The role of leukocytes in the formation and rupture of intracranial aneurysms

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ABSTRACT

Ruptured intracranial aneurysms (IAs) affect a small proportion of the population; however, the morbidity and mortality is disproportionally high. Although little is known about IA formation, progression, and rupture, mounting evidence suggests that inflammation may play an important role in IA pathogenesis. There is emerging evidence to suggest that leukocytes play a key role in generating and maintaining a pathologic inflammatory response that leads to aneurysm formation and rupture. We present the current literature pertaining to the role of leukocytes in aneurysm formation, progression, and rupture. The contributions of individual cell types are detailed, with special attention paid to the cytokine and molecular profiles. The role of magnetic resonance imaging as a means by which to evaluate aneurysm-associated inflammation is reviewed. Finally, we discuss leukocytes as potential targets of pharmacologic intervention.

Key words: Aneurysm, inflammation, inflammatory cells, leukocytes, lymphocytes, macrophages, mast cells, neutrophils

INTRODUCTION

Stroke is the fourth leading cause of death in the United States and is a prominent cause of long-term disability.[1] The prevalence of stroke among adults age 20 or older is estimated at 6.8 million, with 795,000 individuals experiencing a new or recurrent stroke annually.[1] Subarachnoid hemorrhage (SAH), secondary to ruptured intracranial aneurysms (IAs) comprises 1-7% of all strokes.[2] On an average 3.6-6% of the adult population harbor IAs; however, the rate of rupture is estimated to be between 0.05% and 0.5%.[3] The small number of IAs that do rupture have a poor prognosis with a mortality rate of roughly 50%.[3] Of those that survive the initial hemorrhage, approximately 30% remain severely disabled, resulting in a poor quality of life.[4]

The mechanisms of aneurysm genesis, maturation, and eventual rupture remain incompletely defined, yet new studies highlight multiple genetic and environmental factors that may contribute to the pathogenesis. Chronic hypertension, binge drinking, and cigarette smoking have all been linked to aneurysm development and rupture.[5-7] Inflammation represents a potential common endpoint through which these diverse environmental stimuli enact pathologic changes in the intracranial vasculature, thus leading to aneurysm formation.

Animal aneurysm models, as well as analysis of human aneurysms, suggest that inflammation is a key mediator in the formation, progression, and rupture.[5,8-19] Multiple studies have demonstrated the inflammatory response to be associated with persistent pathologic vascular remodeling in response to an insult to the vessel wall. Abnormal blood flow, chronically elevated blood pressure, and shear stress have all been linked to the induction of the inflammatory response as well as IA pathogenesis.[6,12,20-29] Central to the process of inflammation-driven vascular remodeling is endothelial and vascular smooth muscle cell (VSMC) dysfunction resulting in vessel weakening.[30] The inflammatory response associated with vascular remodeling is composed of multiple complex cellular and biochemical processes. VSMCs, endothelial cells, and inflammatory cells participate in intercellular signaling, resulting in the recruitment of immune cells, such as leukocytes, to the vessel walls.

We review the current literature pertaining to the role of leukocytes in aneurysm formation, progression,

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and rupture. The contributions of individual cell types are detailed, with special attention paid to the cytokine and molecular profiles. The role of magnetic resonance imaging (MRI) as a means by which to evaluate aneurysm-associated inflammation is reviewed. Finally, we discuss leukocytes as potential targets of pharmacologic intervention.

DETECTION AND INVESTIGATION OF LEUKOCYTES IN HUMAN INTRACRANIAL ANEURYSM PATHOGENESIS

Currently, the literature suggests that leukocyte infiltration of the intracranial vasculature may play various roles in the prolonged formation and acute rupture of IAs. Frösen *et al*.[12] reported that IA walls obtained less than 12 h after rupture demonstrated T-cell and macrophage infiltration, as well as VSMC proliferation, indicating a chronic process that preceded rupture. In addition, the observation of leukocytes spread throughout aneurysm walls supports their role in the global deterioration of the vessel and suggests that their prominence in ruptured aneurysms is not an acute response to the sudden event.[31]

