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Inflammatory targets of therapy in sickle cell disease

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Abstract

Sickle cell disease (SCD) is a monogenic globin disorder characterized by the production of a structurally abnormal hemoglobin (Hb) variant Hb S, which causes severe hemolytic anemia, episodic painful vaso-occlusion and ultimately end-organ damage. The primary disease pathophysiology is intracellular Hb S polymerization and consequent sickling of erythrocytes. It has become evident over several decades that a more complex disease process contributes to the myriad of clinical complications seen in SCD patients with inflammation playing a central role. Drugs targeting specific inflammatory pathways therefore offer an attractive therapeutic strategy to ameliorate many of the clinical events in SCD. In addition they are useful tools to dissecting the molecular and cellular mechanisms that promote individual clinical events, and for developing improved therapeutics to address more challenging clinical dilemmas such as refractoriness to opioids or hyperalgesia. Here, we discuss the prospect of targeting multiple inflammatory pathways implicated in the pathogenesis of SCD with a focus on new therapeutics, striving to link the actions of the anti-inflammatory agents to a defined pathobiology, and specific clinical manifestations of SCD. We also review the anti-inflammatory attributes and the cognate inflammatory targets of hydroxyurea, the only FDA approved drug for SCD.

Keywords

Inflammation; pathway; hyperalgesia; therapeutic; pathobiology

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CONFLICT OF INTEREST STATEMENT

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1. Introduction

Inflammation is a cardinal component of the pathophysiology of sickle cell disease (SCD). The spectrum of SCD inflammation is broad, impacting many pathways in virtually all organ systems. Recent articles have reviewed inflammation related to coagulation and hypoxia in SCD; these topics will not be discussed here. We discuss emerging therapies targeting inflammation caused by extracellular heme, the inflammasome, NF-kappa B (NF-kB), 5′-lipoxygenase, mast cells and the invariant natural killer (iNKT) lymphocyte inflammatory pathways. And, we discuss the inflammatory pathways targeted by hydroxyurea, the only FDA approved therapy for SCD.

By its simplest definition SCD is an inherited single gene disorder that produces abnormally shaped *sickle* erythrocytes that have an increased propensity to lyse and adhere to other cells within the intravascular space. These two cellular anomalies (i.e. increased lysis and adhesion) underlie the pathophysiology of the disease, commonly characterized by painful vaso-occlusive episodes (VOE) (1). It is thought that VOE result in ischemia/reperfusion injury (IRI), tissue hypoxia and organ damage (1). Studies in transgenic SCD mice indicate adhesion molecules orchestrate the interaction of blood cells with the endothelium to promote vaso-occlusion (2–6), aided by an inflamed sickle vasculature (7).

Systemic inflammation in patients is evident by elevated steady-state concentrations of several inflammatory markers, notably interleukins (IL); IL-1, IL-6 and IL-8 in the plasma, (8) as well as raised plasma Tumor Necrosis Factor-α (TNF-α), which are elevated further during acute illness (9, 10). Increased leukocyte count is associated with the development of many severe complications of SCD including acute chest syndrome (ACS) and stroke, and with a higher mortality rate (11). Acute inflammation in major organs is common; the most widely described is a condition in the lung known as ACS, which shares many similarities with acute respiratory distress syndrome (ARDS). ACS is the second most common reason for hospital admission (12), and the leading cause of referral to intensive care units (13). At the time of diagnosis, patients typically have hypoxemia, hemolysis and multi-lobar pneumonia/lung infiltration (14). The lung injury is characterized predominantly by edema, which affirms the role of inflammation in this event (15). Chronic inflammation is typified by an unremitting low-grade inflammation that is associated with many difficult clinical events in SCD that have no satisfactory therapeutic solutions. For instance, chronic pain in some patients is not amenable to traditional non-steroidal anti-inflammatory drugs (NSAIDS) even thought this same intervention maybe effective in the acute setting. Impaired cognitive function is linked to chronic inflammation in SCD children who otherwise have normal brain magnetic resonance imaging (16).

Extracellular heme is a potent inflammatory agonist and oxidant (17), and a classic damage-associated molecular pattern (DAMP) molecule. In healthy subjects, destruction of senescent red blood cells occurs predominantly in the spleen, circulating blood contains virtually no detectable heme as it is bound instantly with high affinity by plasma proteins, notably hemopexin (Hx) (18, 19). The heme-Hx complex is transported to the liver and removed via CD91-mediated endocytosis. In SCD patients, however, hemolysis is unrelenting; it is estimated that approximately 30g of Hb is released per day from

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hemolyzed erythrocytes in patients with SCD (20) with 30% of the total hemolysis being intravascular (21). Haptoglobin (Hp) and Hx (which scavenge cell free Hb and heme respectively), are depleted (19), thus, the plasma of patients with SCD contains excess cell-free Hb and heme (20, 22). Together with other DAMPs, heme can drive sterile inflammation sufficiently severe to cause tissue damage, and promote acute organ failure in SCD (23). The danger posed by heme and other inflammatory agonists can potentially be neutralized by a variety of therapies, which are discussed in this review.

2. Intravascular hemolysis and extracellular heme

Introduction

In the physiologic state, the iron within the heme pocket of intracellular hemoglobin (Hb) exists largely in the ferrous state; oxidation to ferric iron yields methemoglobin (MetHb) and superoxide ion (O2-) (24). Reduction by MetHb reductase reverses Hb oxidation while catalysis of O2- by superoxide dismutase produces hydrogen peroxide (H_2O_2), and ultimately to water through the action of catalase. Thus, the ferrous state of heme ensures that intracellular Hb binds to oxygen, as well as securing heme in the prosthetic Hb pocket (25). Hb S undergoes auto-oxidation at an accelerated rate compared to Hb A (26). The hydrogen peroxide intermediate generated in the O2- dismutation can oxidize Hb to ferryl Hb and globin free radicals; binding by haptoglobin inhibits this pseudoperoxidase activity. Deoxygenated Hb S polymers cause mechanical stress in the erythrocyte membrane. Enhanced physical interaction of deoxy Hb S with Band 3 promotes the release of spectrin and actin from the erythrocyte membrane resulting in various membrane defects in the erythrocyte (27, 28). These membrane-destabilizing events coupled with the high oxidant stress typical of sickle erythrocytes, increase their susceptibility to lyse in the intravascular space.

