

Macrophage Activation in Atherosclerosis: Pathogenesis and Pharmacology of Plaque Rupture

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Abstract: Atherosclerosis is still an important disease. It accounts for 39% of deaths in the U.K. and 12 million U.S. citizens have atherosclerosis-associated disease. Atherosclerosis may exert clinical effects by slow narrowing, producing stable angina or dramatic rupture, producing acute coronary syndromes such as unstable angina or myocardial infarction and death. Macrophages are abundant in ruptured atherosclerotic plaques. Macrophages are innate immune effectors, *i.e.* they are activated without antigenic specificity. This may make them liable to indiscriminate tissue damage, since they are less selective than lymphocytes. Macrophages are recruited and activated by many signals and have an impressive armamentarium of molecules to promote tissue damage. Macrophage recruitment by abnormal endothelium over developing atherosclerotic plaques, is aided by endothelial expression of adhesion molecules (ICAM-1, VCAM, ELAM). Use of knockout mice has implicated the chemoattractant cytokine (chemokine) MCP-1 in attracting macrophage recruitment in atherosclerosis. Macrophage-activation stimuli associated with atherosclerotic risk factors include oxidised low density lipoprotein (oxLDL, 'bad cholesterol'), advanced glycosylation end products (AGEs) of diabetes, angiotensin II and endothelin. Substantial work has clarified macrophage activation by OxLDL via macrophage scavenger receptors (MSRs), especially MSRA and CD36. Activated macrophages express effector molecules that kill cells and degrade extracellular matrix. These include Fas-L and nitric oxide (NO). Macrophage NO is derived from the high output inducible nitric oxide synthase (iNOS) pathway and upregulates vascular smooth muscle (VSMC) cell surface Fas, priming them for apoptosis. Activated macrophages express surface Fas-L, similar to cytotoxic T-lymphocytes and natural killer cells. Since VSMCs promote plaque stability, VSMC apoptosis may promote plaque rupture. Macrophages express multiple metalloproteinases (*e.g.* stromelysin) and serine proteases (*e.g.* urokinase) that degrade the extracellular matrix, weakening the plaque and making it rupture prone. Macrophages secrete numerous other effectors including reactive oxygen species, eicosanoids, tumour necrosis factor alpha and interleukin-1. Macrophage-derived transforming growth factor beta promotes fibrosis. Existing cardiovascular treatments including angiotensin II receptor antagonists and angiotensin converting enzyme inhibitors, aspirin, cholesterol reduction agents especially statins may inhibit macrophages. The interaction of NO-donors with macrophages and apoptosis is complex and bifunctional. Traditional anti-inflammatory agents such as glucocorticoids and cyclophosphamide have very serious side effects and are probably inappropriate. Novel anti-inflammatory agents *e.g.* new immunosuppressives and anti-TNF therapy may have an improved cost-benefit ratio.

ATHEROSCLEROSIS

Atherosclerosis is an increasingly important disease worldwide [1,2]. Although in developed countries age-specific mortality has fallen as a result of improved medical care [3], this is reflected in cardiovascular mortality at older ages, rather than deaths from other causes [4]. Cardiovascular disease (mainly atherosclerosis) accounts for 35% of deaths in the U.S.A. [5] and similar Western countries. Atherosclerosis can cause slow coronary narrowing, producing stable angina, predictable pain on exertion. Alternatively the plaque may rupture, producing acute coronary syndromes, unstable angina, myocardial infarction or sudden death [1].

ATHEROSCLEROSIS IS AN INFLAMMATORY DISEASE

Serum C-reactive protein (CRP), a marker of inflammatory activity is a better predictor of coronary risk

than low-density-lipoprotein (LDL) cholesterol [6]. Moreover, atherosclerotic plaques contain a characteristic inflammatory infiltrate, including monocytes, numerous monocyte-derived macrophages; modified lipid-laden macrophages (foam cells); and T-lymphocytes [1,7]. Thus, macrophages are critical to atherosclerotic inflammation [1]. Plaque T-lymphocytes have an interesting immunophenotypic pattern. Plaque T-lymphocytes express activation markers. Many plaque T-lymphocytes are a mix of CD4-positive T-helper cells that secrete interferon- and tumour necrosis factor- (Th1 cells). The remainder are CD8-positive cytotoxic T-lymphocytes that contain pro-apoptotic perforin and Fas-Ligand [7]. Plaque T-lymphocytes are enriched (about 30%) in the normally rare -T-cell subset [7]. Importantly, atherosclerotic plaques only rarely contain either neutrophils or B-lymphocytes, which are usually found in the majority of forms of chronic inflammation [7].

