SPECIAL ARTICLE

Macrophage function and stability of the atherosclerotic plaque: Progress report of a European project

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Introduction

Some degree of atherosclerosis is an almost invariable accompaniment of aging in developed countries, but by no means all individuals with atherosclerosis suffer its devastating thrombotic complications of myocardial infarction and stroke.

Over the last few years, it has become clear that thrombotic occlusion does not generally occur in the region of full-blown fibrotic and calcified atherosclerotic plaques, but rather in the vicinity of smaller and often cell-rich regions in which a thin fibrous cap overlies a central core of extracellular lipids. These regions have been called "culprit lesions" (1, 2). Infra-red thermography has shown that such vulnerable plaques are metabolically active, and histological examinations has revealed them to be particularly rich in macrophages (3). The reasons for the rupture of culprit lesions are not entirely clear, but it is known that the prefinal event of plaque fissuring is the outcome of a complex metabolic drama in which inflammation, apoptosis and lipid metabolism are intimately intertwined (Fig 1).

Excessive degradation of the various components of the extracellular matrix of the fibrous cap is considered to be an important causative factor of plaque weakening (4). It is likely that collagenases and other matrix metalloproteinases (MMPs) secreted by various cell types within the atherosclerotic plaque, especially macrophages (5, 6), play a central role in this degradation. As the oxidized low-density lipoproteins (LDL) (7) and pro-inflammatory cytokines found in atherosclerotic lesions, such as tumour necrosis factor α (TNF-α) and interleukin 1-β (IL-1β) (8), induce MMP secretion by macrophages *in vitro*, one possible avenue to plaque stabilisation is to inhibit MMP secretion and extracellular activation.

The effects of a number of compounds on MMP activity have been tested, including naturally occurring substances

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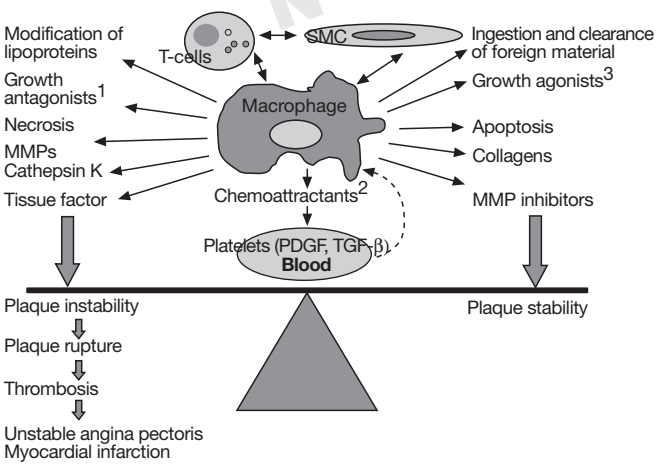
FIGURE 1

