INFLAMMATION IS A CRUCIAL FEATURE OF ATHEROSCLEROSIS AND A POTENTIAL TARGET TO REDUCE CARDIOVASCULAR EVENTS

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Summary

Atherosclerosis, and its devastating complications cerebral and myocardial infarction and gangrene of the extremities, is the leading cause of death. Atherogenesis in humans develops over many years, often reckoned in decades. Early lesion formation may even occur in adolescence. Lesion progression depends on genetic make-up, gender and certain well-recognized risk factor as well as a number of non-traditional risk factor that are currently the subject of intense investigation.

Our concepts of atherogenesis have evolved from vague ideas of inevitable degeneration to a much better defined scenario of molecular and cellular events. As we enhance our understanding of its fundamental mechanism, we can begin to approach atherogenesis as a modifiable rather than ineluctable process. Recently, inflammatory and immunological mechanisms have been increasingly implicated in the pathogenesis of this disease. Postulates for the involvement of innate and adaptive immunity with cellular as well as humoral components have met with considerable experimental support. It is now well recognized that atherosclerotic lesions contain activated, immunocompetent cells, including T lymphocytes and monocyte/macrophages. Inflammatory and/or immune mechanisms appear to be particularly active when plaques are activated and rupture and they may therefore cause acute coronary syndromes or stroke. Increasing knowledge of the basic mechanisms enables us to understand how current therapies for atherosclerosis may act. Insights derived from recent scientific advances should aid the discovery of new therapeutic targets that would stimulate development of novel treatment. Such new treatments could further reduce the considerable burden of morbidity and mortality due to this

modern scourge, and reduce reliance on costly technologies that address the symptoms rather than the cause of atherosclerosis. Eventually, mastery of the cell and molecular biology of atherosclerosis may permit development of novel strategies for mitigating this prevalent disease. Our research group has produced several evidences to shown new mechanism of immune cell cross-talk and activation. In this review I will describe some anti-inflammatory agents capable reducing these inflammatory processes and atherosclerosis.

Introduction

Health trends in the next 25 years will be determined by the ageing of the world's population, the decline in age-specific mortality rates from communicable, maternal, perinatal, and nutritional disorders, the spread of HIV, and the increase in tobacco-related mortality and disability. Today, ischemic heart disease is the leading cause of death in developed countries with more than 3 million death/year, followed by stroke (1.6 million) and lung cancer (0.7 million) [1]. By 2020, based on current trends, ischemic disease will be the leading cause of disease burden worldwide, followed by unipolar major depression, road traffic accidents, stroke and chronic obstructive lung disease. For worldwide ischeamic disease only, the number of Disability-Adjusted Life Years or DALYs estimated for 2020 is about 82.3 millions per year [2]. Thus, despite changes in lifestyle and the use of new pharmacological approaches, atherosclerosis and its devastating clinical complications such as ischemia and infarction of the heart, the brain and other vital organs, ruptured aortic aneurysm and peripheral vascular insufficiency, continue to account for the majority of mortality and morbidity in the adult population of industrialized countries, and would be so worldwide by 2020 [3].

Atherosclerosis is a progressive disease process that generally begins in childhood and has clinical manifestations in middle to late adulthood. Atherosclerosis focally affects the aorta, carotid, coronary, iliac and femoral arteries. This disease is characterized by lesions (called atheroma), localized in the intima of the vessel wall. From the initial phases

of leukocyte recruitment, to eventual rupture of the vulnerable atherosclerotic plaque, inflammatory mediators appear to play a key role in the pathogenesis of atherosclerosis [4]. Thus, atherosclerosis is not any longer considered to be a degenerative process because of the accumulation of lipid and necrotic debris in the advanced lesions. Indeed, already in 1856 Virchow proposed that "A form of low-grade injury to the artery wall resulted in a type of inflammatory insudation, which in turn caused increased passage and accumulation of plasma constituents in the intima of the artery"[5]. Three fundamental biological processes generate atherosclerotic lesions: 1) LDL-cholesterol infiltration and activation within the intima of arteries; 2) endothelial activation and dysfunction, with adhesion molecule expression; 3) leukocyte recruitment from the blood stream with the consequent production of pro-inflammatory cytokines, chemokines and proteases, responsible for the maintaining of these inflammatory process within the vessel wall. These soluble mediators induce also the proliferation on smooth muscle cells and the production of acute phase reactants by the liver [6]. This complex process induces the developing of a systemic pro-inflammatory state in which different soluble factors and cellular players are involved. Accumulating evidence suggests that inflammatory processes play a fundamental role in each of these stages in atherogenesis. Although the first event of atherogenetic cascade remains unknown, the inflammatory processes have to be considered as key components of the developing of the disease and promising targets for novel and more selective treatments. Therefore, the discovery of new molecular mechan-isms, which could be contrasted by novel therapeutic strategies, represents an important possibility for humans to mitigate atherosclerosis. In this regard, the development of murine models of atherosclerosis has revolutionized the approach to evaluating potential roles of specific factors in lesion development. These models are based on targeted disruption of the low-density lipoprotein (LDL) receptor or apolipoprotein E (apoE) genes [7]. Feeding of these knock out mice with cholesterolrich diet results in massive hypercholesterolemia and, thus, to atherosclerosis development. The implication of various inflammatory mediators in atherosclerosis has been clarified by crossing these animals with mice that overexpress or lack possible candidate genes.