Pathobiology

Intravascular hemolysis releases Hb from a living space of ~80×10^{-15}L within the erythrocyte into a comparatively oceanic plasma milieu. The cell-free Hb tetramer can scavenge nitric oxide (NO) and perturb the anti-inflammatory effect conferred by NO on the endothelium (29, 30). Low steady-state plasma Hp in SCD patients reflects the chronicity and severity of this process, as large amounts of Hb released into plasma overwhelm the Hp scavenging capacity. The aforementioned colossal volume transition associated with intravascular hemolysis inevitably promotes dissociation of the Hb tetramer into its $\alpha1\beta1$ dimers, with consequent oxidation of heme and its displacement from globin, a process that is enhanced by Hp deficiency (25, 31, 32). However, plasma Hx is not depleted in all intravascular hemolytic conditions(19), which suggest a role for Hb S in the excess plasma heme in SCD.

Despite extensive knowledge about the deleterious effects of heme, the precise role played by this molecule in the pathobiology of SCD was largely unknown. Indeed, intra-peritoneal (i.p.) injection of hemin was reported to improve vaso-occlusion in transgenic mice with SCD (33). In 2010, our study employing an alternative experimental approach that challenged SCD mice with intravenous (i.v.) hemin overcame this impasse (34).

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Importantly, this approach allowed us to ask a simple question that had not been investigated previously in this field, namely what is the sequelae due to excess extracellular heme in SCD? We discovered that infusion of a relatively modest amount of purified hemin that had no adverse effect in transgenic sickle-trait mice caused a complete exhaustion of plasma Hx, and a lethal acute lung injury (ALI) reminiscent of ACS, in littermates with SCD(34, 35)

Two independent large-scale genetic association studies support the experimental finding linking heme to ACS. In a study of 945 children of the silent infarct transfusion (SIT) cohort, a polymorphism that enhances expression of heme oxygenase-1 (HO-1), the rate-limiting heme degradation enzyme, was associated with lower rates of ACS (36). A second study of >1,500 patients from the Cooperative Study of Sickle Cell Disease, replicated the association between ACS risk and the HO-1 gene, on this occasion, involving a polymorphism in the 3′UTR (37). The same study found a stronger association ($P= 4.1 \times 10^{-7}$) with a single nucleotide polymorphism (rs6141803) ~8 kilobases upstream of COMMD7, which is involved in regulating the activity of nuclear factor-kappa B. Interestingly, hemin alters the expression of COMMD in endothelial cells enhancing the notion that inflammation involving heme is involved in the pathogenesis of ACS. In our murine ACS studies, we discovered that hemin-induced lung injury required TLR4 expression in non-hematopoietic tissues, thus establishing for the first time the heme-TLR4 inflammation paradigm in SCD (35). Subsequently, hemin was shown to cause stasis in various transgenic SCD also in a TLR4-dependent manner (38). Neutrophil extracellular traps (NETS) are chromatin entities released by activated neutrophils covered with histones and granulocyte enzymes elastase and myeloperoxidase that form long fibers that are microbicidal (39). SCD plasma induces NET formation (NETosis) dependent on heme-iron, reactive oxygen species (ROS) and prior priming of neutrophils by cytokines (40); this process is linked to lung injury and sudden death in TNF-alpha treated SCD mice. In summary, a wave of experimental findings, some with corroborating genetic association datasets, have provided a causal link between extracellular heme and multiple inflammatory events in SCD.

Therapeutics

Extracellular heme therapeutics fall into two major types: a) direct heme antagonist, and b) heme signaling antagonists. The major heme scavenger Hx is a glycoprotein produced by the liver, and to a lesser extent by kidney mesangial cells, neurons, glial cells and skeletal muscle. Its primary function is to sequester heme for delivery to the liver for degradation. Mice deficient in Hx challenged with i.v. hemin show decreased NO synthesis (from uncoupling of Nitric oxide synthase), increased oxidative stress and endothelial activation, which are all corrected by Hx infusion (41). Hx inhibits cytokine release from hemin-stimulated macrophages (42, 43) and it induces hepatocyte HO-1 expression (44). In preclinical studies Hx prophylaxis prevented ACS(35) and attenuated stasis (38) in i.v. hemin challenged SCD mice. Importantly, Ghosh *et al* showed that Hx but not TAK-242 (a TLR4 inhibitor) stopped SCD mice with early signs of ACS from developing respiratory failure(35). However, there are potential deleterious effects of Hx therapy. For instance, the protease activity of Hx may be responsible for proteinuria in nephrotic syndrome (45). And in SCD, accelerating heme delivery to the liver in the acute setting may cause acute hepatic

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injury. There are currently no preparations of Hx available for clinical trials (46), however pre-clinical studies to better define the risks of both on- and off-target effects of this biologic are warranted.

The TLR4 signaling inhibitor TAK-242 (Resatorvid) prevented ACS(35) and blocked NFκB activation, endothelial adhesion molecule expression, leukocyte rolling and lethality in SCD mice infused with hemin(38). There are no clinical trials of TLR4 inhibitors in SCD, however, in individuals with severe sepsis, a randomized placebo controlled double blind study of TAK 242 showed no reduction in IL-6 levels or 28-day mortality rate (47). Other potential drugs to neutralize heme include, Calphostin C, NADPH oxidase inhibitors, as well antioxidants (e.g. n-acetyl cysteine), which attenuate venular stasis in i.v. hemin challenged SCD mice (38). Several antioxidant therapies elicit anti-inflammatory responses that maybe effective in attenuating downstream heme effects. For instance, Niacin reduces ROS production and increases HO-1 expression in cultured human coronary endothelial cells via induction of the Nuclear Factor erythroid-derived 2 related factor 2 (Nrf2) in rabbits fed a Niacin-supplemented diet (48). A randomized double- blinded placebo-controlled trial of extended release Niacin in SCD patients showed a modest HDL cholesterol increase but no improvement in endothelial function (49). There are currently several studies evaluating the efficacy of various anti-oxidants to reduce inflammation in SCD. A trial of Alpha Lipoic acid and Acetyl L Carnitine (NCT01054768), and two trials of N-acetylcysteine, a precursor of glutathione are currently enrolling participants (NCT 01800526 and NCT01849016), while a study of daily broccoli sprouts homogenate ingestion (as a source Sulphoraphane to activate Nrf2) completed recently (NCT01715480).