Role of macrophage and macrophage gene products in plaque stability. It is thought that in most patients, myocardial infarction and unstable angina pectoris are due to erosion or rupture of the fibrous cap overlying the lipid core of the atherosclerotic plaque. The degree of stability of an atherosclerotic lesion in the coronary arteries is the result of a dynamic process in which both factors that increase plaque stability and others that destabilize the plaque are constantly active. It is also likely that small ruptures occur continuously, but that these are stabilized at the stage of thrombosis, and that this thrombosis becomes integrated into the lesion without catastrophic occlusion of the vessel. Such events are thought to be the pathophysiological correlate underlying the clinical syndrome of unstable angina pectoris. Macrophages have a Janus-like role in plaque stability, and display a wide range of both stabilizing and destabilizing functions. Whether the net effect of the macrophage is stability or instability depends on the status of the cell. Indeed it is possible that two macrophages in close proximity to each other may have quite different net effects on plaque stability. The phenotype of macrophages is intimately controlled by the cells, including T cells and smooth muscle cells, within their immediate environment. These cells also interact in mutual fashion. Macrophages may modify lipoproteins, contributing to inflammation within the lesion, but also ingest and clear foreign material including altered lipoproteins. Macrophages produce a wide range of growth agonists, but also produce an equally broad array of growth antagonists. Macrophages also secrete a large number of chemoattractants that entice not only monocytes, but also T-cells and mast cells into the plaque. These T-cells interact with macrophages, producing as they do granulocyte-macrophage colony stimulating factor, tumor necrosis factor α, and γ-interferon that activate and maintain the viability of macrophages, while macrophages release interleukin 2, which induces proliferation of T-cells. Macrophages may undergo necrosis, which by the uncontrolled release of cellular content has the potential to destabilize the lesion, while regulated apoptosis of macrophages may not have this effect. Macrophages produce a wide range of matrix metalloproteinases (MMPs), including MMP-1 (collagenase), MMP-3 (stromelysin) and MMP-9 (gelatinase) as well as cathepsin K, which by digesting the fibrous cap destabilize it. However, we have shown that macrophages also produce type VIII collagen, which may well have the opposite effect. In addition, a range of tissue inhibitors of matrix metalloproteinase activity (TIMPs), which are produced by both macrophages and smooth muscle cells, modulate MMP activity. Macrophages are also the main source of tissue factor, which by activating the clotting cascade causes thrombosis to occur in the vessel overlying the lesion. When a thrombus is initiated, blood factors such as platelet-derived growth factor (PDGF) stimulate the migration and proliferation of SMCs, while transforming growth factor β (TGFβ), which is also released from platelets, stimulates the synthesis of intracellular matrix. (55, 56). [1]Growth antagonists produced by macrophages: granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage-CSF (M-CSF), heparin-binding epidermal growth factor-like growth factor (HB-EGF), insulin-like growth factor-1 (IGF-1), vascular endothelial growh factor (VEGF), basic fibroblast growth factor (bFGF), interleukin-1 (IL-1), tumor necrosis factor α (TNFα), transforming growth factor α (TGFα), transforming growth factor β (TGFβ), platelet-derived growth factor (PDGF). [2]Chemoattractants produced by macrophages: GM-CSF, M-CSF, VEGF, bFGF, macrophage chemotactic peptide 1 (MCP-1), TGFβ, PDGF, oxidized low density lipoprotein (oxLDL). [3]Growth antagonists produced by macrophages: interferon γ (IFNγ), IL-1, TGFβ.



such as transforming growth factor-β (TGF-β) corticosteroids and heparin, and synthetic or semi-synthetic compounds such as tetracycline antibiotics, anthracyclines and synthetic peptides. However, as none of these has shown convincing signs of therapeutic usefulness (9), treatments aimed at stabilising atherosclerotic plaque have so far been indirect and have largely concentrated on attempts to reduce the lipid core by means of lipid-lowering drugs, mainly the inhibitors of hydroxymethylglutaryl coenzyme A reductase (statins) (10). There is therefore a great need to develop therapies that directly stabilise atherosclerotic lesions.

In response to this challenge, the Macrophage Function and Stability of Atherosclerotic Plaque (MAFAPS) consortium was founded in June 1999 to bring together European experts in various areas with the aim of identifying targets of macrophage origin whose modification could increase the stability of vulnerable atherosclerotic lesions. The consortium, which officially began its research activities in June 2000, is funded by a grant from the European Commission's Fifth Framework Programme "Quality of Life and Management of Living Resources". This report describes the consortium's research aims and summarises the results of its first scientific symposium held at the University of Münster in December 2000.

Project plan

Figure 2 shows a flow-chart from which it can be seen that the aim of the project is to identify macrophage-derived gene products and potential mechanisms for stabilising vulnerable atherosclerotic plaques.

