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Over expression of ACE in myeloid cells increases immune effectiveness and leads to a new way of considering inflammation in acute and chronic diseases.

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Abstract

Purpose of Review—To review recent studies exploring how myeloid cell over expression of angiotensin converting enzyme (ACE) affects the immune response and to formulate an approach for considering the effectiveness of inflammation in cardiovascular disease.

Recent Findings—While it is widely appreciated that the renin-angiotensin system affects aspects of inflammation through the action of angiotensin II, new studies reveal a previously unknown role of ACE in myeloid cell biology. This was apparent from analysis of two mouse lines genetically modified to over express ACE in monocytes/macrophages or neutrophils. Cells over expressing ACE demonstrated an increased immune response. For example, mice with increased macrophage ACE expression have increased resistance to melanoma, methicillin resistant *S. aureus*, a mouse model of Alzheimer's disease, and ApoE knockout induced atherosclerosis. These data indicate the profound effect of increasing myeloid cell function. Further, they suggest that an appropriate way to evaluate inflammation in both acute and chronic diseases is to ask whether the inflammatory infiltrate is sufficient to eliminate the immune challenge.

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Conflict of Interest

The authors declare no conflicts of interest relevant to this manuscript.

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

Summary—The expression of ACE by myeloid cells induces a heightened immune response by these cells. The over expression of ACE is associated with immune function beyond that possible by wild type (WT) myeloid cells. A heightened immune response effectively resolves disease in a variety of acute and chronic models of disease including models of Alzheimer's disease and atherosclerosis.

Keywords

angiotensin converting enzyme; myeloid cells; macrophage; neutrophil; immunity; atherosclerosis

Introduction

Wolfgang Amadeus Mozart died on the 5th of December, 1791. In 2009, Zegers *et al.* considered the known clinical features of Mozart's final illness (fever, severe edema, possible rash) in the context of the prevalent lethal diseases in 1791 Vienna [1]. They concluded that likely diagnoses included streptococcal infection with post infectious glomerulonephritis and streptococcal induced scarlet fever. While we may never know the exact cause of Mozart's untimely death, morbidity and mortality following streptococcal infection was often due to the negative effects of the immune response provoked by the infection. Even today, the effect of inflammation is a constant in life irrespective of age. Lack of an immune response is catastrophic. Conversely, post-infectious valvular disease, forms of vasculitis, glomerulonephritis or any one of a number of other diseases resulting from an aberrant and/or exuberant immune response can be equally calamitous. In fact, inflammation plays an important pathological role in cardiovascular diseases such as atherosclerosis and hypertension, the leading causes of death in the modern world [2, 3].

Here, we wish to propose a new way to think about inflammatory processes. This resulted from studies of how expression of angiotensin converting enzyme (ACE) affects the function of macrophages and neutrophils. We begin with a short introduction to the immune response.

Innate Immunity.

Typically, the first system to respond to an immune challenge, it is an ancient system that is present in all multicellular organisms. As summarized by Turvey and Broide, cells of the innate immune response (macrophages, neutrophils, eosinophils, natural killer cells and natural killer T cells) use pattern recognition receptors to respond to challenge by microorganisms [4]. These receptors can be placed into three categories: those detecting common, conserved elements expressed by a wide variety of microbes (as example: toll-like receptors), intracellular receptors detecting tissue damage associated molecules (as example: NOD-receptors), and receptors detecting missing self-proteins, molecules normally made by healthy tissue but are lacking in cells infected with organisms (as example: killer-cell immunoglobulin-like receptors (KIRs)). The innate immune system also includes several soluble components including the enzyme lysozyme, antimicrobial peptides, acute phase proteins, and the complement system [5]. In vertebrates, cytokines (interleukins, interferons, tumor necrosis factors etc) play a very important role in innate immunity and antimicrobial defenses [6].

Adaptive immunity.

Only present in vertebrates, adaptive immunity chiefly concerns the function of T and B cells. The central feature of adaptive immunity is genomic rearrangement that produces an enormous number of highly diverse cell surface receptors [7]. In B cells, this generates antibody diversity, while in T cells it produces diverse T cell receptors. Two crucial features are thymic selection which removes reactivity to self-antigens, and the ability of T and B cells to clonally expand following ligand binding to their cell's receptors. Thus, while the innate immune response is rapidly initiated, the adaptive response generally lags while the rare cells possessing cell receptors that bind antigenic epitopes expand in number to the point of being clinically effective.