My main research field was focused on leukocyte recruitment to inflammatory sites and inflammatory cell activation. Then, I identified promising therapeutic strategies (statins, anti-chemokines and cannabinoids) to reduce these inflammatory processes and thus the progression of atherosclerosis.

Inflammation

Inflammation is a protective reaction against a variety of exogenous (microbial, chemical, physical) or endogenous (immunological, neurological) disturbances, which is characterized by the accumulation of specific subsets of leukocytes to sites of infection or tissue damage, and their subsequent activation [8]. The attraction of leukocytes to tissues is essential for inflammation and the host response to infection [9]. This migration is a directional, nonrandom and selective process. The process of leukocyte trafficking manifests itself as inflammation with four classic cardinal signs: redness, swelling, heat and pain. In order to recruit leukocytes, the capillary blood flow and the vascular permeability are increased. This allows for enhanced migration of the leukocytes through the vascular endothelium, which is the boundary between the capillaries and the tissue, towards the site of inflammation. Depending on the cause, inflammation can resolve rapidly or develop into a complex process involving different leukocytes as well as endothelial and mesenchymal cells. When an infection or a lesion appears, the organism acts as quickly as possible in order to get rid of the injury. Among the first immune cells to arrive at the lesional site are neutrophils, which initiate a rapid, nonspecific phagocytic response [10, 11]. These cells produce toxic substances including proteases and oxygenic radicals that suppress the pathogen quickly but non-specifically. Whilst this process is efficient, a more specific antigenic recognition mechanism has evolved. In the specific antigen recognition process, antigen-presenting cells migrate to the site where antigen is present, followed by specific subsets of T and B lymphocytes [12]. The combination and activation of leukocytes generates the antigen-specific immune response, which results in the production of appropriate antibodies and activation of cytotoxic T lymphocytes [8].

There are two classes of inflammation: acute inflammation, which is of short duration and is characteristically accompanied by plasma fluid exudates and neutrophils accumulation; and chronic inflammation which involves other leukocyte types such as monocytes/macrophages, T cells, eosinophils, basophils, mast cells and dendritic cells. This class of inflammation is of longer duration and is characterized by dense cellular infiltrates. Emerging evidence supports involvement an implication of chronic inflammation as the crucial cornerstone of atherogenesis [13–17].

Atherosclerosis and inflammation

A role for inflammation has become well established over the past decade or more in theories describing the atherosclerotic disease process. From a pathological viewpoint, all stages, ie, initiation, growth, and complication of the atherosclerotic plaque might be considered to be an inflammatory response to injury. In a variety of animal models of atherosclerosis, signs of inflammation occur hand-in-hand with incipient lipid accumulation in the artery wall. For example, blood leukocytes, mediators of host defenses and inflammation, localize in the earliest lesions of atherosclerosis, not only in experimental animals but in humans as well. The basic science of inflammation biology applied to atherosclerosis has afforded considerable new insight into the mechanisms underlying this recruitment of leukocytes. The normal endothelium does not in general support binding of white blood cells. However, early after initiation of an atherogenic diet, arterial endothelial cells begin to express on their surface selective adhesion molecules that bind to various classes of leukocytes. Interestingly, the foci of increased adhesion molecule expression overlap with sites in the arterial tree particularly prone to develop atheroma. Considerable evidence suggests that impaired endogenous atheroprotective mechanisms occur at branch points in arteries, where the endothelial cells experience disturbed flow. For example, absence of normal laminar shear stress may reduce local production of endothelium-derived NO. This endogenous vasodilator molecule also has anti-inflammatory properties [18]. In addition to inhibiting natural protective mechanisms, disturbed

flow can augment the production of certain leukocyte adhesion molecules [19]. Augmented wall stresses may also promote the production by arterial smooth muscle cells of proteoglycans that can bind and retain lipoprotein particles, facilitating their oxidative modification and thus promoting an inflammatory response at sites of lesion formation.