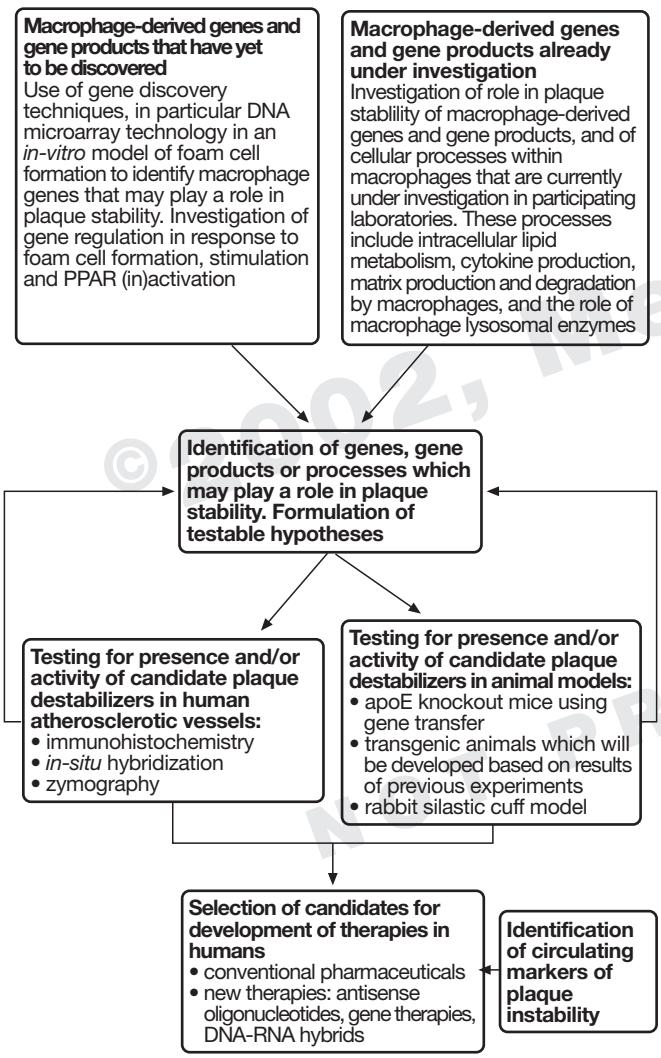
Circulating markers of plaque instability

The main clinical correlate of plaque rupture is acute coronary syndrome, a medical emergency that is usually due to platelet aggregation at the site of an ulcerated atheroma (11). Acute coronary syndrome is difficult to manage clinically and often lethal (12). For this reason, many affected patients require emergency coronary bypass surgery. However, one major limitation of the current treatment of plaque instability is the fact that its imminence can be predicted on the basis of only a few symptoms, signs, laboratory tests or investigative procedures.

The clinical and histological features of atherosclerosis indicate a process of inflammation. Some antigens, such as

FIGURE 2

Flow-chart showing the basic research plan of the MAFAPS Consortium. In addition, the consortium is also carrying out research aimed at identifying circulating markers of plaque instability. MAFAPS: Macrophage Function and Stability of the Atherosclerotic Plaque; PPAR: peroxisome proliferator-activated receptors.



oxidised LDL, have already been identified as causing inflammation within the plaque (13), but it is likely that a number of other local and systemic auto- or foreign antigens also play a role (13, 14). However, it is not known if the inflammation is antigen-specific, if it is confined to the plaque, or if circulating inflammatory markers, such as activated T cells, and/or soluble factors, such as C-reactive protein (CRP) and adhesion molecules, reflect inflammation within the plaque or elsewhere (15).

There is evidence that systemic inflammation increases the chances of an acute atherosclerotic event. The risk of myocardial infarction and stroke is higher in patients with chronic or recurrent infections (16-18), and increased CRP levels, which are indicative of an acute phase response, predict cardiovascular risk in healthy individuals and a poor outcome in acute coronary syndromes (19).

Macrophage, T-cell and mast cell-rich inflammation is a classical feature of plaque rupture and superficial erosions or flaps (20, 21). The proportion of macrophages, T cells, mast cells and HLA-DR+ cells in the culprit lesion increases with the severity of the acute coronary syndrome (22-24). Furthermore, systemic monocyte activation is a feature of unstable angina (25), and there is also evidence that circulating T-cells are also activated (26). For example, the blood of patients with unstable angina contains more interferon (IFN)-γ producing T-cells than that of patients with stable angina (22, 27, 28), although this increase declines after 7-14 days and there is a return to baseline values after three months (29).

The aim of the studies being performed by Andrew Exley at Papworth Hospital in Cambridge is to identify further circulating markers of plaque instability as a step towards determining the probability of plaque rupture in patient groups, and thus to generate relative risk calculations for patients with acute coronary syndromes. This group is collecting coronary sinus blood intra-operatively from patients with acute coronary syndrome, and using patients undergoing elective coronary surgery as controls. The samples will be tested for the presence of high levels of plaque-derived soluble factors (systemic markers of inflammation) and the presence of macrophage-activating T cells. Given the lack of a good animal model of plaque rupture, the detection of circulating markers of plaque instability is important for identifying surrogate end-points that can be used in clinical studies of therapies aimed at modifying this condition.