Rupture-prone (vulnerable) atherosclerotic plaques have increased inflammation [1]. Vulnerable plaques usually have a large soft lipid core (necrotic core) with a thin fibrous cap

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[1]. The fibrous cap is formed of collagen and vascular smooth muscle cells (VSMCs) and endothelial lining, separating the thrombogenic lipid core contents from the blood in the lumen [1]. The fibrous caps of ruptured atherosclerotic plaques have more macrophages than those of non-ruptured atherosclerotic plaques [1,8]. This circumstantially implicated macrophages in plaque rupture [1]. Macrophages have numerous effector mechanisms that may promote atherosclerosis and plaque rupture [1].

Macrophages are innate immune effectors, that is they are activated without antigenic specificity [7]. This may make them more prone than T-lymphocytes to indiscriminate tissue damage that could promote plaque rupture. Macrophages are recruited and activated by many signals and have an impressive armamentarium of molecules to promote tissue damage, tissue repair, coagulation and fibrinolysis and to communicate with the rest of the immune system.

MACROPHAGE RECRUITMENT

Macrophage recruitment by abnormal endothelium over developing atherosclerotic plaques, is aided by endothelial expression of the inflammatory adhesion molecules for leukocytes [ICAM, VCAM, ELAM, P-Selectin, E-Selectin. ICAM, VCAM and ELAM]. These are upregulated by multiple atherosclerotic risk factors acting directly on the endothelium, including oxLDL, smoking, hypertension and diabetes. In particular, they are modulated by haemodynamic factors, arterial pressure and shear stress that are important in localising atherosclerotic lesions [9]. Shear stress high areas are protected from atherosclerosis, which forms preferentially in low shear stress areas at branches and bends [9]. Endothelial adhesion molecules are upregulated in a complex way by inflammatory cytokines (reviewed in [10]). Different inflammatory cytokines produce different time-courses of expression, different adhesion molecules have different avidity for different leukocyte subsets, and different adhesion molecules may be dependent on either protein synthesis or redistribution. Thus there are overlapping cytokine-specific patterns of activation. This has led to the concept that leukocytes home to specific sites depending on the combination of adhesion molecules, rather like the letters that form an address [11]. The importance of these molecules is evidenced by knockout mice. ICAM-1 knockout mice are resistant to atherosclerosis [12] and mice with reduced VCAM-1 have less atherosclerosis [13] (true VCAM-knockouts cannot be studied as the deletion is embryonic lethal).

Use of knockout mice has implicated the chemoattractant cytokine (chemokine) MCP-1 in attracting macrophage recruitment in atherosclerosis [14]. Atherosclerosis is essentially abolished in MCP-1^{-/-} mice indicating that MCP-1 is absolutely required for atherosclerosis from its earliest stages [14]. Anti-MCP-1 gene therapy can promote the regression of advanced plaques in mice, indicating that that MCP-1 plays a role in advanced plaques. MCP-1 acts via its receptor CCR-2, and correspondingly, CCR2^{-/-} mice are also resistant to atherosclerosis [15]. Bone marrow transplant studies have confirmed that it is monocyte CCR-2 that mediates this role [16]. Since CCRs are 7-transmembrane

segment G-protein-linked receptors, they are very good targets for the development of small molecule antagonists, of which several are now available [17]. It remains to be seen whether they are effective in reducing atherosclerosis. MCP-1 is a chemoattractant for human monocytes, is expressed in human atherosclerotic plaques, and is reduced by statins [18]. However, its true role in atherosclerosis in humans is uncertain as yet and it must be emphasised that in reality *in vivo* leukocyte recruitment is the complex resultant of multiple pleiotropic cytokines and chemokines acting on endothelium and leukocytes.

MACROPHAGE DIFFERENTIATION

When monocytes exit from the circulation, they migrate across endothelium into tissues and alter phenotype to become macrophages. This is variably termed maturation, differentiation or activation. Moreover, it is increasingly apparent that there are different sorts of macrophage differentiation depending on different stimuli, corresponding to different macrophage phenotypes. Some authors draw a distinction between differentiation, which they define as effects of IFN- and activation, which they define as effects of bacterial lipopolysaccharide.