3. The Inflammasome

Introduction

DAMP molecules trigger the formation of inflammasomes (50), which promote inflammation through the induction of interleukins and other pro-inflammatory cytokines (51, 52). The levels of uric acid, a classical DAMP molecule that stimulates inflammasomes correlates with endothelial dysfunction and markers of hemolysis in SCD patients (53). In a study of inflammation and brain injury following induced subarachnoid hemorrhage in rats, Greenhalgh et al. were the first to identify heme as a danger-associated molecular pattern driving inflammation through interleukin-1 (54). Inflammasomes consist of a sensor molecule, an adaptor protein and caspase-1(55). Proteins required to assemble this complex are typically found in immune cells; construction begins typically with a NOD like receptor (NLR); NLRP1 (NOD-, LRR- and pyrin domain-containing 1), NLRP3, NLRP6, NLRP7, NLRP12 or NLRC4 (NOD-, LRR- and CARD-containing 4; also known as IPAF) (50, 56). Others include pyrin and HIN domain-containing protein (PYHIN), family members absent in melanoma 2 (AIM2) and IFNγ-inducible protein 16 (IFI16) (57). The hallmark of the NLR family is the presence of a central nucleotide binding and oligomerization (NACHT) domain, which is usually flanked by N-terminal caspase activation and recruitment (CARD) or pyrin (PYD) domains, and C-terminal leucine-rich repeats (LRRs) (51). The NACHT domain has ATPase activity, which enables the activation of the signaling complex by ATP-dependent oligomerization. The LRRs regulate the sensing of ligands, whereas CARD and

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PYD domains mediate homotypic protein binding for downstream activation and signaling (50, 56). An adaptor protein, apoptosis-associated speck-like protein containing a CARD (ASC), which contains a PYD and CARD domain is often necessary for the recruitment of caspase-1 after ligand stimulation (57).

Pathobiology

Heme activates the NLRP3 inflammasome in LPS-primed macrophages with subsequent release of IL-Iβ via the canonical pathway requiring ASC and caspase-1 (58). In that study, Dutra *et al*., demonstrated a role for NLRP3, ASC Caspase-1 and IL-R in hemolysis-related lethality in an injury model induced by intraperitoneal injections of phenylhydrazine (PHZ). Although the cause of death was not defined and the direct role of heme was not tested with rescue experiments, the data suggest the animals succumbed to multiple organ failure that appeared dependent on the inflammasome machinery after the 3rd day of the PHZ challenge. Multiple organ failure is common in SCD particularly towards the terminal stages of ACS. Thus, the inflammasome may be a potential target to develop salvage therapy for ACS, and other instances of severe intravascular hemolytic crisis in SCD.

Therapeutics

There are several inhibitors of the inflammasome that can be developed for SCD. Figure 1 shows the possible sites within the inflammasome that can be targeted for therapeutic intervention. A small molecule NLRP3 inhibitor MCC950 specifically inhibits inflammasome activation in a dose dependent manner. This drug was tested and proved to be efficacious in preclinical model of multiple sclerosis (59, 60). Other classes of compounds have been developed which can target one or more molecules in the inflammasome activation and signaling pathway with varying degrees of potency and success. Caspase-1 cleavage inhibitors and IL-1β antagonizing agents have shown efficacy in both preclinical and clinical settings (61). For example, rats given an inhaled peptide caspase-1 inhibitor Ac-YVAD-CHO were protected from LPS-induced endotoxaemia and had reduced pulmonary and systemic release of pro-inflammatory mediators (61, 62). A monoclonal antibody that selectively targets IL-Iβ, Canakinumab is effective in treating chronic inflammation related to cryopyrin-associated periodic syndromes (CAPS) (63), and maybe efficacious in preventing hemolysis-related end-organ damage in SCD. Compounds antagonizing P2X7 have shown promise in Phase II/III clinical trials in rheumatoid arthritis and Crohn’s disease (61, 64), however inflammasome activation in SCD maybe unrelated to this receptor since P2X7 null mice displayed similar susceptibility to hemolysis induced lethality as wild-type mice(58).

4. The Nuclear Factor Kappa B Pathway

Introduction

The NFκB family of inducible transcription factors regulates an array of biological functions, and they have been implicated in the vascular inflammation of SCD (7). In mammals, NFκB is composed of five structurally homologous transcription factors: p105/p50 (NF-κB1), p100/p52 (NF-κB2), p65 (RelA), RelB, and c-Rel (Rel) (65, 66). Additional processing of p105 and p100 is necessary to yield the functional proteins p50 and

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p52 respectively. NFκB proteins share ~300 amino acid N-terminal Rel homology domain (RHD) that bind DNA (67). RelB, c-Rel and p65 contain C-terminal transcription activation domains (TAD) that facilitate the recruitment of co-activators; alternatively NFκB1 and NFκB2 up-regulate transcription of target genes through the formation of heterodimers with p65, c-Rel, RelB, or other TAD containing proteins. They are typically sequestered in the cytoplasm by inhibitor of kb (IkB) proteins (i.e. IκBa, IκBb, IκBe, IκBg, BCL-3, and IκBDNS (IκBd) until activated by specific biological and biochemical signals (67) through canonical and non-canonical pathways (68). DAMPs activate the canonical pathway resulting in the expression of inflammatory and immune response genes (69). Activation of NFκB and its subsequent binding to the DNA response element is depicted in Figure 2.