Once adherent to the endothelium, monocytes and T lymphocytes penetrate into the intima. Chemoattractant molecules appear to be responsible for the direct migration of monocytes and T lymphocytes into the intima at sites of lesion formation [20–23]. Once resident in the arterial wall, the blood-derived inflammatory cells participate in and perpetuate a local inflammatory response. The macrophages express scavenger receptors for modified lipoproteins, permitting them to ingest lipid and become foam cells. Several pro-inflammatory mediators, such as macrophage colony-stimulating factor (M-CSF) contribute to the differentiation of circulating monocytes into the macrophage foam cell [24]. T lymphocytes likewise encounter signals that cause them to elaborate inflammatory cytokines, such as interferon-γ, interleukins, or tumor necrosis facto-α, which in turn can stimulate macrophages as well as vascular endothelial cells and SMCs [25]. Inflammatory processes not only promote initiation and evolution of atheroma, but also contribute decisively to precipitating acute thrombotic complications of atheroma. Most coronary arterial thrombi that cause fatal acute myocardial infarction arise because of a physical disruption of the atherosclerotic plaque. The activated macrophage abundant in atheroma can produce proteolytic enzymes capable of degrading the collagen that lends strength to the plaque's protective fibrous cap, rendering that cap thin, weak, and susceptible to rupture. Interferon arising from the activated T lymphocytes in the plaque can halt collagen synthesis by SMCs, limiting its capacity to renew the collagen that reinforces the plaque [8]. Macrophages also produce tissue factor, the major procoagulant and trigger to thrombosis found in plaques. Inflammatory mediators regulate tissue factor expression by plaque macrophages, demonstrating an essential link between arterial inflammation and thrombosis [14–16].

Anti-inflammatory effects on atherogenesis
CD40

More than a decade ago, discovery of the B lymphocyte receptor CD40, which mediates B cell activation, proliferation and survival when engaged by antibodies, initiated a search for its respective native ligand. Due to the observation that interaction with T lymphocytes mimics the process observed in B lymphocytes after engagement of CD40 by an activating antibody, several groups independently identified the CD40 ligand (also referred to as gp39, TRAP, or TBAM), recently renamed CD154, as the native ligand [26–28]. CD154 was characterized as an integral membrane protein restricted to activate CD4+ helper T lymphocytes. Subsequent studies established that the interaction of CD154 with its receptor CD40 is essential for appropriate thymus-dependent humoral immune response [29–31]. The fundamental role for CD40/CD154 receptor-ligand dyad in these responses was further established by the demonstration that mutations in the gene encoding for the ligand result in the X-linked immunodeficiency hyper IgM syndrome, which is characterized by drastic or complete inhibition of the T lymphocyte-helps humoral immune response [32, 33]. Studies of CD40- and CD154-deficient mice further outlined the essential function for this receptor-ligand dyad in secondary immune responses. However, it has become evident in recent years that both CD154 and CD40 are expressed on other leukocytes and, even more interestingly, on non-hematopoetic cell types as well [34]. The discovery of a broad distribution pattern naturally implied the potential importance of CD40/CD154 interactions in processes other than originally consider-ed. Indeed, recent findings revealed elevation of CD40 and CD154 in chronic inflammatory diseases, such as rheumatoid arthritis [35], systemic lupus erythematosus [36], multiple sclerosis [37], and graft-versus-host disease [38, 40]. This suggested to us to study the implication of CD40-CD40 ligand interaction in inflammatory processes involving leukocytes and vascular cells and governing atherosclerotic processes. We first demonstrated that CD40 and CD154 were highly expressed (most predominantly in the shoulder region of the plaque, the border between the lesion and the unaffected portion of the artery) by atheroma-associated cells in tissue-section obtained from of human carotid atheroma [41].

Then, we showed that, by inducing the expression of matrix-degrading proteinases and of tissue factor procoagulant, CD40 signalling in macrophages may contribute to the triggering of acute coronary events [42]. Furthermore, also smooth muscle cells are activ-ated by this system in an inflammatory microenvironment of atherosclerotic plaques [43]. Also T lymphocytes are activated through CD40 triggering [44]. The most important evidence of an involvement of this interaction in atherosclerotic process was strongly support by reduction of atherosclerosis progression in mice treated with an antibody blocking CD154 signalling [45]. Therefore, we observed that CD40-CD40 ligand interaction is crucial for several events in atherosclerotic processes.