While an immune response is often characterized as innate or adaptive, this differentiation is somewhat artificial in the sense that there are many overlapping interactions between components of the two systems. It may be better to envision the immune response as a layered model or perhaps a three-dimensional matrix response. What this means is that there are many overlapping immune systems to defend against immune challenge. This is particularly true in outbred animals (eg. humans) where the genetic loss of one portion of the immune response can have a muted effect, as compared to the same genetic change in an inbred mouse (see the discussion of IRAK4 and MyD88 deficiency in reference 4).

A second important point is that macrophages, in particular, play a central role in the interface between innate and adaptive immunity; macrophages and dendritic cells play a crucial role in interfacing with T and B cells both through the presentation of antigenic peptides and through cytokine production [5, 8]. While neutrophils are properly envisioned as the prototypic innate cell, even these cells are now thought to influence adaptive immunity [9].

A final point concerns the observation that the response to an immunologic challenge necessitates 1) signals to start the immune response, 2) both cellular and soluble factors that eliminate the immune challenge, and 3) mechanisms to clean-up residual damage and terminate the immune response. While an obvious observation, we believe too little emphasis has been placed on understanding the exact differences between an acute immune challenge, such as that by infectious disease, and a chronic immunologic challenge, such as those present in atherosclerosis or Alzheimer's disease (AD). In chronic inflammation, something goes amok. Whether it is the impossibility of completely resolving the immune challenge or the inability to prevent recurrence of the immune challenge (for example, inflammation due to hypertension or to high serum lipids in the context of atherosclerosis), the immune system converts from a series of cell and humoral factors that efficiently resolve immune challenge into a chronically activated system that often contributes to the pathologic characteristics of a disease.

How best to approach the role of immunity in cardiovascular disease such as atherosclerosis? Our insight came from studying angiotensin converting enzyme (ACE) and its role in influencing the immune behavior of myeloid cells. As we describe below, these observations lead to the conclusion that most descriptions of inflammation such as the number of cells, the type of cells, the level of cytokines, inadequately measure the

effectiveness of the response in eliminating the underlying immune insult. This becomes clinically important in considering whether it is advantageous to increase or suppress the immune response.

RAS and Inflammation.

Preclinical and clinical studies have revealed that the renin-angiotensin system (RAS) plays an important role in the initiation and maintenance of the inflammatory processes associated with the development of atherosclerosis, hypertension, and other cardiovascular diseases [10]. Indeed, the therapeutic efficacy of RAS blockers, such as ACE inhibitors and angiotensin receptor blockers, (ARBs) in treating many cardiovascular diseases relies not only on their direct antihypertensive effect but also, in their ability to suppress inflammatory processes [11]. Data indicate that RAS signaling induces inflammatory responses in several disease states [12–14]. Until recently, most studies focused on angiotensin II stimulation of the AT1 receptor which leads to the production of proinflammatory cytokines, chemokines and adhesion molecules by resident cells, thereby promoting an inflammatory response [15–17]. However, ACE metabolizes a variety of peptides besides angiotensin I. Further, as discussed below, many immunomodulatory effects of ACE are mediated by peptides apart from angiotensin II.

Characteristics of ACE.

ACE is a well-known peptidase that is produced by virtually all cells in the body [18]. The best known actions of ACE are as part of the RAS in which angiotensin I is converted to the active agent, angiotensin II, by the removal of the C terminal two amino acids. In addition, ACE is well known for its actions in degrading the potent vasodilator bradykinin. However, ACE is a relatively nonspecific dicarboxypeptidase that has been shown to cleave hundreds of substrates varying in size from as small as three amino acids to as large as amyloid β 1–42 [19]. While a single polypeptide chain, ACE contains two catalytic domains, each of which binds zinc as a crucial requirement for catalytic activity [20–22]. The two domains are the result of an ancient gene duplication as ACE-like enzymes containing two homologous catalytic domains can be found in many species throughout the earth's diverse biome [18].

As was indicated, many tissues make ACE but the organs typically associated with high expression levels are the lung in which high endothelial expression results in the conversion of blood angiotensin I to angiotensin II with only a single pass through this organ, kidney in which high expression by proximal tubular epithelium cleaves filtered peptides into angiotensin II and other products that affect the reabsorption of sodium by tubular transporters, and the gut in which high ACE expression by epithelial microvilli probably plays a role in the digestion of peptides. Perhaps the organ that best demonstrates the diverse function of ACE is the testis. Here, interstitial cells make the somatic form of the enzyme found throughout the body while developing male germ cells expressing very high levels of an ACE isozyme called testis ACE, composed of only the single C-terminal domain of ACE [18]. While the role of ACE production by interstitial cells is not well understood, ACE expression by developing male germ cells is essential for the normal reproductive function of mature sperm. Here, it is not angiotensin II that is critical but another unidentified peptide substrate or product [23].