Pathobiology

The main function of NFκB in the canonical pathway is to serve as a transcriptional regulator that can receive pathogen or damage recognition signals and propagate pro-inflammatory cytokine and chemokine production to the site of infection or injury (69). Un-abated activation of NFκB typically leads to chronic inflammation as seen in SCD and is stimulated by IRI as well as heme-induced TLR4 activation (38, 70). This is evident by the presence of chronically elevated levels of C-reactive protein, cytokines, leukocytes, abnormal activation of granulocytes and monocytes, and the up-regulation of VCAM-1, ICAM-1 and selectins on the surface of endothelial cells (4). Activation of NFκB is increased in the lungs of transgenic SCD mice and treatment of these animals with sulfasalazine, an inhibitor of NFκB activation and endothelial activation, reduced endothelial oxidant generation and NFκB activation, with a marked decrease in leukocyte adhesion and improved microvascular blood flow (4). More recently it was shown that free heme can activate NFκB through TLR4, subsequently causing the induction of adhesion molecules, and additionally, heme activation of NFκB through TLR4 in monocytes and macrophages enhanced the secretion of TNFα, causing vaso-occlusion, stasis and exacerbating inflammation

Therapeutics

Several classes of established NFκB inhibitors including glucocorticoids, immunosuppressants, and some non-steroidal anti-inflammatory drugs have shown promise in the treatment of many chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease (71). Sulfasalazine blocks IκBα degradation thus attenuating NFκB activation and nuclear translocation (72, 73). This agent inhibited NFκB activation, and caused a significant decrease in circulating endothelial cell activation, and in expression of VCAM-1, ICAM-1 and E-selectin, as well as significantly improving blood flow in transgenic SCD mice (4, 74). Glucocorticoids potently inhibit NFκB by inducing IκBα, and also by the interaction of the glucocorticoid receptor with the p65 subunit of NFκB (75–77). In transgenic sickle mice, pretreatment with the synthetic glucocorticoid dexamethasone, significantly inhibited hypoxia-induced activation of NFκB, vaso-occlusion and adhesion molecule expression in the endothelium of the lungs, liver, and skin (2).

Glucocorticoids change the phenotype of macrophages involved in uptake of the Hb-Hp complex by upreguating CD163 and enhancing iron recycling (78). The use of

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corticosteroids for ACS remains controversial. In a study by Griffin et al., children and adolescents with SCD and painful VOE were randomized to receive either methylprednisolone or saline on admission and 24 hours later together with morphine and or oral opiates. Methylprednisone significantly reduced the duration of opiate therapy and hospitalization (41.3 vs 71.3 hours, P = 0.03) but was associated with a higher rate of rebound pain (79, 80). Bernini replicated the study in patients with ACS using dexamethasone and obtained 40% reduction in duration of pain and hospitalization but reported rebound pain requiring readmission in 6 out of 7 patients that was not statistically different in treated and untreated groups (p=0.095)(80, 81). A prospective multicenter randomized clinical trial of dexamethasone in 12 SCD patients showed the drug shortened the duration of hospitalization by a mean of 20.8 hours but not the duration of ACS; the risk of readmission for painful VOE increased (82). The rebound pain described in these studies occurred in both groups but was more severe in patients who received dexamethasone. This was attributed, in part to raised adhesion molecules as patients who developed rebound pain had higher plasma L-selectin 24 hours after receiving dexamethasone. Uncertainty about corticosteroids is a major concern in SCD, limiting their use even in patients with acute asthma exacerbation.

Other inhibitors of NFκB signaling that have shown preclinical efficacy in transgenic sickle mice include polyhydroxyphenyl hydroxamic acid derivatives, Didox (N-3,4-trihydroxybenzamide) and Trimidox (N-3,4,5 tetrahydroxybenzenecarboximidamide HCl). These drugs act as antioxidants but also potently abrogate NFκB activation by selectively inhibiting the phosphorylation and degradation of Iκbα (83). Both agents decreased leukocyte adhesion, stasis, and improved blood flow in transgenic SCD mice (84). In a pilot study, sulfasalazine has shown promise in SCD patients when it was administered orally for 1–4 weeks. Treated patients showed a marked decrease in circulating endothelial cell expression of VCAM, ICAM, and E-selectin (74). Though the preclinical efficacy of NFκB inhibitors in reducing inflammation is well documented, and reports of clinical efficacy are also widespread in the literature, as yet there is no FDA approved drug targeting this pathway in SCD.

5. The 5-Lipoxygenase Pathway

Introduction

Leukotrienes are fatty acid-derived mediators of inflammation implicated in the pathogenesis of several chronic inflammatory disorders (85). Arachidonic acid is metabolized by 5-Lipoxygenase (5LPO) generating several forms of hydroperoxyeicosatetranoic acid known as HPETE (86). In a two-step reaction, 5LPO together with its co-activator 5LPO-activating protein (FLAP) act on arachidonic acid to generate 5 (S) HPETE and ultimately yields the epoxide of 5HPETE known as leukotriene A4 (LTA4) (86). LTA4 is rapidly hydrolyzed by LTA4 hydrolase to leukotriene B4 (LTB4) or converted to leukotriene C4 through addition of glutathione by Glutathione S Transferase (86, 87). LTC4 together with its derivatives leukotriene D4 (LTD4) and leukotriene E4 (LTE4) are referred to as the cysteinyl leukotrienes (87). Leukotrienes are formed in and released as mediators of inflammation from neutrophils, eosinophils, mast cells, B

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lymphocytes, dendritic cells (88), and under the influence of erythroid cell–derived placental growth factor, from monocytes/macrophages (89).

Pathobiology

Complications resulting from 5LPO activation include airway hyper-responsiveness (90) and remodeling of smooth muscle leading to increased severity of pre-existing asthma (91, 92), pulmonary arterial hypertension (93) and stroke (94). In SCD the organs directly impacted by increased production of leukotrienes through the 5LPO are the vascular endothelium (95, 96), cells of the innate immune system (97), the lung and the nervous system. Generally, leukotrienes are implicated in several clinical events that are common in SCD; bronchoconstriction, mucus production, edema and inflammation, airway hyperactivity (95, 98), and enhanced sensitivity of peripheral nociceptors to smaller stimuli that are otherwise not noxious (99). LTE4 is elevated in steady state and increases further during VOE, and it is associated with increased risk of pain episodes requiring hospitalization (100–102). LTB4 is chemotactic to neutrophils (103, 104), and it promotes adhesion of sickle erythrocytes to endothelium via vitronectin (95, 97, 105). Others have implicated activation of 5LPO in VOE, (100, 101) allodynia (99), bronchial hyper-reactivity (91, 106, 107), ACS (91, 92, 101, 108) and neuropathic pain.