Chemokines

Chemokines were first identified as a family of chemoattractant cytokines, over 10 years ago [46]. Chemokines constitute a large family of small proteins (8-10 kDa) that are involved in both the basal leukocyte trafficking and the activation and recruitment of specific cell populations during disease. Chemokines have a broad spectrum of biological activities [47]. To date, these proteins have been shown to be implicated in a wide range of immune inflammatory responses:
By 2006, the use of either conventional cloning techniques or signal sequence trapping methods brought the number of human chemokine sequences to around 60, most of them identified in past few years. With this high number of distinct chemokines known, the extent of complexity in the chemokine superfamily is unrivaled within the field of cytokines. If the estimate of 100,000 for the total number of distinct human genes which are transcribed into mRNAs (and ultimately translated into proteins) is accurate, then it is likely that the entire number of cDNA fragments characterized to date represent probably only 25–35% of the total. On this basis, it is possible that the complete number of chemokines, when known, could exceed 100 [48].

The chemokines act through receptors belonging to a superfamily that span the membrane seven times [49–51]. The number of chemokine receptors has recently increased significantly. As there are so many

ligands with apparently similar chemoattractant properties, a highly active field of research has been to try to identify their respective receptors. The explosion in the number of ligands identified has meant that many orphan receptor sequences have been finally paired up with at least one cognate ligand.

Due to the growing evidence that inflammatory cells migrating from the circulation to the vascular wall are key players during atherogenesis, there are increasing reports demonstrating expression and implication of chemokines and their receptors in the atherosclerosis process [52]. Few years ago, the chemokine MCP-1 and IL-8 has been detected within human and animal atherosclerotic lesions, expressed by lesional macrophages-derived foam cells and by endothelial cells or SMCs at several different stage of the disease [53]. Both of these chemokines were not present in normal artery. Then, the chemokines MCP-4 and RANTES were also identify within atherosclerotic plaques [54]. In the same time, the chemokines receptors CCR2 and CXCR1, were also identified at the surface of monocyte-macrophages within lesions. But the strong evidence that chemokines and their receptors play important role in atherogenesis came in 1998 with two reports using animal model to block the CCR2-MCP-1 pathway. In the first study, the investigators generated a CCR2 knock-out (-/-) mice, which they then crossed to ApoE-/- mice, a well-known and documented mice model of atherosclerosis. Compared to ApoE-/-mice, the CCR2-/- / ApoE-/- present an overall decrease in atherosclerotic lesion size and fewer macrophages were noted in the aortas, without any effect on cholesterol plasma levels [55].

Despite these increasing evidence for involvement of T lymphocytes in atherogenesis, the mechanism of T lymphocyte recruitment within the vascular atherosclerotic lesions remains incompletely defined. In this prospective, we studied the expression of the three IFNγ-inducible CXC chemokines IP-10, Mig and I-TAC, known to exert potent chemotaxis on activated-Th1 lymphocyte in other inflammatory dise-ases such as psoriasis or sarcoidosis [56]. All three chemokines signal through a common receptor, CXCR3, expressed by activated-Th1 lymphocyte, but not by monocytes or neutrophils. Using immunohistochem-istry and Western

blot techniques we were able to demonstrate a differential expression of IP-10, Mig and I-TAC by atheroma associated cells [57]. Interestingly, we found a high expression of the receptor CXCR3 by all T lymphocytes within human atherosclerotic lesions. In vitro experiments showed that the pro-inflammatory cytokines TNFα and IL-1β, as well as CD40 ligand potentate IP-10 expression from IFNγ-stimulated endothelial cells. Moreover, nitric oxide (NO) treatment decreased IFNγ induction of IP-10. These findings suggest that the differential expression of IP-10, Mig and I-TAC by atheroma associated cells plays a role in the recruitment and retention of activated T lymphocytes observed within vascular wall lesion during atherogenesis. We also showed that blocking chemokine signalling in vivo through deletion of the chemokine receptors CCR2 and CXCR3 has differential effects during atherogenesis with particular consequences for the regulatory T lymphocytes during early atherogenesis [58]. On the basis of these evidences, we have also investigated the role of other chemokines on atherosclerotic plaque development, showing a crucial role for CCR5 (Fig. 3) [59]. Furthermore, we were able to demonstrate for the first time that chronic treatment with a chemokine receptor antagonist (Met-RANTES) limits the progression of atherosclerosis in vivo [60].

