed an extra non-germline band not present in a polyclonal population (unpublished work). Although this may be an in vitro phenomenon, it indicates a specifically selected clonal expansion in both cases. Preliminary work using PCR has also revealed an increased presence of specific Vβ regions in the tissue infiltrate when compared with the patient's blood, indicating clonal dominance. Of course, further studies must be carried out to confirm and expand these results.

Concluding remarks

Chronic periaortitis is a local complication of advanced atherosclerosis. The findings of the studies presented here may have no relevance to processes occurring in atherogenesis, as they appear to be a consequence rather than a cause of human atherosclerosis. However, they are likely to be of relevance in the progression of the disease.

It is hoped to extend these findings by examining more biopsy specimens from patients with a spectrum of chronic periaortitis who present surgically. Both immunohistochemical studies in situ and lymphocyte culture studies in vitro are presently in progress.

Genetic and environmental modulation of low-density lipoprotein catabolism

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Introduction
There is now incontrovertible evidence to link plasma total cholesterol concentrations and, more specifically, low-density lipoprotein (LDL) cholesterol to the development of coronary heart disease. This association has obvious and important clinical and therapeutic implications which have stimulated numerous studies of the control of LDL metabolism. The regulation of plasma LDL is clearly multifactorial and is currently believed to be the result of the compounding influences of environmental and genetic factors.

LDL particles are spherical complexes consisting of a lipid core which is predominantly esterified cholesterol, and a polar shell composed of free cholesterol, phospholipid and protein. The protein component has both structural and functional importance. Over 90% of the protein present in each LDL particle is in the form of a single molecule of the huge apolipoprotein B (molecular mass 550 kDa). This protein maintains the structural integrity of the LDL particle and serves as the ligand for the specific cell surface LDL receptor. In normal individuals approximately half of LDL catabolism is mediated through this specific receptor pathway while the balance is removed by non-specific receptor-mediated uptake. The mechanism involved in this process is not yet established. Control of LDL catabolism may be discussed in terms of LDL receptor modulation and variation in ligand binding.

Modulation of receptor activity and function
(a) Environmental influences. The quality and quantity of dietary fat and carbohydrate determine, in part, the expression of hepatic LDL receptors. Diets rich in saturated fat result in increased production of LDL and decreased LDL receptor activity. Receptor-ligand binding is specifically influenced by saturated fatty acids [1]. A high carbohydrate diet is as effective in lowering an elevated LDL level as a high monounsaturated fat diet [2] or a standard modified fat diet [3]. The effects of dietary cholesterol on the activity of the LDL receptor have also been examined. Cholesterol feeding promotes LDL synthesis and suppresses receptor-mediated catabolism [4], resulting in an increased plasma LDL level. However, recent studies summarized by McNamara [5] using only moderately increased dietary cholesterol loads, have shown that there is a subset of the population (20–25%) which exhibits an exaggerated response to dietary cholesterol. Nutritional education is the first line therapy for all primary hyperlipidaemias and the guidelines followed by most clinicians are based on these metabolic observations.

Following the publication of the findings from the Lipid Research Clinics Coronary Primary Prevention Trial [6] and the Helsinki Heart Study [7] there has been great clinical interest in the pharmacological modulation of lipoprotein levels. These studies, for the first time, showed a reduction in coronary morbidity associated with lipid lowering drug therapy.

At least six distinct classes of lipid lowering drug are now available in the clinical armamentarium and three of these, the bile acid sequestrant resins, the 3-hydroxy-3-methyl glutaryl coenzyme A (HMG CoA) reductase inhibitors and the fibrates have been shown to exert their hypolipidaemic effect by influencing indirectly the LDL receptor. The resins interrupt the entero-hepatic circulation of bile acids and deplete the intrahepatic sterol pool, which is in turn replenished by the up-regulation of hepatic LDL receptors. The HMG CoA reductase inhibitors, by suppressing de novo synthesis of cholesterol, also result in a diminished intrahepatic sterol pool which is corrected in a similar fashion. The fibrates have been shown to suppress HMG CoA reductase activity in vivo leading to the stimulation of receptor-mediated uptake of LDL. The mechanism involved in this process is not yet established.

(b) Genetic influences. Receptor function as an inherited characteristic was recognized by Goldstein & Brown [8] who received the Nobel Prize in 1985 for their pioneering work. They studied patients with the autosomal codominant trait familial hypercholesterolaemia (FH) which is associated with abnormally high LDL levels and increased risk of premature coronary heart disease. Defects in the LDL receptor gene resulted in poor uptake of LDL by cells and accumulation of LDL in the plasma. Studies of cultivated fibroblasts from these patients and identification of the molecular lesions within the gene sequence encoding the LDL receptor have contributed to the understanding of the function of different regions of this receptor molecule. There are many documented defects in the LDL receptor itself which have been described at the molecular level [9]. These include deletions and insertions which are sometimes explained by unequal cross-over between homologous repetitive elements within the introns. Distinct abnormalities of receptor number and function have been described under the heading of FH whose overall prevalence in the population is approximately 1 in 500. As many as 1 in 20 of those individuals referred to specialist lipid clinics with elevated LDL levels will have receptor defects.

Abbreviations used: LDL, low-density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; FH, familial hypercholesterolaemia; RFLP, restriction fragment length polymorphisms.