Therapeutics

BRP-7, a benzimidazole derivative that inhibits 5-LPO activating protein (FLAP) suppressed cellular leukotriene formation, prevented co-localization of FLAP with 5 LPO and attenuated inflammation in animal models of pleurisy and peritonitis (109). Although several FLAP inhibitors have been tested pre-clinically in many inflammatory disease models none are currently in clinical use (110). Zileuton, a structural analog of hydroxyurea which inhibits 5 LPO-catalyzed synthesis of leukotrienes is FDA approved for individuals aged 12 years and above with asthma. Of relevance to SCD, we showed that this drug not only inhibits leukotriene production but also induces γ-globin gene expression and increased fetal hemoglobin levels in erythroid progenitors derived from SCD patients (111). Also, Zileuton reduced secretion of IL-13, which can promote VCAM-1 expression by activated lymphocytes (112). A Phase 1 study of Zileuton in children and adults showed the drug was well tolerated in SCD (ClinicalTrials.gov identifier NCT01136941), and more importantly, it reduced many inflammatory mediators (e.g. sVCAM-1, sICAM-1, IL-6) (113). Other drugs include Montelukast and Zafirlukast; leukotriene receptor antagonists, and Pranlukast, a competitive inhibitor of LT C4, D4 and E4 cysteinyl leukotrienes. A placebo controlled phase II study of Montelukast in combination with hydroxyurea is currently ongoing to determine if Montelukast confers additional benefit in preventing VOE. The study endpoint is plasma levels of soluble VCAM-1 (ClinicalTrials.gov identifier NCT01960413).

6. Mast cell activation

Introduction

A small population of hematopoietic cells transits the blood into tissues where they undergo terminal differentiation into mast cells (114, 115). Fully matured mast cells are located primarily in mucosal surfaces, and in peri-neural and perivascular regions of the skin, lung

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and intestine as well as in the CNS where they are implicated in the development of migraines (116–119), a common phenomenon in SCD (120, 121). Mast cells express the receptor for stem cell factor (SCF) c-kit (CD117), the FcεR1 receptor (115) and they secrete bioactive substances including Substance P (SP) and Tryptase upon degranulation (122–124); the presence of c-kit is critical for their survival and function (125, 126). Secreted tryptase excites nociceptors on unmyelinated C fibers through activation of Protease activated receptor 2 (PAR2) and Transient Receptor Potential Vanillioid 1 (TRPV1) (127–129). The C fibers transmit signals of painful stimuli to the central nervous system via afferent fibers to the dorsal root ganglion, and they can also induce neurogenic inflammation mediated by CGRP and SP (128). In addition, mast cells can release cytokines without degranulation in response to specific stimuli; this is exemplified by the release of IL-6 in response to IL-1 stimulation (130). Figure 3 shows the effect of mast cell degranulation on immune cells and the vasculature and sites in the pathway of mast cell activation targeted by therapeutics.

Pathobiology

Multiple pro-inflammatory perturbations in SCD promote mast cell activation (128). Nitric oxide (NO) has an inhibitory effect on mast cell activation and inflammation. However, as discussed earlier SCD is characterized by reduced NO bioavailability; thus an inhibitory NO effect on the production of IgE dependent cytokines such as TNF α, IL 4, IL-6 by mast cells is diminished (118, 131). Conversely, IRI, oxidative stress and TLR4 activation, which are all enhanced in SCD promote mast cell activation (132, 133) and are associated with increased FcεR1 expression (134, 135). In agreement with these associations, skin biopsies of transgenic SCD mice express higher levels of tryptase, substance P and c-kit/CD117 than control mice, and mast cells obtained from the skin of these animals release more tryptase in culture and stain positive for c-kit, tryptase and FcεRI (128). These phenotypes were absent in a hybrid mutant sickle mice harboring a mutation in the murine c-kit gene, and in transgenic SCD mice pre-treated with imatinib (128). Ironically, morphine, a common analgesic used to treat pain in SCD induced hyperalgesia and repeated dosing caused allodynia in transgenic SCD mice (128). The data in mice is supported by findings of raised serum SP that increases further during VOE in children who have SCD (136). Moreover, a subset of SCD patients with pain symptoms similar to that seen in individuals with mast cell activation syndrome respond to mast cell stabilizing therapy (137).

Therapeutics

Imatinib and Cromolyn Sodium dampen neurogenic inflammation in preclinical models of SCD. Both agents inhibited release of SP and CGRP from the skin and dorsal root ganglion preparations of transgenic SCD mice (128). It has previously been established that cromolyn sodium exerts anti-sickling and possibly membrane stabilizing effect on sickle erythrocytes *in vitro* (138). Inhalation and intranasal administration of cromolyn sodium (*cromoglicate*) markedly reduced the number of sickle erythrocytes in patients (139). These promising studies led to a pilot prospective single blind crossover clinical trial over two years in 17 patients who received: a) placebo nasal spray alone, b) hydroxyrea combined with cromolyn sodium nasal spray, c) hydroxyrea combined with placebo nasal spray and d) cromolyn sodium nasal spray only. The combination of cromolyn sodium and hydroxyurea produced

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the largest improvement in pain scores, and the largest reduction in the number of sickle erythrocytes in an *ex vivo* assay (140).

7. The invariant Natural Killer T cell–Adora2a Receptor pathway

Introduction

Natural killer T (NKT) lymphocytes recognize mammalian, as well as microbial molecules found in the cell wall of bacteria; a dual recognition of self and microbial ligands that underlies their innate-like antimicrobial function that is enhanced by rapid cytokine release (141, 142). Invariant NKT (iNKT) cells are either CD4+ or CD4− and have a CD1d-restricted T cell receptor (TCR) that is activated by lipids bound to CD1d on antigen presenting cells (APC)(141, 143). Activation of iNKT cells is dependent on phosphorylation of p65 NFκB (144, 145), stimulating the release of cytokines including interferon γ (IFNγ), and IL-4 (146, 147). Activated iNKT cells cause proliferation of other cells of the adaptive immune response (B and T lymphocytes as well as dendritic cells). Cytokines secreted by activated APCs such as IL-12 (147) and IL-18 (146) further activate iNKT cells independent of CD1d (146, 147). IFNγ induces chemokines like CXCR3 (148), which is chemotactic to neutrophils and macrophages (141). Studies show that concurrent iNKT cell activation and rapid induction of adenosine ADORA2a (A2aR) receptors is triggered primarily by IRI and occurs in an NFκB-dependent manner (144, 149). A2aR activation exerts anti-inflammatory effects, limiting the duration of the inflammatory response (143, 144, 150, 151).