Transcriptome analysis of control vessels and IAs demonstrates upregulation of gene expression of the pro-inflammatory cytokines associated with leukocyte infiltration within aneurysm walls.[15,32-36] Weinsheimer *et al*.[35] in an analysis of IAs obtained from autopsies within 24 h of death, showed upregulation of several pro-inflammatory genes, including, adherens junction, the mitogen-activated protein kinase pathway, and Notch signaling. Krischek *et al*.[36] investigated gene expression on 10 IAs (6 ruptured and 4 unruptured) and determined that the most significantly upregulated pathway was antigen processing. Shi *et al*.[15] interrogated 6 IAs using the illumina microarray platform and determined focal adhesion, extracellular matrix receptor interaction, cell communication, inflammatory response, and apoptosis to be the most significant functional pathways involved in IA pathogenesis. These findings indirectly implicate leukocyte infiltration as a major contributor to aneurysm genesis and progression.

Finally, immunohistochemical analysis of the animal model and human aneurysms has repeatedly demonstrated leukocytes within the aneurysmal walls. Chyatte *et al*.[20] reported the presence of macrophages and T-lymphocytes within the walls of unruptured aneurysms. Ruptured aneurysms have also been found to harbor T-lymphocytes and macrophages within their walls.[12,31] In a study of ruptured and unruptured IAs, Kataoka *et al*.[31] observed leukocyte infiltration, particularly macrophages, in 50% of the unruptured and in all of the ruptured IAs. Using electron microscopy, the authors were able to demonstrate an association between advanced deterioration in the wall of ruptured aneurysms and the infiltration of leukocytes and macrophages.[31] Frösen *et al*.[12] also observed more prominent leukocyte infiltration in ruptured IAs when compared to unruptured IAs. These findings suggest that the structural architecture of ruptured aneurysms differs from that of unruptured aneurysms. Furthermore, leukocyte invasion appears to be a mediator of this change and a potential driving impetus behind the progression to aneurysm rupture.

ROLE OF MACROPHAGES

Animal and clinical studies have identified macrophages as important contributors to the formation and rupture of aneurysms. These cells participate in the synthesis and secretion of matrix metalloproteinases (MMPs) and elastases, which play significant roles in the degradation of the extracellular matrix and internal elastic lamina. Histopathological analysis of both unruptured and ruptured aneurysms has repeatedly identified macrophage infiltration within the aneurysm walls.[12,20,37] In addition, Ruzevick *et al*.[38] observed the pro-inflammatory haptoglobin 2-2 genotype to be associated with larger aneurysms and increased macrophage infiltration within the aneurysm walls.

Macrophage-depleted mice have been shown to have a moderate protective advantage from aneurysm formation and rupture, suggesting macrophages play a critical role in the aneurysm pathogenesis.[13,39] Corroborating this hypothesis are two animal studies investigating the role of monocyte chemoattractant protein 1 (MCP-1), an important macrophage chemoattractant that has been studied in atherosclerosis and abdominal aortic aneurysms (AAA).[40,41] By using MCP-1 knockout (KO) mice, both Aoki *et al*.[9] and Kanematsu *et al*.[13] were able to demonstrate a decrease in aneurysm formation and macrophage accumulation. Aoki *et al*.[9] also reported that inhibiting MCP-1 activity using a dominant negative mutant of MCP-1 resulted in the inhibition of aneurysm progression in rats. MCP-1 deficient mice also demonstrated decreased macrophage accumulation and expression of MMP-2 and MMP-9.[9] A recent study conducted by Chalouhi *et al*.[42] surveyed the cytokines and chemokines in aneurysm lumen blood and found an increase in chemoattractant cytokines interleukin-7 (IL-7), IL-8, and MCP-1, suggesting active recruitment of inflammatory cells into the aneurysm.