ACE and the Immune Response.

In 1975, a clinical observation first suggested a role for ACE in the inflammatory response. This was the finding by Lieberman that patients with active sarcoidosis have high serum levels of ACE [24]. Remission of sarcoidosis was associated with reduced serum levels of ACE which again rose upon reactivation of the disease. It is now known that this ACE is the product of the macrophages and giant cells (themselves derived from macrophages) that comprise the core of the sarcoid granuloma [25]. Further, we now know that the macrophages comprising the granuloma of virtual all granulomatous diseases make elevated levels of ACE, including histoplasmosis, silicosis, schistosomiasis, miliary (non-caseating) tuberculosis, and granulomatosis with polyangiitis (Wegener's granulomatosis) [25–28]. Even macrophages from zebrafish are reported to increase ACE expression in a granuloma [29]. Increased ACE expression by macrophages and other myeloid cells is not restricted to just granulomatous disease. For example, increased ACE expression is found by macrophages in both the early and late stages of human atherosclerotic lesions [30, 31]. ACE increases when the human monocytic cell line THP-1 is differentiated into macrophages [30]. When human peripheral blood monocytes are differentiated to macrophages *in vitro*, these cells increase ACE activity 9-fold [31]. Macrophages in Gaucher's disease make abundant ACE [32]. In mice, ACE expression in splenic neutrophils, macrophages and dendritic cells rapidly increases following experimental infection with either *S. aureus* or *L. monocytogenes* [33–34]. In part, these observations indicate that activation of macrophages is associated with increased ACE expression. However, the more fundamental question of why myeloid cells respond to immunologic challenge by up-regulating ACE is not well understood.

ACE Over Expressing Mice.

Our group made a series of mice in which gene manipulation was used to increase ACE expression in subsets of myeloid cells. One of the most important lines is termed ACE 10/10, in which ES cell targeting was used to insert the *c-fms* promoter immediately 5' of the coding regions of the natural mouse ACE gene [35]. The effect of the DNA insertion was that the tissue specificity of the natural ACE promoter was eliminated and replaced by the specificity of the *c-fms* promoter resulting in mice with high levels of ACE expression by monocytes and macrophages. Depending on how and when ACE is measured, the macrophages of these animals express from 16-fold to 25-fold normal levels of ACE. Other myelomonocytic cells, such as neutrophils and dendritic cells, also over-express ACE, but at only 4% and 17% of macrophage levels. ACE expression by T or B cells is very low, similar to WT. In contrast, ACE 10/10 mice lack ACE expression by endothelial cells, renal epithelial cells and other tissues which do not recognize the *c-fms* promoter. Despite the change in ACE expression, ACE 10/10 mice have normal body and organ weights, serum ACE levels, blood pressure, renal function, bone marrow and peripheral blood characteristics.

ACE 10/10 mice were studied following subcutaneous injection of mouse B16-F10 melanoma cells. This is an aggressive mouse tumor and the implantation of 10^6 cells results in a visible tumor at the end of two weeks. To our surprise, ACE 10/10 were much more resistant to the growth of melanoma (Figure 1) [35]. Tumors in WT mice averaged 540

mm³; ACE 10/10 mice averaged only 90 mm³. Histologic examination of the small tumors present in ACE 10/10 mice confirmed many more inflammatory cells (myeloid and lymphoid derived cells) within the tumor blood vessels and the tumor itself. The increased resistance to tumor growth is dependent on the action of CD8⁺ T cells, emphasizing the interaction of myeloid and lymphoid cells in this model of adaptive immunity. The increased resistance to tumor of the ACE 10/10 mice was eliminated by treating the mice for 1 week with an ACE inhibitor. In contrast, losartan, an angiotensin II receptor blocker had no effect. Indeed, creation of ACE 10/10 mice genetically lacking angiotensinogen retained an increased immune response [35]. These observations, and equivalent observations with models of infection described below, indicate that it is not angiotensin II or any angiotensin peptide that is responsible for the enhanced immune response.