Pathobiology

It is thought that repeated bouts of IRI secondary to microvascular occlusions activate iNKT cells to drive chronic inflammation in multiple tissues in SCD (141, 144, 152). Compared to race-matched controls, SCD patients have higher numbers of circulating iNKT cells (141, 144), these numbers increase further during VOE (144), and moreover the activation of CD4+ iNKT cells appear to be restricted by CD1d lipid antigen presentation (144, 153). Adenosine binds to four trans-membrane G protein-coupled receptors A1, A2a, A2B and A3 (154). Adenosine ligation of the A2a and A3 receptors has long been recognized as an attractive anti-inflammatory therapeutic strategy (154). Anti-inflammatory A2aR signaling occurs through cyclic AMP and protein kinase A (143) which inhibits NFκB activation, the main effect being inhibition of T cell activation including iNKT cells. Secondary effects include decreased cytokine production, decreased neutrophil oxidative burst, and decreased adhesion molecule production. However, adenosine signaling via the A1 and A2B receptors is pro-inflammatory (155), and it produces several cardiovascular effects that may exacerbate SCD complications (154–156). A2B receptor activation on mast cells results in cytokine and IgE mediated bronchospasm (155–157), as well as priapism resulting in penile fibrosis (156, 158, 159). In particular, there is some evidence suggesting A2BR signaling promotes sickling of erythrocytes by increasing the production of intracellular 2, 3, Diphosphoglycerate concomitant with reduced Hb S oxygen affinity(160).

Therapeutics

Several approaches are currently being explored to prevent iNKT cell activation in SCD. They include the use of Regadenoson, antibodies against CD1d, and iNKT cell depletion

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with humanized monoclonal antibody. While the effectiveness of A2aR activation in reducing ischemic injury in several organs is well documented (161–165), the identity of the specific effector cells involved has been poorly defined. Studies of several murine models now show that regardless of the widespread distribution of A2a receptors on various cells, it is primarily the receptors on T cells that are responsible for the anti-inflammatory response limiting IRI (166–168). The A2aR agonist ATL146e improved several markers of inflammation in the lungs of NY1DD sickle mice (169). Regadenoson is a selective A2a agonist that has been used to induce cardiac hyperemia in cardiac stress testing for individuals unable to exercise. A two-stage multi-center phase 1 dose-escalating and safety study of Regadenoson in SCD children and adults in steady state utilizing three different doses administered as a continuous infusion (0.24, 0.6 and 1.44μg/kg/hour) for 12 hours showed the drug was well tolerated and no toxicity (hypotension and reflex tachycardia) was noted (ClinicalTrials.gov identifier NCT01085201. Regadenoson reduced markers of iNKT cell activation (CD69, CXCR3, and IFN-γ) with high levels of A2aR and low levels of IκBα emerging as superior markers of iNKT cell activation. The highest dose of 1.44μg/kg/hr was administered over 24 hours without toxicity (170), and in the third stage of the trial, the drug was administered to 6 patients with painful VOE in whom activated iNKT cell number was reduced without evidence of toxicity (171). Currently, a multicenter phase II randomized placebo-controlled trial to determine the efficacy of 48 hour-long infusion of Regadenoson in reducing iNKT cell numbers and activation during painful VOE and ACS is underway (ClinicalTrials.gov identifier NCT01788631).

8. HYDROXYUREA

Introduction

Hydroxyurea (HU) is the only FDA approved drug for SCD. It reduces many of the clinical events including painful VOE, hospitalizations, ACS and the need for blood transfusions (172, 173). Many of these benefits are associated with increased production of gamma globin, which is incorporated largely into an asymmetric hemoglobin (Hb) tetramer (FS; $\alpha_2\gamma\beta^S$) that limits intra-erythrocytic Hb S polymerization (174, 175), and consequently sickling. However, HU also reduces the number of leukocytes and platelets, and the concentrations of multiple cytokines and adhesion molecules in the plasma. These other changes highlight anti-inflammation as a major mechanism of HU's action in SCD patients. For instance, whole blood of such patients flows with relatively higher velocities and forms fewer micro-channel obstructions in a microfluidic endothelialized platform (176), reflective of the inhibitory effect of HU on vaso-occlusion. In addition, several markers of intravascular hemolysis are reduced in SCD patients on HU therapy suggesting that the drug targets inflammatory pathways that promote adhesion and hemolysis, the two central tenets of SCD, to improve overall disease outcome.

Inhibition of inflammation in vaso-occlusion

Low neutrophil count is widely acknowledged to lessen the frequency of VOE in SCD patients (177). The most widely acclaimed effect of HU is ribonucleotide reductase inhibition, which promotes cell cycle arrest and cell death. This accounts for the lowering of leukocyte and platelet counts in SCD patients. Absolute neutrophil count of 2000/ul and/or

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platelet count of 80,000/ul is typically used as a yardstick to determine the maximum tolerated dose for individual patients. In SCD patients, there is increased expression of multiple adhesion molecules, including Mac-1, LFA-1 and VLA-4 (178), greater spontaneous neutrophil chemotaxis and increased leukocyte survival due partly to increased cyclic Adenosine Monophosphate-Protein Kinase A (cAMP-PKA) signaling and decreased caspase activity (179, 180). The platelets of SCD patients have an activated phenotype at baseline, defined by enhanced expression of multiple surface receptors that promote aggregation and spreading, and increased secretion of inflammatory mediators such as IL-1β, sCDL-40, IL-6 (181). Studies in transgenic SCD mice indicate neutrophil adhesion to the endothelium initiates VOE (182). Neutrophil activation increases membrane exposure of phosphatidylserine (PS) on sickle erythrocytes, which promotes retention of the leukocytes in the lung (183). HU markedly inhibits this process in part by disrupting the neutrophil-mediated sickle erythrocyte PS exposure (184). Thus, down regulation of neutrophil adhesion molecule expression, and a consequent impact on cognate receptors on other cell types in the circulation may contribute to the ameliorating effect of HU on VOE.