CRP

Recent clinical trials showed that C-reactive protein (CRP) is a powerful independent predictor of future cardiovascular events [61]. CRP is also the best physiological marker for measuring inflammation, the occult killer in cardiovascular disease. CRP, the major acute-phase reactant in humans, is mainly produced by hepatocytes in response to interleukin-6 (IL-6) and is then secreted into systemic circulation. Recent studies reported that, besides its predictive role in determining cardiovascular risk, CRP could exert a direct pro-atherogenic role. Indeed, it has been shown that CRP accelerates the progression of atherosclerosis in Apolipoprotein E-deficient mice [64]. Evidence for the pro-atherogenic role of CRP is further provided by in vitro studies reporting that CRP modulates the activity and expression of multiple factors implicated in atherogenesis. CRP downregulates endothelial

nitric oxide synthase (eNOS), resulting in decreased release of NO, and thus facilitation of endothelial cells apoptosis and inhibition of angiogenesis. In addition, CRP stimulates the production of the vasoconstrictor endothelin-1 (ET-1) and the inflammatory marker IL-6 by endothelial cells [63]. Furthermore, CRP increases the expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin and monocyte chemoattractant protein-1 (MCP-1), resulting in enhanced leukocyte transmigration [64].

The statin connection

In primary and secondary prevention, the lipid-lowering drug statins (HMG-CoA reductase inhibitor) have shown great efficacy in reducing cardiovascular events and deaths. Since the results of the Scandinavian Simvastatin Survival Study (4S) were first reported over 10 years ago, many other trials have corroborated the beneficial effects of this class of drugs [65]. Some results of these trials indicate that statins may have anti-inflammatory effects. In 2005, the Pravastatin or Atorvastatin Evaluation and Infection Therapy (PROVE-IT) trial clearly demonstrated that CRP is a marker of CV risk in primary and secondary prevention [66]. The PROVE-IT investigators also showed that some statins might have greater power to reduce CRP than others. In this trial, atorvastatin, 80 mg/day, reduced CRP significantly more than pravastatin, 40 mg/day, within the remarkably short interval of 30 days.

Pleiotropic effects of statins target CRP

There is no doubt that statins have lipid-lowering effects, but these agents also appear to have pleiotropic effects (beyond their lipid lowering properties). We recently demonstrated for the first time that statins regulate IL-6-induced CRP expression in human hepatocytes, its site of production (Figure 1) [67]. This reduction in CRP expression occurs both at the protein and the mRNA level, indicating that statins exert their effect at the transcriptional level. In our in vitro model, statins were used at doses corresponding to those measured in human plasma (Figure 1). We observed that, similar to the results in humans, atorvastatin seems to be the most potent inhibitor of CRP release. Although numerous clinical studies have reported that statins lower plasma levels

of CRP, it is the first study showing a direct effect of statins on IL-6-induced CRP expression in hepatocytes. In our study, we also show that the statin-mediated reduction of CRP release can be mimicked by GGTI (geranylgeranyl transferase inhibitors). Thus, it seems that the effect of statins on CRP release occurs via the inhibition of protein geranylgeranylation.

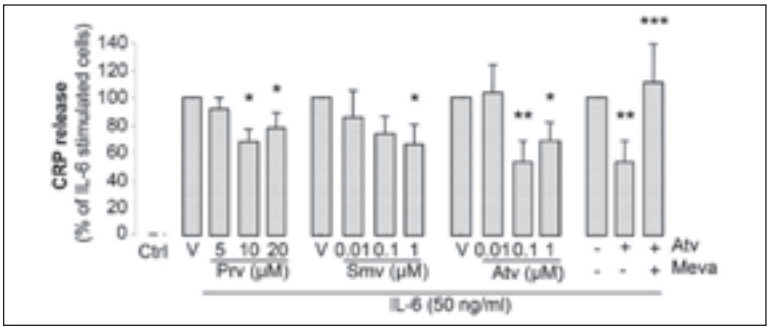

Figure 1
Statins reduce IL-6-induced CRP in hepatocytes.
*CRP release measured by ELISA in supernatants of hepatocytes cultured for 24 hours in normal media (Ctrl=control); activated by 50 ng/mL IL-6 alone (V=vehicle of statin); or in the presence of pravastatin (Prv 5, 10, and 20 μmol/L); simvastatin (Smv 0.01, 0.1, and 1 μmol/L); atorvastatin (Atv 0.01, 0.1, and 1 μmol/L), or 0.1 μmol/L atorvastatin; and 400 μmol/L L-mevalonate (Meva). *P<0.05 vs IL-6; **P<0.001 vs IL-6; ***P<0.05 vs IL-6+Atv. [67].*