We have also tested the resistance of ACE 10/10 mice to acute infection with either *L. monocytogenes* (listeria) or methicillin resistant *S. aureus* (MRSA) as a means of assessing innate immunity [36]. As with melanoma, the immune response of the ACE 10/10 to infectious challenge was substantially increased. For example, four days after challenge with MRSA, the skin lesions in ACE 10/10 mice were much smaller and contained roughly 50-fold fewer viable bacteria as compared to lesions in WT mice. Results with MRSA were particularly significant because mice are far more resistant to pathogenic MRSA than humans. Nonetheless, despite an effective innate immune in WT mice, the immune response of ACE 10/10 was markedly better, indicating an immune response significantly better than that possibly achievable by WT mice.

A second line of mice was created via transgenic insertion of a construct consisting of the *c-fms* promoter driving ACE cDNA. We examined several founders until one was identified in which ACE expression was increased 12 to 18-fold only in neutrophils; in these mice, macrophage ACE expression is very similar to WT mice [33]. The mice, called NeuACE, were also studied using models of infection. Whether tested with MRSA, *Klebsiella pneumoniae*, or *Pseudomonas aeruginosa*, the resistance of the NeuACE mice was much better than that observed in WT animals. Neutrophils kill bacteria by a variety of means, but central is the generation of superoxide by NADPH oxidase. The over expression of ACE in either neutrophils or macrophages increases NADPH oxidase production of superoxide by increasing phosphorylation of the critical regulatory components that regulate oxidase activity [33]. As discussed above, these effects of ACE over expression are due to a peptide other than angiotensin II.

We have also tested the ability of the ACE 10/10 mice to resist a model of chronic disease: brain amyloid β 1–42 (A β 42) over expression that in mice mimics several aspects of human Alzheimer's disease [37]. By eight months of age, the mice over expressing A β (APP_{SWF}/PS1_{E9}) present with cortical and hippocampal amyloid plaques, chronic brain inflammation and demonstrable reduction in the ability to learn and retain the solution to a maze. When mating was used to create A β 42 mice over expressing mice that were also ACE 10/10 (ie they overexpressed ACE in monocytes and macrophages), there was a marked reduction of pathology. This was characterized by far fewer amyloid brain plaques, significantly less chronic inflammation and, most importantly, retention of cognitive abilities that was nearly identical to that of control mice not over expressing A β . Histologic

evaluation of these mice confirmed the infiltration of the brain by bone marrow derived macrophages and the apparent ability of such macrophages to surround and phagocytize plaque material.

Atherosclerosis.

The model of AD in mice bears resemblances to the typical model of atherosclerotic disease in rodents. For example, AD-like pathology is induced by the over expression of A β 42, which is functionally equivalent to the high levels of blood lipids present in ApoE knockout or LDL receptor knockout mice. Further, in the AD model, brain microglia and infiltrating macrophages are unable to effectively alleviate A β deposition, leading to an ineffective and deleterious immune response in which both inflammatory cells and cytokines contribute to neural injury and death. This is reminiscent of rodent models of atherosclerosis where the inability of macrophages and other cells to effectively alleviate the burden of intravascular oxidized low density lipoprotein contributes to a toxic environment in which cell cytokines and cell death intensify vascular pathology.

To investigate the ability of ACE 10/10 mice to ameliorate a model of atherosclerosis, ApoE knockout (KO) mice were bred with ACE 10/10 mice such that the ApoE KO animals now over expressed monocyte/macrophage ACE [38]. Such mice maintained the C57BL/6 background normally used in this model. These mice were compared to ApoE KO mice with normal ACE expression using two models of atherosclerosis: the administration of a western diet or the administration of a western diet plus uninephrectomy, subcutaneous implantation of a slow release DOCA tablet, and 1% NaCl drinking water (referred to as DOCA salt). While a western diet did not affect the blood pressure of the mice, the DOCA salt model does induce hypertension. After treatment for 8 weeks, disease was assessed by measuring the percent of the thoracic and abdominal aorta showing atherosclerotic plaque. With both the western diet and the western diet plus the hypertension associated with the DOCA salt protocol, there was significantly less atherosclerosis in the mice over expressing macrophage ACE (Fig.2) [38]. Similar to the AD model, there was reduced vascular wall inflammation and remodeling, largely because of an increased ability of the ACE over expressing macrophages to resolve the inflammatory challenge of elevated lipids.