HU therapy is associated with lowering of the absolute count, adhesion and the degranulation of eosinophils (185). Interestingly, HU does not impact eosinophil expression of eotaxin-2, 3 and RANTES, or the production of ROS suggesting that the major influence of the drug on this cell is adhesion. However, it reduces the expression of several adhesion molecules including E- and P-selectin, I-CAM, PECAM-1 and VCAM-1 (186–189) that have been implicated in VOE (190) and are predominantly expressed by endothelial cells and leukocytes (191, 192). Down regulation of E-selectin expression protected SCD mice from a lethal pneumococcal infection by attenuating leukocyte-endothelial interactions (193). Thus, HU may protect SCD patients from invasive bacterial infections and decrease the lethal consequences of a heightened inflammatory response to such infections. The plasma of SCD patients contains raised TNF-α, IL-6, IL-1β, IL-17, and IL-8 and reduced IL-10 (194–197). While evidence of HU decreasing TNF-α and increasing IL-10 levels in SCD plasma is compelling (198–200), reports of its effects on other cytokines are inconsistent. Increased TNF-α promotes VOE by increasing endothelial cell adhesion (201), highlighting a clear mechanism for lessening VOE that is supported by *in vitro* studies (202).

Inhibition of hemolysis associated inflammation

Induction of IL-10 by HU in cultured cells (203) suggests the drug contributes to the increased levels of this anti-inflammatory cytokine in SCD patients receiving HU therapy (198, 199). Interestingly, IL-10 potently increases the expression of HO-1(204). In a murine model of sepsis, HO-1 inhibition reversed the inhibitory effect of IL-10 production on TNF-α in a process involving carbon monoxide, a byproduct of heme catabolism (204). IL-10 induction may explain HU's beneficial effect in ACS. We described earlier a new model of ACS pathogenesis-*The Heme Hypothesis*, which posits that excess intravascular heme released from entrapped sickle erythrocytes during VOE triggers ACS(35). This disease model is supported by experimental studies in transgenic SCD mice and genomics findings in patients. In addition, steady-state plasma concentration of heme is markedly higher in children who have multiple episodes of ACS than in age-matched counterparts in the same

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clinic who have never had an ACS event (205), The murine model offers a robust platform to directly test the proposed HU mechanism on ACS. Our preliminary studies using this model show dramatic reduction of IL-10 in the blood, lung and bronchoalveolar lavage fluid in transgenic SS mice experiencing severe ACS (Jackson *et al*., unpublished). In support of this link, SCD patients on HU demonstrate markedly raised IL-10 expression in neutrophils (198), which are a major source of the acute lung damage seen in ARDS.

HU's anti-inflammatory effects may be attributable in part to its actions as a NO donor. Analogues of HU form NO in vitro in the presence of an Fe3+ porphyrin complex and an oxygen donor (206). HU reacts with deox-Hb, oxy-Hb and metHb, generating nitrosyl-Hb (207). HU caused a significant increase in plasma nitrosylated Hb, nitrate and nitrite consistent with generation of NO, which peaked 2 hours after drug administration in SCD patients (208). In addition, the NO-soluble guanylase cyclase pathway has been implicated in the mechanism of gamma globin induction by HU (209). Almeida et al. recently showed that a single dose of HU inhibits onset of acute inflammation induced by hemolysis in C57BL6 mice(210). Studies focused on defining the key inflammatory molecules and cognate pathways altered by HU to confer protection from ACS, VOE and anemia may help to improve how the drug is administered, and to identify potential adjuvants to increase overall efficacy.

9. Conclusion

Inflammation plays a cardinal role in many of the clinical events of SCD, most notably in ACS. We have highlighted specific inflammatory mediators both cellular and molecular that are influenced by HU to lessen the severity of SCD. In addition, there is a growing number of drugs, old and new, targeting many inflammatory pathways including those discussed in this review that can be repurposed for SCD. However, the introduction of any new drug to the SCD population is most appropriately accomplished through a systemic preclinical approach, for several reasons. First, SCD patients are physiologically fragile, so no drug, even if tested in other populations or normal mice, can be assumed to be safe in SCD. Second, renal function and hepatic metabolism is often abnormal in SCD, and accordingly, drug metabolism and excretion may not be normal. Third, it is imperative to test new drugs in the most appropriate disease models; historically this has been a problem in this field as hypoxia has summarily been adopted as a default model of disease complications. For a preclinical model of multiple organ failure for instance, a low-to-moderate dose (15–25 μmoles/kg) of purified hemin given intravenously to transgenic SCD mice maybe more appropriate than hypoxia. The former causes hepatic failure within ~24 hours with about 45% lethality by 72 hours in transgenic SCD mice(35). Using this low-grade i.v. hemin model, evidence of inflammation and the anti-inflammatory effects of putative drugs in SCD can be assessed noninvasively by monitoring alterations in plasma concentrations of appropriate markers in mice. The large number of anti-inflammatory agents in the drug discovery pipeline coupled with emerging preclinical models of SCD inflammation auger well for developing effective therapies for this prototypical molecular disease in the era of precision medicine.