In our investigations we also showed that statin pretreatment significantly reduced STAT3 phosphorylation on serine, but not on tyrosine residues. The signaling pathways leading to STAT3 phosphorylation are well known and differ among tyrosine or serine residues. In the first case, the tyrosine phosphorylation is mediated by the tyrosine kinase activity of the stimulated IL-6 receptor complex (IL-6Rα/gp130/JAK). In the other case, recent reports indicate that the serine phosphorylation is mediated by protein kinase C-delta, which in turn is activated by a signal transduction pathway consisting of Vav, Rac-1, MEKK and SEK-1. In our study, we have shown that statins reduce IL-6-induced CRP expression *via* the inhibition of protein geranylgeranylation. STAT3 is known to be regulated by Rac-1, a member of the Rho family, which needs to be geranylgeranylated to activate downstream

cascade. In conclusion, we hypothesize that, by suppressing the geranylgeranylation of Rac-1 in hepatocytes, statins reduce IL-6-induced phosphorylation of STAT3, thus resulting in reduced CRP expression (Figure 2). Such direct anti-inflammatory consequences may improve the understanding of the clinical effects of statins on cardio-vascular events and mortality.

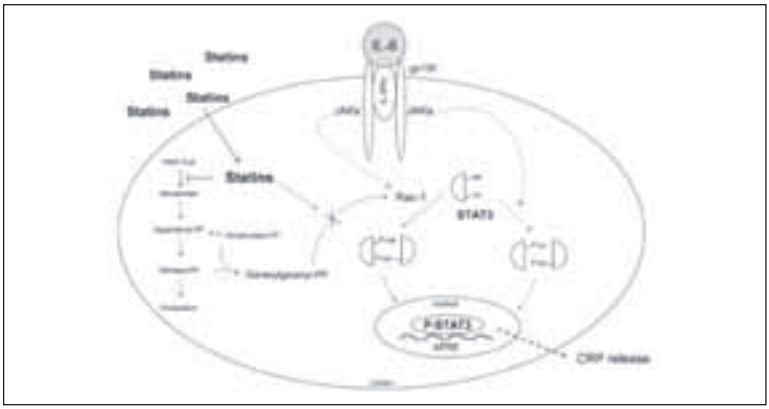

Figure 2
Proposed signaling pathways leading to statin-induced reduction of CRP release in hepatocytes (JAK indicates Janus kinase; APRE: acute phase response element; P-STAT3: phospho-STAT3) [67].

The endocannabinoid system

The discovery of membrane receptors that bind the psychoactive compound of marijuana, Δ9-tetrahydrocannabinol (THC) and their endogenous ligands has led to the description of the endocannabinoid system. At present, the system is composed of two receptors that have been cloned, and endogenous ligands or endocannabinoids including anandamide, 2-arachidonoylglycerol, and others. All endocannabinoids identified so far are derivatives of long-chain polyunsaturated fatty acids and exhibit varying selectivity for the two cannabinoid receptors [68].

Both cannabinoid receptors are G protein-coupled receptors that modulate second messengers and signaling components such as adenylate

cyclase, mitogen-activated protein kinases or members of the NF-κB family. The tissue distribution of the two receptors is likely to account for the well-known psychotropic and peripheral effects of THC. Cannabinoid receptor 1 (CB_1) is expressed predominantly in the central and peripheral nervous system, while cannabinoid receptor 2 (CB_2) is present on immune cells [69]. Thus, CB_2 receptors may have physiological importance in immune response, inflammation and chronic pain.

Immunosuppressive and anti-inflammatory properties

The development of selective agonists, antagonists, and transgenic mice lacking CB_1 and CB_2 receptors has contributed to broaden our current understanding of cannabinoid biology. As a consequence, the capacity of cannabinoids to regulate immune function is now well established. In vitro, THC treatment of human immune cells inhibits secretion of pro-inflammatory cytokines and chemokines and triggers the differentiation into a Th2 phenotype [70]. As demonstrated, a CB_2-specific antagonist abrogates the majority of these immunomodulatory effects [70]. Moreover, THC-mediated inhibition of T helper cell activation is absent in CB2-deficient mice, supporting the hypothesis that the immunomodulatory effects of cannabinoids are CB2-dependent [71].

Cannabidiol, the major non-psychotropic constituent of the Cannabis sativa plant, has been reported to ameliorate chronic inflammation in murine collagen-induced arthritis, a mouse model of rheumatoid arthritis, by inhibiting antigen-specific lymphocyte proliferation and interferon-(IFN)-γ secretion [72]. More recently, marijuana-derived cannabidiol was shown to inhibit macrophage chemotaxis in vitro and in vivo in a CB_2 receptor-dependent manner [73].

CB2 is expressed within plaques

To investigate whether CB2 receptor was expressed in atherosclerotic lesions, we visualized the receptors in human coronary and mouse aortic plaques using immunostaining techniques. We observed abundant CB2 receptor expression in both mouse and human plaques, while none was found in normal arteries [74]. Double immunofluorescence staining revealed that the CB2 receptor is mainly expressed on macrophages and CD4+ T cell lymphocytes within atherosclerotic plaques.