Macrophage differentiation *in vitro* may be followed with HLA-DR (which presents antigen) and other surface markers including CD16 (Fc-γRIII) [19]. CD16 is a receptor for the crosslinked form of antibody IgG, such as found on microbes or in pathological antibody deposits (immune complexes). It is unclear whether CD16 is found on human plaque macrophages. However, intriguingly, immune complexes and microbes have been identified as atherosclerosis risk factors. We have shown that macrophage differentiation of a weeks culture *in vitro* is associated with the acquisition of a macrophage pro-apoptotic effect towards co-cultured VSMCs. Apoptosis is a regulated mode of cell death, and several groups have demonstrated increased apoptosis in atherosclerotic plaques using a variety of techniques. We found that macrophage-induced VSMC apoptosis required cell-cell contact and was mediated by Fas-L [19]. This indicates that this phenomenon, previously thought to be specific for cytotoxic T-lymphocytes (CTL), is shared by macrophages. Notably, CTL are also associated with apoptosis in human plaques, but in CTL the induction of apoptosis is tightly restricted by antigen and self-MHC. Since macrophages do not detect specific antigens or MHC, some other mechanisms must regulate macrophage-induced apoptosis. At least one such pathway involves nitric oxide (NO), which potentiates macrophage-induced VSMC apoptosis by upregulating macrophage Fas-L and VSMC Fas [19,20].

Macrophage phenotypes are an interesting but confused area. Riches suggested three macrophage phenotypes histotoxic, reparative and inflammatory [21]. However, there is overlap between these (*e.g.* pro-inflammatory and histotoxic phenotypes) and there are other macrophage functions, such as antigen presentation, that his schema does not cover. Rees has extended this work to demonstrate macrophage programming, where activation and by one cytokine reduces subsequent responses to other cytokines, indicating phenotypic commitment. One important

specialised phenotype of the macrophage-lineage of emerging importance in atherosclerosis is dendritic cells (DCs) [8]. DCs are specialised for antigen presentation to initiate an immune response [22]. Immune phenomena are increasingly recognised in atherosclerosis [1]. Human atherosclerotic plaques contain CD4-positive T-helper lymphocytes that recognise oxidised LDL as an antigen [23]. CD80, which is essential to initiate a successful T-lymphocyte reaction, is found in human atherosclerotic plaques, and human carotid plaques contain cells immunoreactive for other DC markers including DC-SIGN [22]. Thus, macrophage differentiation to specialise for antigen presentation is emerging as important in atherosclerosis.

Adhesion and mechanical stimuli also regulate macrophage differentiation. Indeed, when added to culture, contact with the culture plastic itself has a massive effect on the profile of macrophage transcriptome profile, via the NF- κ B and Jak/STAT pathways [24]. There is evidence that this particularly applies to atherosclerosis. Mechanical strain alters several important macrophage properties including expression of scavenger receptors for atherogenic OxLDL [25,26]. This may partially explain the synergistic interactions between hypercholesterolaemia and hypertension. Transendothelial migration itself alters macrophage activation [27] and may promote their differentiation towards an antigen-presenting DC phenotype.

MACROPHAGE ACTIVATION

In addition to differentiation, macrophages may be activated by a number of stimuli. The exact difference between differentiation and activation is a moot semantic point, reviewed in part by Riches [21].

More recently, multiple stimuli associated with atherosclerotic risk factors have been shown to activate macrophages. These include OxLDL (so-called bad cholesterol), advanced glycosylation end products (AGEs) of diabetes, angiotensin II, and endothelin.

It is widely recognised that LDL although an epidemiological risk factor for atherosclerosis, is probably not the atherogenic form. Instead, it is oxidised, possibly by plaque macrophage themselves, to ox-LDL, which is atherogenic [28]. Substantial work has clarified macrophage activation by ox-LDL via macrophage scavenger receptors (MSRs) especially MSR-A and CD36 (the main MSR-B). However, there are a large number of other putative MSRs defined by their ability to bind the many forms of OxLDL. These include CD68, LOX and the receptors for AGEs (RAGE), MSRs and RAGEs have several commonalities. Essentially, AGEs are over-glycosylated proteins in diabetes that constitute modified / degenerate forms of normal proteins, that are recognised and phagocytosed as 'junk' for clearance [29]. Indeed, classical MSRs may act as RAGEs, especially CD36 [30]. The best characterised of the RAGEs, simply called RAGE, activates macrophages and is found in sites of chronic inflammation including atherosclerosis [31] (reviewed in [32]).

The vasopressor peptides AII and endothelin may directly activate macrophages *in vivo* [33,34] and *in vitro*

[35,36]. While largely of unexplored significance, this could provide another pathogenetic link between hypertension and atherosclerosis.

MACROPHAGE ALTERNATIVE ACTIVATION

More recently Gordon has mooted the alternative activation of macrophages, to describe the macrophage phenotype induced by Th2 cytokines [37]. This contrasts with the phenotype(s) induced by microbial agents or by the Th1 cytokine IFN- γ , in particular in that alternative activation is associated with upregulation of the macrophage mannose receptor [37]. Other phenotypic differences may become apparent with wider (*e.g.* proteomic) analysis. The alternative activation paradigm roughly corresponds with the M1-M2 paradigm promoted by some other authors [38], which is associated with an altered pattern of chemokines [38].