Nuclear factor-κB (NF-κB) is a family of transcriptional factors involved in regulating the expression of a variety of inflammatory factors including MCP-1. Aoki *et al*.[43] investigated the role of NF-κB in the

initiation and progression of IAs using animal models. The authors were able to demonstrate that NF-κB participates in the initiation of IA formation through transactivation of many downstream genes related to macrophage recruitment and vascular inflammation, such as MCP-1, vascular cell adhesion molecule 1 (VCAM-1), MMP-2, MMP-9, IL-1β, and inducible nitric oxide synthase (iNOS).[43] In addition, NF-κB decoy oligodeoxynucleotides (ODNs), which inhibit NF-κB, abrogated the upreguation of inflammatory factors, including MCP-1. In an additional study conducted by the same group, Aoki *et al*.[44] linked MCP-1 expression in VSMCs with Ets-1, a transcription factor implicated in many vascular inflammatory diseases. Ets-1 binds to the promoter region of MCP-1 resulting in increased Ets-1 expression. Utilizing the knowledge obtained from previous studies on the role of NF-κB and Ets-1, Aoki *et al*.[45] showed that treating rats with chimeric decoy ODNs, designed to simultaneously inhibit NF-κB and Ets-1, reduced aneurysm size while thickening aneurysm walls of preexisting aneurysms. Furthermore, decreased expression of MCP-1 and reduced macrophage infiltration was observed in rats treated with the decoy ODNs.

Additional molecular signaling molecules associated with macrophage-induced aneurysm formation include tumor necrosis factor alpha (TNF-α) and stromal cell-derived factor-1 (SDF-1). Several studies have suggested that TNF-α is a key mediator in aneurysm development through the activation of several cytokines and MMPs.[17-19,46] TNF-α has been shown to upregulate MCP-1, which in return attracts macrophages, thereby leading to additional TNF-α expression in a positive feedback loop.[18] Using TNF-α KO mice, Starke *et al*.[18] demonstrated a reduction in IA formation and rupture. Additional studies using a synthesized TNF-α inhibitor 3, 6'dithiothalidomide (DTH) substantiated the results from the KO experiments. Starke *et al*.[18] also showed DTH to inhibit IA progression with fewer ruptured IA in the treatment group compared to the control group. Furthermore, using tumor necrosis factor receptor superfamily member 1a (TNFR1) deficient mice, Aoki *et al*.[46] demonstrated suppressed IA formation with decreased NF-κB activation, reduced MCP-1 and cyclooxygenase 2 (COX-2) expression, and fewer infiltrating macrophages. These results suggest that TNF-α/TNFR1 signaling is critical in IA pathogenesis.

Stromal cell-derived factor-1 is an important chemokine that promotes inflammation directly as well as through angiogenesis.[47] Macrophage recruitment and retention around new blood vessels has been shown to be mediated by SDF-1.[48] Expression of SDF-1 in IAs was recently evaluated in a study conducted by Hoh *et al*.,[47] wherein SDF-1 was present in the walls of both human and mouse aneurysms. Hoh *et al*.[47] also found SDF-1 promotes aneurysm wall angiogenesis through endothelial cell tube formation and macrophage infiltration. Inhibiting SDF-1, using anti-SDF-1 blocking antibodies, supressed murine aneurysm wall angiogenesis and resulted in the development of significantly fewer IAs compared to control mice.

Macrophages mediate flow-induced vascular remodeling, in part, through the release of MMPs, a process that under physiologic conditions, preserves vascular integrity and health.[39,49] However, increased levels of MMP expression, particularly MMP-2 and MMP-9, have been reported in IAs.[10,11,50,51] Studies using broad-based MMP inhibitors, such as doxycycline, have shown significant reductions in the incidence of IAs in animal models.[49,52] Tolylsam, a selective inhibitor for MMP-2, -9, and -12 also abolished the progression of IA, although it did not reduce the incidence of total aneurysmal changes.[10] Using more refined inhibition techniques, a greater understanding for the role of MMPs has materialized. Nuki *et al*.[52] showed that MMP-9 KO mice, but not MMP-2 KO mice, diminished the incidence of IAs. A separate study by Ota *et al*.[49] also demonstrates a reduced incidence of IAs in MMP-9 KO animals but not in MMP-12 KO animals.