Cannabinoids and atherogenesis

To investigate whether THC treatment might reduce the burden of atherosclerotic disease, we placed apolipoprotein E (apoE)-knockout mice on a high-fat cholesterol diet. After 5 weeks on the diet, THC was administered to the treatment-randomized mice and these were compared to controls 6 weeks later. In the control mice, lesions increased on the high-cholesterol diet; however, lesion progression decreased significantly in the mice treated with a very low dose of oral THC (Figure 3).

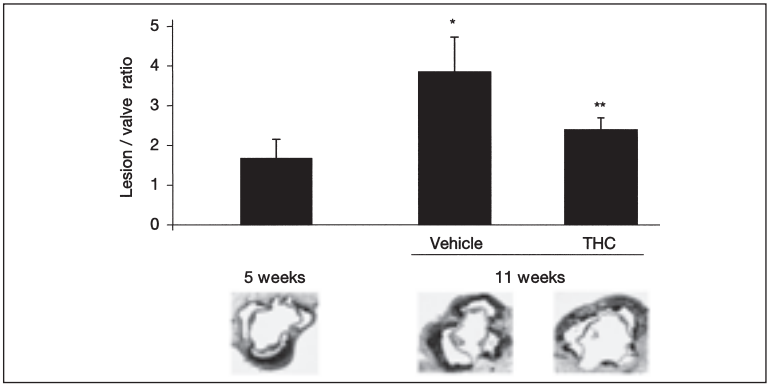

Figure 3

*Treatment with THC reduces atherosclerotic plaque development in ApoE-/-mice. Atherosclerotic lesions within aortic roots were analysed by Sudan IV staining for lipid deposition. Quantification of lipid deposition (immunostaining) was performed by computer image analysis. *P<0.05 compared with ApoE$^{-/-}$ mice at 5 weeks; **P<0.05 compared with ApoE$^{-/-}$ mice at 11 weeks without THC (vehicle) [75].*

When we added a specific CB2 receptor blocker, the protective effect of THC was completely abolished, indicating that the atheroprotection was due exclusively to the CB2 receptor pathway. T cell activation and proliferation as well as IFN-γ production declined in the lymph nodes of THC treated mice, but no effect was found on anti-inflammatory cytokine production. We also demonstrated reduced migration of peritoneal cavity macrophages from the same THC-treated apoE-knockout mice, in vivo, with stimulation by IFN-γ or tumor necrosis factor (TNF)-α. This effect was also clearly linked to the CB2 receptor, since migration was unaffected in CB2 knockout mice [75]. CCR2 (ß-sub-

class) receptors are among the most important chemokine receptors responsible for macrophage attraction from the circulation to vascular tissue and plaques. CCR2 expression was strongly reduced in splenocytes from apoE-knockout mice treated with THC, in vitro, at doses corresponding to effective in vivo dosages (Figure 4). This could explain the reduction, in vivo, of macrophage migration and atherogenesis. It is particularly noteworthy that the observed in vitro and in vivo effects of THC were dose-dependent. The dose dependency showed a U-shaped curve, where both higher and lower doses were inactive. The effective dose was lower than the dose usually associated with psychotropic effects of THC.

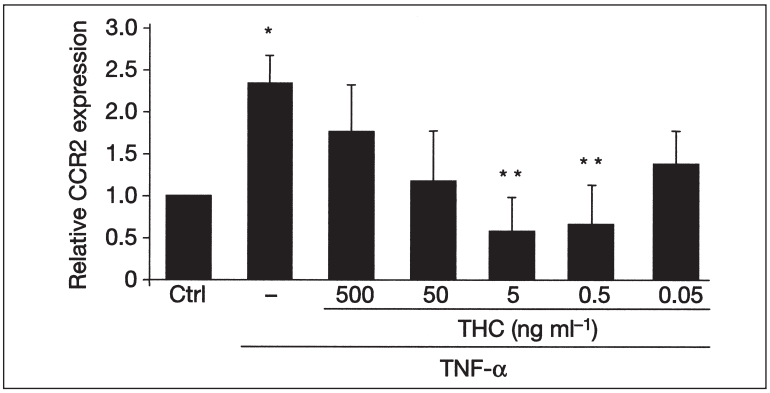

Figure 4
Treatment with THC reduces CCR2 mRN A expression in vitro.
*Isolated splenocytes obtained from ApoE$^{-/-}$ mice were stimulated with 10 ng/ml TNF-α in the presence or absence of different doses of THC. Relative expression levels of CCR2 messenger RNA were determined by quantitative real-time PCR. Data represent mean values + s.e.m. *P<0.05 compared with control, unstimulated cells; **P<0.05 χομπαρεδ ωιτη ΤΝΦ-α-stimulated cells [75].*