Although IL-10 $^{-/-}$ mice are more susceptible to atherosclerosis [39], IL-10 is a broadly anti-inflammatory cytokine and the role of Th2 cytokines such as IL-4 in atherosclerosis has not been specifically studied [37].

MACROPHAGE ACTIVATION BY CD40

Macrophage express CD40, a member of the TNF-R superfamily, which is activated by CD154 (CD40L) a member of the TNF superfamily (reviewed in [40]). Anti-CD40 antibodies reduce atherosclerosis *in vivo* [41]. Macrophage CD40 expression is induced by oxLDL [40]. CD40L is expressed by several cell types, but principally by CD4 $^{+}$ T-helper cells. Thus, CD40L/CD40 is one route of macrophage activation by plaque T-lymphocytes [40]. CD40 stimulates effector functions including chemokine release [40], angiogenesis induction [40], enhanced procoagulant activity due to upregulated tissue factor [40], Clinical trials of humanised anti-CD40 antibodies are underway [40].

MACROPHAGE EFFECTOR FUNCTIONS

Macrophage antigen presentation has been briefly described. Macrophages express an enormous battery of effector molecules that are too numerous to comprehensively detail in one review [7]. Overall, these make macrophages well placed to lyse host tissues, including atherosclerotic plaques, which would be expected to promote plaque rupture [42]. These include metalloproteinases [1,43,44], serine proteases [43,44], aspartate and cysteine proteases [43,45], eicosanoids [46], inflammatory cytokines [47], reactive oxygen species, reactive nitrogen species (RNS) [20], and death-inducing molecules including Fas-L [20].

Metalloproteinases

(MMPs) have transitional metal cations at the active site [48]. MMPs have been given a systematic numerical nomenclature [48]. The list of metalloproteinases described in atherosclerosis is continuously expanding, its growth now being assisted by array-based methods including transcriptional profiling. At the time of writing MMPs implicated in macrophages in atherosclerosis include MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9 and MMP-13 [49]. MMPs are induced by cytokines and by mechanical

strain [26]. Although most research attention has recently focussed on collagenolysis, the extracellular matrix also includes other components notably elastin and proteoglycans. Both elastinolysis and proteoglycanolysis are accomplished by MMP isozymes. MMPs reciprocally activate and are activated by, other macrophage proteases especially urokinase (via plasmin) [49,50] (reviewed in [43]). Although MMPs clearly play a critical role in matrix degradation, the full picture of the different MMP isoforms and their interplay *in vivo* with each other and with other macrophage proteases is still largely uncertain [51].

Eicosanoids

Most recent work in this area has focussed on the respective role of isoform of phospholipase A₂, secretory (sPLA₂) and cytoplasmic (cPLA₂) [52]. Despite its name, cPLA₂ more probably has a membrane-associated subcellular location, where it is the isozyme responsible for classical activities such as leukotriene biosynthesis.

Macrophages secrete sPLA₂, where it may bind to the extracellular matrix [52]. Although *in vivo* functions of sPLA₂ are uncertain; degradation of bacteria, LDL and apoptotic cells have been shown *in vitro* [52]. It is postulated that matrix-bound sPLA₂ may modify subendothelial LDL to an atherogenic form [53]. Inflammatory cytokines induce sPLA₂ [52]. In addition, sPLA₂ has autocrine effects on macrophages, upregulating Fas-L [54].

Nitric Oxide

Paradoxically, although NO, the active product of nitrovasodilators, is seen as beneficial in vascular disease, NO and its products (collectively called reactive nitrogen species (RNS)), are usually seen as deleterious in chronic inflammation. At least part of this depends on concentration. For example, endothelial NO is derived from the low output eNOS isozyme and is probably of net benefit whereas macrophage NO is derived from the high output iNOS isozyme [20]. Although human iNOS produces less NO than rodent iNOS, adjacent to source macrophages NO probably reaches sufficient concentration to be histotoxic, even in humans [20,55].