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Definition of abbreviations

LFA-1	Leucocyte function associated antigen
VLA–4	Very Late Antigen-4
ICAM-1	Intercellular Adhesion Molecule 1
VCAM-1	Vascular Cell Adhesion Molecule
PECAM	Platelet Endothelial Cell Adhesion Molecule

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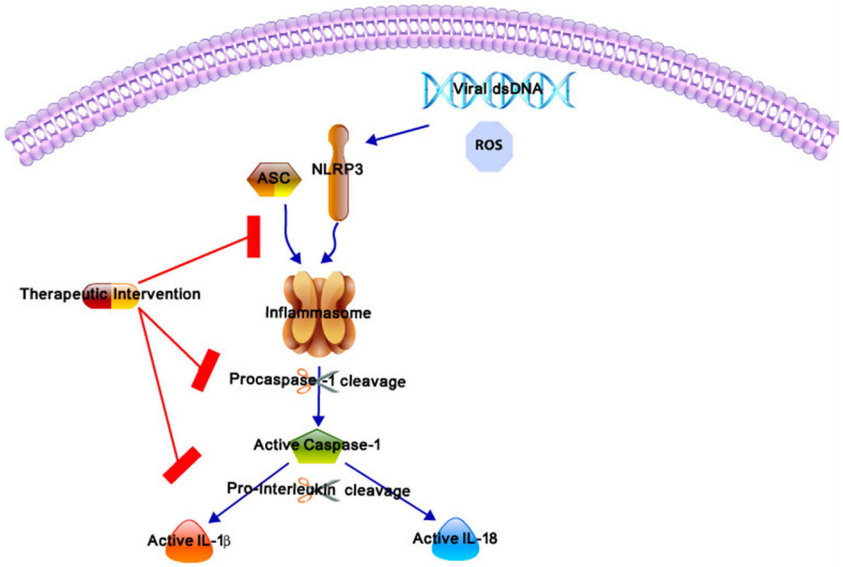

Figure 1. The Inflammasome

NLRP3 senses PAMP and DAMP molecules leading to the recruitment of ASC and inflammasome assembly. The inflammasome mediates caspase cleavage, which facilitates the cleavage and activation of IL-18 and IL-1b which promote inflammation. Several druggable targets in this pathway have been established and have proven efficacious in an array of inflammatory diseases.

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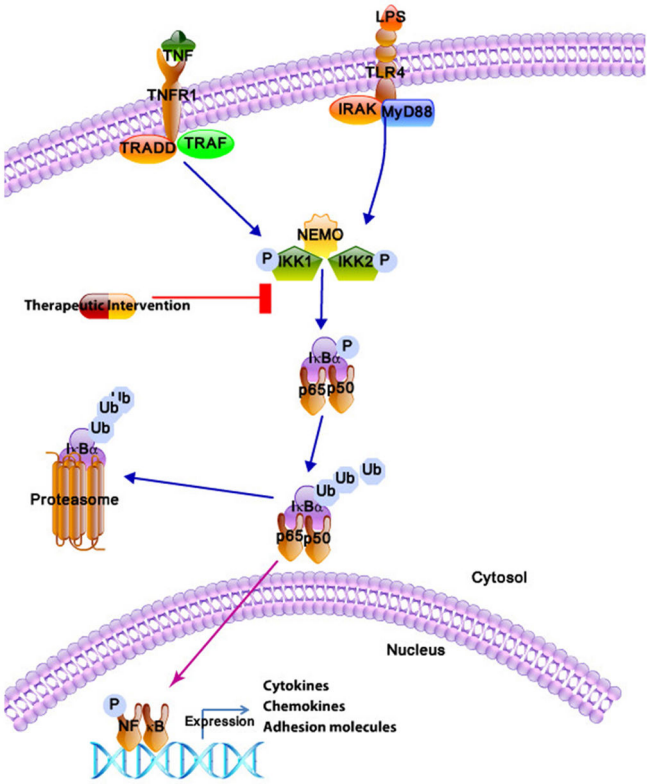

Figure 2. The NFκB Pathway

Cytokine signaling and activation of TNFR and TLR is propagated through adaptor molecules such as TRADD, TRAFF, IRAK and MyD88 leading to phosphorylation and activation of the IKK kinase complex. The IKK complex phosphorylates the NFκB Inhibitory protein IκB which promotes polyubiquitination and proteasomal degradation. NFκB will subsequently translocate to the nucleus and bind its DNA response elements known as κB sites, thus leading to mRNA expression of target genes. Anti-Inflammatory therapeutic interventions in SCD that inhibit the phosphorylation of IκB have proven efficacious at inhibiting NFκB activation. (TRAFF, TNF receptor-associated factor 1; TRADD, Tumor necrosis factor receptor type 1-associated DEATH domain protein; IRAK, Interleukin-1 receptor-associated kinase 1; MyD88, Myeloid differentiation primary response protein).

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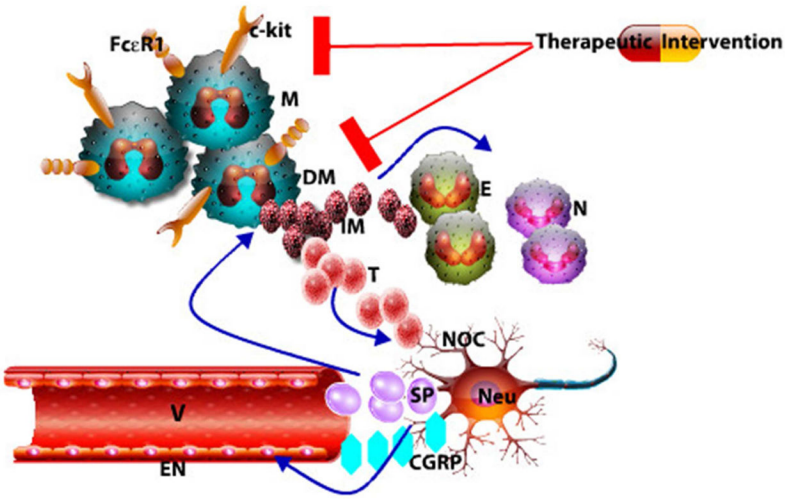

Figure 3. Mast cell activation

Mast cells (M) require the trans-membrane tyrosine kinase receptor C-kit (c-kit) to function properly. Activation of mast cells occurs through binding of ligand to the Fc epsilon R 1 receptor (FcεR1) or independent of it. Activated or de-granulating mast cells (DM) release pro-inflammatory mediators (IM), which attract cells of the innate immune system such as eosinophils (E) or neutrophils (N). Tryptase (T) released from DM excites nociceptors (NOC) on neurons (NEU) to produce the neuropeptides Substance P (SP) and calcitonin gene related peptide (CGRP) whose effect on the endothelial cells (EN) of blood vessels (V) is increased vascular permeability and inflammation. SP causes further mast cell activation and degranulation.