Investigation of known macrophage-derived genes and gene products

As shown in Figure 2, one of the main aims of the MAFAPS project is to investigate the role of known genes and gene products, particularly those involved in the regulation of intracellular lipid metabolism, in macrophage homeostasis.

Intracellular lipid metabolism in macrophages

It has recently become clear that various members of the adenosine triphosphate (ATP) binding-cassette transporter (ABC) gene family are intimately involved in cholesterol transport in macrophages and other cell types (30). ABCG1 (formerly known as ABC8) seems to play a role in transporting cholesterol out of cells (30-32), whereas ABCG8 and ABCG5 are intimately involved in the regulation of sterol absorption in the gut (33, 34). In addition to its role in cholesterol metabolism, ABCA1 also contributes to the secretion of IL-1β from monocytes and the engulfment of apoptotic cells by macrophages, as reported by Von Eckardstein *et al* (35). The results of other studies by this group indicate that ABCA1 also affects another major pathway of cholesterol efflux in macrophages, namely that mediated by apolipoprotein E (apoE) (35). Macrophage-derived apoE particles also inhibit the migration and proliferation of smooth muscle cells (36), and inhibit the expression of vascular cell adhesion molecule 1 (37). The ABC gene family (and ABCA1 in particular) may therefore have a number of functions that might be exploited in the future development of new anti-atherogenic therapies.

One of the hallmarks of atherosclerosis is the presence of cholesterol ester-laden macrophages within the plaque, and it has been shown that a number of pharmacological agents can affect cholesterol metabolism in these cells. Cignarella (38) of the University of Milan has reported that statins reduce the esterification of cholesterol in macrophages possibly by blocking intracellular cholesterol trafficking, and Bernini *et al* (39) of the University of Parma have studied the effects of calcium antagonists on cholesterol esterification and found that lacidipine (a third-generation dihydropyridine calcium antagonist) inhibits this process in cultured macrophages and the aortas of cholesterol-fed rabbits. More recent studies have also shown that lacidipine and statins reduce the release of MMP 9 from macrophages (39-41), thus suggesting that these drugs may play a role in promoting plaque stability.

Peroxisome proliferator-activated receptors (PPARs) in macrophages

Over the last five years, it has been found that the PPAR family of nuclear receptors plays a significant role in lipid and glucose metabolism (42). The two main members of this family are PPAR-α, which is mainly expressed in the liver, but is also found in kidney, heart, muscle and the cells of the arterial wall, and PPAR-γ, which is most strongly expressed in white adipose tissue where it triggers adipocyte differentiation. PPAR-α is activated by the fibrate class of lipid-lowering drugs, fatty acids and eicosanoids, whereas PPAR-γ is activated by 15-deoxy-Δ (12, 14) prostaglandin J_2 (15-d PGJ_2), oxidised fatty acids and glitazone antidiabetic agents (43, 44). Both PPAR-α and PPAR-γ are produced in macrophages, where they regulate the genes involved in inflammatory response, modulate differentiation, and promote TNF-α/IFN-γ)-induced apoptosis (45-47).

Chinetti *et al* (48) described the results of recent studies performed at the Pasteur Institute in Lille on the role of PPARs in the formation of macrophage-derived foam cells. PPAR-γ ligands inhibit the transcriptional activation of type A scavenger receptors and enhance the expression of both type B1 scavenger receptors (a ligand for high-density lipoprotein) and the CD36 molecule, which binds oxidised LDL. It was therefore reasonable to suspect that PPAR-α and PPAR-γ ligands affect foam cell formation in human primary macrophages, but a series of careful studies have shown that this is not the case: neither of the ligand types

TABLE 1
Principal achievements of the MAFAPS Consortium to date.