Apoptosis

An emerging area is macrophage-induced apoptosis of normal vascular cells [19,20]. It has been previously shown that macrophages are cytotoxic to tumour cells. Although other leukocyte subsets, particularly cytotoxic T-lymphocytes may induce apoptosis inappropriately, e.g. in autoimmunity, macrophages had not formerly been recognised to induce pathological apoptosis in normal cells. However, prolonged culture of human peripheral blood monocytes allowed maturation to a pro-apoptotic phenotype after 5-8 days culture [19,20]. These macrophages induced apoptosis in cocultured (bystander) VSMCs, irrespective of origin from human coronary media, aortic media or carotid plaque [20,19]. A series of experiments demonstrated that the mechanism required a combination of at least Fas-L and NO [20,19]. These 2 mediators were both essential, because

blockade of either abrogated apoptosis. This was traced to induction of VSMC surface Fas by NO, and to induction of macrophage surface Fas-L by NO [20]. This enhanced the efficiency of Fas/Fas-L interactions, shown by increased responsiveness of NO-stimulated VSMCs to Fas-L [19,20]. Since NO activates P53 [56], and P53 induces the translocation of Fas to the cell surface [56], NO-induced Fas activation is likely to be by a P53-dependent mechanism.

EXISTING CARDIOVASCULAR THERAPEUTICS

Some of the evidence presented here suggests that several cardiovascular drugs already used in clinical practice may act, in part, by modulating macrophage function. For example, since AII activates macrophages, AII receptor antagonists and CEIs may suppress macrophage activation. Aspirin, which inhibits cyclooxygenase has a clear link with plaque inflammation as a mode of action in addition to its effects on vascular homeostasis. Cholesterol reduction agents especially hydroxy-methyl-glutaryl-coenzyme-A (HMG-CoA) reductase inhibitors (statins) may inhibit macrophages by an interesting mechanism. Cholesterol enriched membrane microdomains (so-called lipid rafts) essential for leukocytes signal transduction, and comprise cell membrane regions enriched in receptors, cholesterol and palmitoylated and farnesylated signalling proteins. Statins have effects on atherosclerosis in excess of their effects on cholesterol-lowering, inhibit leukocyte signalling and reduce fatty acyl (as well as cholesterol) synthesis, so it is tempting to speculate that these features are causally related. As already discussed, the interaction of NO-donors (e.g. nitrovasodilators) with macrophages and apoptosis is complex and bifunctional [57].

NOVEL ANTI-INFLAMMATORY AGENTS

Traditional anti-inflammatory agents such as glucocorticoids and cyclophosphamide have very serious side effects making them almost certainly inappropriate for atherosclerotic inflammation. However, a series of new immunosuppressive agents with much improved side effect profiles are becoming available (developed for transplantation). Furthermore, anti-TNF- monoclonal antibody therapy is now licensed for use in severe chronic inflammation of the gut and joints. These have an improved cost-benefit ratio. I think that there is a clear need for an agent that will directly tackle plaque inflammation with sufficiently few side effects that it can be given long term.

CONCLUSIONS

Macrophages are an interesting and versatile lineage, with numerous associated effector functions that implicate them in several features of atherosclerosis including lipid phagocytosis, plaque repair, plaque rupture, and the autoimmune phenomena associated with atherosclerosis. There are documented influences of atherosclerotic risk factors on the recruitment and activation of macrophages in atherosclerotic plaques. Macrophages may, in part, be involved in the anti-atherosclerotic effects of several cardiovascular agents. Macrophage effector functions may present useful targets for future cardiovascular therapies.

ABBREVIATION

NO	= Nitric oxide
iNOS	= Inducible nitric oxide synthase
MSRs	= Macrophage scavenger receptors
MSRA	= Macrophage scavenger receptor A
oxLDL	= Oxidised LDL
LDL	= Low density lipoprotein
ICAM-1	= Intercellular adhesion molecule 1
VCAM	= Vascular cell adhesion molecule
AGEs	= Advanced glycosylation end products
MCP-1	= Monocyte chemoattractant protein-1
CRP	= C-Reactive Protein
MHC	= Major Histocompatibility Complex
HLA	= Human leukocyte antigen
Th	= T helper
CD	= Cluster of differentiation number
CTL	= Cytotoxic T lymphocyte
CCR-2	= Chemokine receptor 2
Fc RIII	= Third receptor for C terminal of IgG
DCs	= Dendritic cells
LOX	= Lectin like oxidised low density lipoprotein receptor
JAK	= Just another kinase
STAT	= Signal transducers of activated T cells
IL-4	= Interleukin 4
IFN-	= Interferon-
TNF-	= Tumour necrosis factor
MMP	= Matrix metalloproteinase
RNS	= Reactive nitrogen species
PLA2	= Phospholipase A2
HMG-CoA	= Hydroxy-methyl-glutaryl-CoenzymeA
NF- κ B	= Nuclear Factor kappa B
Rafts	= Short term for cholesterol enriched membrane microdomains

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