Cannabinoids as therapeutic agents

Our study has demonstrated that THC, by acting on CB_2 receptors, reduces inflammation and the infiltration of immune cells into atherosclerotic lesions. Nevertheless, these effects are in conflict with the known adverse effects associated with marijuana consumption. Indeed, the bioactive constituents of the marijuana plant and their synthetic and

endogenous analogs cause not only neurobehavioral, but also cardiovascular effects as demonstrated in humans and animal models [75, 76]. In humans, the most consistent cardiovascular effects of marijuana smoking are peripheral vasodilatation and tachycardia. These effects manifest themselves as an increase in cardiac output, increased peripheral blood flow, and variable changes in blood pressure. In anesthetized rats and dogs, THC produces an acute rise in blood pressure followed by long-lasting hypotension and bradycardia. Based on these hypotensive effects, targeting the endocannabinoid system is likely to offer novel therapeutic strategies in the treatment of hypertension. In a multicenter study performed with 3882 patients, marijuana smoking was identified as a rare trigger of acute myocardial infarction [77]. While some of the cardiovascular effects result from activation of CB1 receptors, recent findings indicate that several cannabinoid effects are not mediated by either CB_1 or CB_2 receptors [78].

Besides the risk of unwanted cardiovascular effects, a broad acceptance of cannabinoids as therapeutic agents is hampered by the fact that they exhibit psychotropic effects. Therefore, particular research interest is focusing on the development and characterization of either synthetic or plant derived cannabinoids with therapeutic value that are non-psychotropic. Among the non-psychotropic plant derived drugs, cannabidiol, a major compound of marijuana, has been shown to have potent anti-inflammatory and neuroprotective properties, as discussed above [70]. First clinical trials performed with THC/cannabidiol plant extracts have demonstrated that the study medications were generally well tolerated and associated with only few side effects. However, larger scale and long-term studies are warranted to confirm a clinically relevant improvement of neurological symptoms [79].

More interestingly, several non-psychotropic synthetic cannabinoids with anti-inflammatory properties have recently been developed from plant cannabinoids, such as ajulemic acid (CT3) and HU-320. Of particular interest is the development of agonists that selectively target CB_2 receptors. In preclinical studies, CB2 receptor agonists, such as HU308 and AM-1241, have been shown to be devoid of effects on the central nervous system and show promise for the treatment of acute and

chronic pain [80]. A promising novel compound designated Sch.336, which belongs to a new class of CB_2-specific ligands, the triaryl bissulfones, blocks leukocyte recruitment in vivo [81]. This effect was demonstrated in three different models of inflammation. Their anti-inflammatory properties suggest that CB2 ligands may serve as novel immunomodulatory agents in the treatment of immune disorders such as atherosclerosis. However, little is known about the molecular mode of action of these compounds and requires further investigation. Finally, it remains unclear whether receptor signaling via endocannabinoids plays a modulatory role in chronic inflammation ongoing during atherogenesis. A recent report demonstrates that CB1 receptors mediate intrinsic protective signals that counteract pro-inflammatory responses in a mouse model of colonic inflammation [82]. Additional studies using selective CB_1 and CB_2 receptor antagonists are warranted to investigate a possible role of the endocannabinoid system during atherosclerosis.

Conclusions

Inflammatory processes play a pivotal role in all stages of atherosclerosis. Many risk factors contribute as triggers of inflammatory reactions and injury to the endothelium. Growing evidence suggests that elevated plasma levels of vascular wall inflammation markers may help to predict future risk of plaque rupture. Prevention and current treatments for atherosclerosis are mainly based on drugs that lower plasma cholesterol concentration and high blood pressure. In particular, statins have proven to reduce the risk of cardiovascular events significantly, not only by their cholesterol-lowering properties, but also by their more recently identified anti-inflammatory and immunomodulatory effects. Nevertheless, atherosclerosis remains the primary cause for heart disease and stroke. Thus, a great challenge for future research would be the identification and development of promising novel anti-inflammatory therapies, such as anti-chemokines or non-psychotropic cannabinoids, to reduce the progression of atherosclerosis. A major challenge of contemporary medicine is to break the traditional compartmentalization that frequently separates different fields. The same holds between medical practice and basic biochemical mechanism. Unexpected link-

ages between different areas of medicine are indeed of particular interest. Unsuspected "bridges" across cardiology practice and molecular immunology, as presented here, are good examples of such a linkage.

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