Whereas MMP-9 is the main gelatinase, MMP-12 is the main elastase secreted from macrophages. Since MMP-12 appears to have no effect on aneurysm formation and rupture, other sources of elastases are likely. Neutrophil elastase is involved in atherosclerotic plaques and AAA and is produced by not only neutrophils but also macrophages and vascular endothelial cells.[53,54] Furthermore, neutrophil depletion studies inhibited AAA development through a non-MMP-2 and non-MMP-9-mediated mechanism, implying other mediators must exist, including the possibility of neutrophil elastase.[55]

Another protease that is of interest is the cathepsin family (B, D, K, and S), which have been shown to be expressed in IAs and promote their progression.[56,57] Specifically, histological analysis of ruptured aneurysms exhibited a cluster of macrophages expressing cathepsin D within the aneurysm wall where there was evidence of collagen erosion.[31] Multiple studies suggest that a polarized macrophage population is associated with a variety of diseases including atherosclerosis, inflammatory lung disease, and inflammatory diseases of the nervous system.[58-62] Two populations of macrophages, the M1 (pro-inflammatory) and M2 (anti-inflammatory) subtypes, have been identified. Predominance of the M1 subtype has been implicated in aneurysm progression and rupture.

The M1 population is pro-inflammatory and secretes high levels of IL-2, IL-23, IL-6, IL-1, and TNF-α.[63] The M2 population is antiinflammatory and secretes high levels of IL-10. Hasan *et al*.[63] examined 10 patients with IAs (5 unruptured and 5 ruptured) for the presence of M1 and M2 macrophage populations. The authors demonstrated a predominance of M1 over M2 macrophages within the walls of ruptured aneurysms and observed an increase in mast cells in ruptured aneurysms compared to unruptured aneurysms. The authors hypothesized that the imbalance between M1 and M2 may be in part due to the effects of mast cells. Given these results, the interplay between M1 and M2 phenotypes appears to be important in the aneurysm pathogenesis and warrants further investigation.

ROLE OF MAST CELLS

Mast cells are resident leukocytes that contain cytoplasmic granules rich in histamine and heparin, as well as, the pro-inflammatory cytokines, TNF-α, IL-1, IL-3, IL-4, IL-5, IL-8 and IL-13, and transforming growth factor-beta.[64,65] Mast cell degranulation and release of cytokines has been linked with vascular inflammatory processes, such as, atherosclerosis and AAAs.[66,67] Recent investigations have targeted mast cells as contributors to IA genesis and progression. In a study conducted by Ishibashi *et al*.,[65] an increase in the total number of mast cells during IA formation was observed using a rat model. Mast cell degranulation inhibitors suppressed IA progression through attenuation of the local chronic inflammatory response, as was evident from decreased NF-κB activation, macrophage infiltration, and expression of MCP-1, MMPs, and IL-1β.[65] In addition, Ollikainen *et al*.[68] demonstrated that mast cells in the wall of IAs were associated with histopathological changes consistent with wall remodeling, lipid accumulation, and inflammatory cell infiltration. Finally, Hasan *et al*.[63] observed increased mast cells in ruptured IAs relative to unruptured IAs. Taken together, these studies indicate that mast cell degranulation play a critical role in aneurysm formation and may contribute to IA rupture.

ROLE OF NEUTROPHILS

Neutrophils are recruited to sites of injury and are a hallmark of acute inflammation. Although the contribution of neutrophils in IA formation is largely undefined, evidence from investigations into AAA pathogenesis offers insight into their role. Animal models have demonstrated progressively increasing neutrophil infiltration into the walls of AAAs over the course of aneurysm development.[55,69]

Neutrophil recruitment to the vascular wall may be associated with macrophage infiltration. Mice treated with an antineutrophil-antibody showed a decreased number of macrophages compared to wild-type (WT) mice.[55] Furthermore, depletion of neutrophils attenuated the size and incidence of AAA. Diminished macrophage infiltration in aneurysms of neutrophil-depleted mice is not associated with a decrease in chemoattracts such as MCP-1 and MIP-1α.[55] This suggests that additional mediators are contributing to this complex interaction. Importantly, there was no difference in expression of MMP-2 and MMP-9, despite a decrease in macrophage infiltration.