To further investigate if it was ACE over expressing myeloid cells that reduced pathology, we performed additional experiments in which the ApoE mice were lethally irradiated and then salvaged by transplantation of either bone marrow from WT mice or from ACE 10/10 mice [38]. Such mice were treated with an atherogenic diet for 8 weeks and then sacrificed. The result was similar to our first experiment in that the mice receiving bone marrow from ACE 10/10 mice (bone marrow over expressing ACE by monocytes and macrophages) proved protective against the development of atherosclerosis as compared to animals transplanted with WT bone marrow.

The role of macrophages in atherosclerosis is complex. The common paradigm of M1 macrophages driving atherosclerosis while anti-inflammatory M2 macrophages protect from disease is clearly an oversimplification. However, without question, chronic inflammation - defined as continuous immunologic provocation with an immune response that is *unable* to resolve the underlying pathology - is not healthy. This is the basis of the inflammatory

hypothesis of atherothrombosis and the recently successful clinical study of anti-IL1 β antibody therapy (Canakinumab Anti-Inflammatory Thrombosis Outcomes Study - CANTOS trial), which demonstrated that decreased vascular inflammation reduced the number of long-term cardiovascular events and death even in the absence of concomitant lipid lowering [39]. However, the inflammatory hypothesis is also probably a simplification. For example, interleukin-6 (IL-6), a well-known pro-inflammatory cytokine that stimulates M1 activation was found to have both pro-and anti-atherogenic features; surprisingly, IL-6 knockout mice on an ApoE-deficient background demonstrated greater plaque lesion areas, although levels of pro-inflammatory cytokines were reduced in this model [40, 41]. The loss of IL-4, a potent anti-inflammatory molecule, does not accelerate atherosclerosis but rather protects from it [42, 43]. Genetic deletion of other pro-inflammatory cytokines, such as IL-18 and IL-12 elicited mixed effects on atherosclerosis, depending on the stage of atherosclerosis [42, 44]. Indeed, atherosclerotic plaques have both pro-inflammatory and anti-inflammatory macrophages, and their pattern of expression may predict risk of lesion rupture [45]. A recent study demonstrated that anti-inflammatory macrophages may actually be responsible for foam-cell formation [46].

We posit that the CANTOS trial succeeds by ameliorating the chronic inflammation resulting from the failure of the natural inflammatory response to clear intravascular lipids. An alternative approach to ameliorating atherosclerosis may be by *boosting* the inflammatory response to reduce lipid deposition and prevent the smoldering inflammatory phenotype which drives the disease. Indeed, we believe it is the enhanced immune response that protects the ACE 10/10 mice against the chronic inflammation and pathology present in Alzheimer's disease and in atherosclerosis. While provocative, the ACE 10/10 data highlight the need for a re-appraisal of the basic understanding of macrophages in atherosclerosis in order to enhance potential treatment effectiveness. Indeed, we would argue that the way to consider inflammation in disease models is to focus on the effectiveness of the response to remove the inciting immune challenge.

Conclusions.

While ACE influences blood pressure regulation by producing angiotensin II and degrading bradykinin, it also has other physiologic effects, including an enhancement of myeloid cell function. This was demonstrated in two separate lines of mice over expressing ACE in macrophages and neutrophils. Increased myeloid ACE increases the immune response with the mice demonstrating resistance to models of tumor, infection, AD, and atherosclerosis. The inflammatory response in ACE 10/10 and NeuACE mice is highly effective in resolving the underlying cause of the inflammation, whether this be due to the detritus of infection, tumor, or the chronic irritation of precipitated and soluble A β 42. As for atherosclerosis, increased levels of oxidized lipids are thought to overwhelm the ability of myeloid cells to take-up lipid and traffic away from the vascular component. Since the inflammatory response in atherosclerosis contributes to the pathology, one theoretical remedy is an *increase* in the capacity and effectiveness of myeloid cells taking-up lipid and surviving within the vascular milieu sufficiently long to exit and traffic lipid back to the liver. We posit that exactly this plays an important role in the resistance of ACE 10/10 mice to atherosclerosis.

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Fig. 1. Tumor size 14 days after injection of B16-F10 melanoma into ACE 10/10, ACE 10/WT heterozygous, and WT mice. Data from individual mice and the SEM.

Fig. 2. ApoE mice that were either WT for ACE (WT/ApoE) or ACE 10/10 (ACE10/ApoE) were treated for 8 weeks with either an atherogenic diet or an atherogenic diet plus DOCA salt. The thoracic and abdominal aortas were analyzed for the lesional area [38]. Over expression of ACE in macrophages reduced the atherosclerotic lesional area.