Achievement	Centre
Establishment of human coronary artery bank	Cambridge Helsinki, Münster
Elucidation of new functions of ABC transporters in macrophage cholesterol metabolism. Investigation of new members of the ABC family.	Münster
Further elucidation of the role of PPARs in macrophage metabolism.	Lille
Role of macrophages in the production of the intracellular matrix within the atherosclerotic plaque	Münster
Establishment of effective gene hunting technology	Lille, Maastricht, Münster
Establishment of a source of transgenic and non-transgenic animal models	Maastricht, Milan
Elucidation of cholesterol metabolism in macrophages	Milan, Münster, Parma
Model system to characterise macrophage-specific NF-κB activators or inhibitors	AstraZeneca

MAFAPS: Macrophage Function and Stability of the Atherosclerotic Plaque; PPARs: peroxisome proliferator-activated receptors; NF-kB: nuclear factor kB.

affected the cholesterol loading of human macrophages or the THP-1 macrophage cell line, but both increased cholesterol efflux by up-regulating the expression of the ABCA1 gene, a transporter that controls apoA-I-mediated cholesterol efflux from macrophages. This was subsequently shown to be most probably due to a PPAR-mediated increase in the expression of the liver-x-receptor α, an oxysterol-activated nuclear receptor that induces the expression of the ABCA1 promoter. These findings are very important given the respective central roles of the activators of PPAR-α (fibrates) and PPAR-γ (glitazones) in the treatment of hyperlipidemia and type 2 diabetes, both of which are associated with premature atherosclerosis.

Nuclear factor κB (NF-κB) in macrophages

The transcription factor NF-κB plays a key role in immune and inflammatory responses, cell survival and stress response. Cell exposure to stressors such as bacterial or viral infections, pro-inflammatory cytokines such as TNF-α or IL-1, or ultraviolet light leads to the degradation of the NF-κB inhibitor I-κB, which allows NF-κB to migrate to the cell nucleus, bind to the κB response element, and direct gene transcription (49). NF-κB is activated in macrophages, smooth muscle cells and endothelial cells, three of the major cell types involved in atherosclerotic lesions (50). The aim of the work of Freeth is to identify and characterise novel macrophage-specific NF-κB activators or inhibitors that may serve as potential therapeutic targets. This is being done by means of functional cloning whereby human macrophage cDNAs are cloned in a retroviral vector downstream of the powerful cytomegalovirus promoter. The result will be the generation of a library of "live" retroviruses, which will be be used to infect a stable reporter cell line containing two different NF-κB-responsive marker genes. The rare cells expressing both marker genes will be isolated by means of high-throughput fluorescence-activated cell sorting and then expanded, after which the inserts will be rescued and identified using sequencing.

The role of macrophages in extracellular matrix production and degradation

Excessive formation of a collagen-rich matrix is one of the main features of the atherosclerotic plaque that causes arterial stenosis. However, an established plaque with a fibrous cap can become a dangerous rupture-prone lesion if collagen degradation is enhanced and collagen synthesis is reduced. Smooth muscle cells are mainly responsible for synthesising the extracellular matrix, but may also produce lytic enzymes (51); by contrast, macrophages, which are mainly seen as specialists in tissue degradation, may also contribute to the formation of the extracellular matrix (52). As reported by Weitkamp *et al* (52) of the Institute of Arteriosclerosis Research in Münster, this may be due to the macrophage secretion of cytokines such as TGF-β (which stimulate matrix formation) and the direct macrophage synthesis of components of the extracellular matrix, such as fibronectin, proteoglycans and type VIII collagen.

Using a combination of immunohistology and *in situ* hybridisation, Weitkamp *et al* (52) have shown that macrophages within the atherosclerotic plaque express both MMP I (collagenase) and type VIII collagen as both were often detected within the same macrophage. Subsequent *in vitro* experiments showed that the synthesis of type VIII collagen and collagenase is differentially regulated: macrophage stimulation with IFN-γ and IL-1 reduced the expression of type VIII collagen but increased that of collagenase. The conclusion drawn from these studies was that macrophages are capable not only of degrading an existing extracellular matrix but also of forming a new provisional matrix. Further research is needed in order to characterise macrophages in terms of their possibly heterogeneous ability to degrade and synthesise the extracellular matrix, and to investigate the interactions between these two activities.

Macrophage lysosomal enzymes and atherosclerosis

One topic that has so far received relatively little attention is the action of macrophage lysosomal enzymes in atherogenesis. The group of Petri Kovanen at Helsinki's Wihuri Research Institute is investigating the expression of cathepsin D and lysosomal acid lipase in human atherosclerotic lesions. both of these enzymes are of central importance in the intracellular degradation of LDL particles. Only small amounts are found in normal arterial intima, where their location is exclusively intracellular (a finding that has been interpreted as reflecting the small number of macrophages in early lesions), but large amounts are found in the macrophages and surrounding extracellular matrix in atherosclerotic lesions. Moreover, the group has found that cultured human monocyte-derived macrophages can secrete active enzyme forms that are capable of modifying LDL, especially if the phago-

cytic activity of the cells is stimulated. It therefore seems that at least some lysosomal enzymes may play an important role not only in the intracellular degradation, but also in the extracellular modification of LDL particles.