The presence of neutrophils was recently reported by Marbacher *et al*.[70] using a decellularized rat aneurysm model. This rat model simulated the loss of mural cells (endothelial and VSMCs), a hallmark of ruptured cerebral aneurysms.[12,31] The ruptured aneurysms displayed marked adventitial fibrosis and inflammation, complete wall disruption, and increased neutrophil accumulation in unorganized intraluminal thrombus.[70] Neutrophils trapped in unorganized thrombus are a major source of matrix-degrading proteases. Intraluminal thrombus is a site of protease and cytotoxic compound release leading to wall inflammation and subsequent matrix degradation.[55,71]

Myeloperoxidase (MPO) is a peroxidase enzyme that catalyzes the formation of a number of reactive oxidant species and is primarily produced by neutrophils.[72] Along with a well-known role in host mechanisms against pathogens, MPO has recently been implicated in the initiation and destabilization of atherosclerotic plaques.[73] In a study conducted by Gounis *et al*.,[74] MPO was detected in all three ruptured IAs and 10 out of 20 unruptured IAs.[74] Additionally, Gounis *et al*.[74] demonstrated that MPO positivity was a significant predictor of 5-year aneurysm rupture rate. An emerging picture suggests a key factor in aneurysm formation and rupture is the ongoing inflammatory process mediated by infiltration of leukocytes. This suggests that MPO may play an important role in the aneurysm pathogenesis. Therefore, MPO may also serve as a potential biomarker.

Neutrophils represent a potential therapeutic target for pharmacologic interventions designed at preventing aneurysm progression and rupture. Hannawa *et al*.[69] demonstrated suppressed AAA formation in L-selectin KO mice. L-selectin, an adhesion molecule expressed on the surface of most leukocytes, is responsible for the recruitment of immune cells.[75-77] Hannawa *et al*.[69] postulated that the diminished AAA formation seen in WT mice compared with the L-selectin KO mice is most likely due to the impaired recruitment of neutrophils

and macrophages. Specifically, since neutrophils are present in the aortic wall before macrophages in WT mice, a decrease in neutrophils in the L-selectin KO mice is most likely due to the lack of L-selectin.[69]

ROLE OF LYMPHOCYTES

The contribution of T and B lymphocytes to IA formation is an additional avenue of exploration. B lymphocytes are rarely detected, and their role in IA pathogenesis is unclear.[20] However, T-lymphocytes have been documented within aneurysm walls[12,20] and CD8+ T-cells have been linked with AAA development.[78] T-lymphocytes have been shown to secrete pro-inflammatory cytokines including TNF-α, IFN-γ, and IL-6.[79] T-lymphocytes were detected within the walls of ruptured aneurysms and were associated with increased infiltration in samples taken < 12 h from rupture. These results indicate that this observation was not reactive.[12] Based on these observations, T-lymphocytes may play an important role in not only aneurysm formation, but also rupture. Additional studies focused on the role of lymphocytes in IA formation and rupture are necessary to further our understanding of the aneurysm pathogenesis.

DETERMINING INFLAMMATORY STATUS USING IMAGING

The apparent relationship between inflammation and aneurysm rupture is of clinical significance and may provide an avenue through which more accurate predictions of aneurysm rupture can be made. MRI is currently being explored as a noninvasive modality with the potential to evaluate the inflammatory state of aneurysms.

Hasan *et al*.[80] have reported on ferumoxytol-enhanced MRI images to evaluate aneurysm walls for macrophage infiltration. Ferumoxytol, which is used to treat iron deficiency anemia in patients with chronic renal failure, is a Food and Drug Administration approved drug consisting of an iron oxide nanoparticle.[81,82] The investigators imaged 19 unruptured aneurysms in 11 patients and determined that images acquired 72 h postinfusion of ferumoxytol were optimal for detecting macrophages within the aneurysm wall.