Searching for regulated macrophage-derived genes

In addition to the studies of the known gene pathways described above, another important aspect of the work of the MAFAPS Consortium involves the use of various screening techniques aimed at identifying regulated genes of interest in macrophages and foam cells. This work is mainly being done by the group of Paul Cullen at the University of Münster's Institute of Arteriosclerosis Research using DNA microarray technology.

Two complementary approaches are being used to detect the macrophage gene products that may contribute to plaque instability. The first involves the use of an *in vitro* system of primary human macrophages and monocyte/macrophage cell lines. To simulate the processes thought to occur within atherosclerotic plaque, these cells are loaded with cholesterol and treated with a variety of agonists. The regulation of gene expression is then studied using DNA array-based technology. The second approach involves the use of laser-capture microdissection to isolate cells from atherosclerotic human coronary arteries, for which there is an extensive tissue bank prepared from the hearts explanted during cardiac transplantation. This tissue has been classified using a modification of the standard Stary classification (53) recently proposed by Virmani *et al* (54) that includes all of the stages of atherosclerotic plaque; stored in a central database, this information will also provide a source of research material for the other members of the consortium. Gene expression in human atherosclerotic tissue will be also investigated using array-based and other techniques. It is hoped that the combination of *in vitro* and *in vivo* gene expression studies will allow hypotheses to be formulated and tested more efficiently than would be possible using either system alone.

Testing hypotheses in animal models and human atherosclerotic tissue

Animal models

Two types of model are available to the MAFAPS Consortium. The first involves the use of an animal model in which a hyperplastic intimal lesion is produced in rabbit carotid artery by means of the implantation of a hollow silicone collar. Although this effect does not depend on a lipid insult, hypercholesterolemia generally leads to a more complicated lesion with abundant extracellular matrix and lipid deposition, the accumulation of cholesterol-laden macrophages in the intimal layer, and more severe intimal thickening. Preliminary experiments using this model have also revealed the up-regulation of MMP expression and an increased cholesterol esterification rate in the cells of the arterial wall.

The second type of animal model involves the generation of transgenic and knockout mouse models aimed at identifying the role of specific gene products in the development of atherosclerosis. These models will be provided by the University of Maastricht's Molecular Genetics Group headed by Marten Hofker, and have the initial aim of making a molecular analysis of early atherogenesis and the role of macrophages in this process. The work can be divided into three parts. The first involves the study of enzymatic lipoprotein modifiers. A mouse model has been generated for this purpose that specifically over-expresses human secretory phospholipase A2 in macrophages using the CD11b promoter. The second part involves the study of the receptors involved in the uptake of modified lipoproteins such as scavenger receptor A and CD36. The third part focuses on the role of inflammation in the initiation and progression of atherosclerosis and, more specifically, on the role of NF-κB for which various mouse models are available. Using these models, the technique of bone marrow transplantation into mice with atherosclerosis-susceptible backgrounds will be applied to the study of the specific role of macrophages in atherogenesis.

Conclusions and aims

Macrophages play a complex role in vulnerable plaques. The traditional view is that they are the culprits. Macrophage foam cells undergo necrosis and apoptosis, and therefore contribute to the growth of the lipid core. Macrophages also secrete LDL-modifying lytic MMPs and enzymes, and they produce a wide range of cytokines and chemotactic substances that contribute to an inflammatory plaque reaction. However, things are not that simple. Macrophages express ABCA1 and apoE, and thus contribute to the the active removal of cholesterol from the lesion. Macrophages ingest and neutralise irritating and toxic substances such as modified LDL, and they secrete a type of collagen that is characteristically found in healing wounds.

The hope of the members of the MAFAPS Consortium is that a better understanding of the pattern of macrophage gene and protein expression will improve our understanding of the role of these cells in unstable plaques and thus allow us to identify potential therapeutic targets. We hope that by the time this project has run its 3-year course, we will have come a step closer to identifying a specific treatment for this life-threatening condition.

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