In a follow-up study, Hasan *et al*.[83] found that early uptake (within 24 h of infusion) of ferumoxytol in unruptured aneurysm walls suggested an active inflammatory process leading to aneurysm instability, ultimately resulting in rupture within 6 months. This hypothesis was validated with increased expression of COX-2 and mPGES-1 and an increased number of macrophages in aneurysms with early MRI signal changes. These results showed similar expression patterns to ruptured aneurysms. Unruptured aneurysms with late uptake (72 h postinfusion), did not rupture or increase in size after 6 months of follow-up. As a result, these studies show that ferumoxytol signal changes may indicate a greater risk of aneurysm rupture and suggest macrophage infiltration as a potential marker of aneurysms more likely to rupture.

Myeloperoxidase-specific paramagnetic magnetic resonance (MR) contrast agents, which are specific for MPO activity, have been evaluated in animal and tissue culture studies to examine their utility for imaging active inflammation.[74,84,85] Rabbit studies have shown promise for the use of an MPO-specific paramagnetic MR contrast agent, di-5-hydroxytryptamide of gadopentetate dimeglumine, in detecting local inflammation.[86] Since MPO has been detected in IAs, specially ruptured IAs, using MPO-specific contrast agents to monitor MPO within IAs will predict active inflammation and may aid in the management of unruptured aneurysms.

FUTURE DIRECTION AND THERAPEUTIC APPROACHES

Despite advances in microsurgical and endovascular therapy, outcomes following IA rupture remain poor. Thus, the identification of indicators of pending rupture and the development of pharmacologic interventions designed at limiting aneurysm progression and rupture are of great clinical interest. A better understanding of the relationship between inflammation and IA pathogenesis is a promising avenue of exploration, as there are multiple cellular and molecular targets for potential exploitation. Pharmacologic interventions targeting inflammation-driven IA formation and progression have shown promise in animal and human studies.[5] These drugs target inflammatory molecules such as TNF-α (DTH),[18] NF-κB (decoy ODN),[43] Ets-1 (decoy ODN),[45] SDF-1 (blocking anti-SDF-1 antibodies),[47] MMPs (tolylsam and doxycline),[10,49,52] MCP1 (7ND),[9] and cathepsins (NC-2300)[56] [Table 1]. In addition, mast cell degranulation inhibitors (tranilast and emedastine difumarate)[65] have also been tested. All these therapeutic agents have shown to decrease aneurysm size in experimental animal models. Ferumoxytol-enhanced and MPO-specific paramagnetic MRI appear to offer a possible means by which to evaluate the inflammatory profile of individual aneurysms. Additional investigations into the role of inflammation and IA formation, progression, and rupture are required to better elucidate potential clinically relevant pathways for intervention.

Table 1: Major leukocyte inflammatory mediators and targeted therapies for intracranial aneurysms

Major molecules	Targeted therapies for intracranial aneurysms
Common inflammatory mediators	
TNF-α	DTH[18]
NF-κB	Decoy ODN[45]
Ets-1	Decoy ODN[45]
VCAM-1	
iNOS	
L-selectin	
MCP-1	7ND[9]
SDF-1	Blocking anti-SDF-1 antibody[47]
Macrophages	
IL-1β, IL-2, IL-6, IL-23	
MIP-1α	
Cathepsins	NC-23000[56]
MMP-2, MMP-9	Tolylsam (selective) and doxycline (broad)[10,49,52]
Neutrophils	
IL-1β	
Neutrophil elastase	
Lymphocytes	
IFN-γ	
IL-6	
Mast cells	
IL-1, IL-3, IL-4, IL-5, IL-8, IL-13	Degranulation inhibitors (tranilast and emedastine difumarate)[65]
TGF-β	

TNF-α: tumor necrosis factor-alpha; DTH: 3,6'dithiothalidomide; NF-κB: nuclear factor-κB; ODN: oligodeoxynucleotide; VCAM-1: vascular cell adhesion molecule; iNOS: inducible nitric oxide synthase; MCP-1: monocyte chemoattractant protein-1; 7ND: N-terminal deletion variant of MCP-1; SDF-1: stromal cell-derived factor 1; MMP: matrix metalloproteinase; MIP-1α: macrophage inflammatory proteins-1-alpha; IFN-γ: interferon gamma; TGF-β: transforming growth factor beta; IL: interleukin

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