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THE ROLE OF SUBSTANCE P, HEMOKININ AND THEIR RECEPTOR IN GOVERNING MUCOSAL INFLAMMATION AND GRANULOMATOUS RESPONSES

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1. ABSTRACT

Granulomas are chronic inflammations that prevent spread of poorly controllable infectious agents. The gut lumen contains enteric organisms that are excluded from the host by leukocytes located in the intestinal lining. Physiological intestinal inflammation and granulomas share some similarities. Both function to confine, but not necessarily abolish potentially harmful factors. Also, both are subject to intense immune regulation to avoid unnecessary tissue injury. Substance P and its natural analog hemokinin are produced at these sites of inflammation and are important components of this regulatory process. They act through a shared receptor (NK-1) expressed on T cells, macrophages, dendritic cells and probably other cell types. One of their functions is to enhance IFN-gamma production and amplify the Th1 response. The NK-1 receptor is an important target for immune regulation. Several Th1 cytokines and T cell antigen receptor (TCR) activation induce NK-1 receptor expression on T cells, while IL-10 and TGF-beta block receptor display. Macrophages also have an inducible NK-1 receptor. Various types of immune cells can make substance P and hemokinin, whose syntheses also are subject to immunoregulation. Thus, substance P and hemokinin are inflammatory cytokines with overlapping functions that help control immune responses in granulomas and at mucosal surfaces, and probably elsewhere.

2. INTRODUCTION

The Tac1 gene encodes a protein called preprotachykinin A, which is enzymatically processed to produce substance (SP). Another molecule called hemokinin 1 (HK) derives from a separate protein named preprotachykinin C, which is a product of a different gene (Tac4). Both SP and HK are eleven amino acid proteins belonging to the tachykinin family of neuropeptides (1, 2, 3). Nerves produce SP and HK, but they also are products of leukocytes. SP and HK stimulate cells via a shared high affinity seven transmembrane, G protein-coupled receptor called neurokinin 1 (NK-1).

Granulomas are specialized chronic inflammatory responses under intense immune regulation (4, 5). They usually form when the host encounters a poorly soluble antigenic factor. For instance, components of bacteria, fungi or parasites can form the antigenic nidus. Examples of granuloma-inducing organisms include *Mycobacteria, Histoplasma* and the ova of *Schistosoma*. Granulomas function to surround and prevent the spread of invading organisms or other potentially harmful substances that resist destruction by a more acute immune response. The cellular composition of the granuloma varies depending the health and genetic makeup of the host, the duration of the inflammation and the nature of the inciting agent. Mature macrophages and activated T cells are a constant feature. Granulomatous responses can persist from months to years. Granulomatous responses are not always beneficial, since they can damage and scar otherwise healthy tissue. There are diseases featuring idiopathic, destructive granulomatous inflammation without known protective function. Such diseases include sarcoidosis, Crohn's disease and Wagener's granulomatosis among others.

The intestine contains large numbers of bacteria and other antigenic substances. These commensal bacteria promote growth and development of the intestinal mucosa, and help with digestion. The mucosal immune system contains and prevents spread of these potentially harmful factors. Thus, in some respects, the physiologic inflammation normally encountered at mucosal surfaces functions similarly to granulomas. Also, both are subject to

intense immunoregulatory control, which limits the immune response and prevents unnecessary tissue injury. Some humans and animals develop chronic, destructive inflammation of the intestines called inflammatory bowel disease, which probably results from a dysregulated mucosal immune response to normal intestinal bacteria (6).

SP and HK are immunoregulatory cytokines that help maintain immune balance at mucosal surfaces and at other sites of chronic inflammation like that of granulomas. The objective of this article is to present evidence derived from human inflammatory bowel disease and from murine models of chronic intestinal and liver inflammation that support this contention.

3. SP AND HK REGULATE INFLAMMATION IN THE LIVER AND INTESTINE

As reviewed below, animal models of inflammation and human disease provide ample evidence suggesting that the NK-1 receptor and its natural ligand SP influence immune responses in the intestines and liver. The newly discovered tachykinin, HK, also is a natural ligand for the NK-1 receptor and is produced at sites of inflammation. Thus, both SP and HK are important immunoregulatory cytokines with overlapping functions.

3.1. Schistosomiasis

Humans can acquire a disease called schistosomiasis. It is caused by parasitic helminthic worms that live in the venous plexus of the intestines or bladder of the host. People with schistosomiasis get chronic granulomatous inflammation resulting from parasite eggs settling in the liver and intestines. Mice can harbor the human schistosome called *Schistosoma mansoni*. As in humans, each ovum that lodges in tissue induces a granulomatous response. The cellular composition of these lesions is about 50% eosinophil, 30% macrophage, 15% T cell, 5% B cell and <1% mast cell. These highly eosinophilic granulomas make large quantities of IL-4, IL-5, IL-13, IgE and thus are classified as Th2-type responses. These granulomas can be isolated from host tissue allowing sophisticated analysis (7).

Bacterial and viral invasion usually triggers IFN-gamma production, which activates macrophages and influences the function of B cells, natural killer cells and T cells. IFN-gamma promotes production of complement fixing antibodies and affects many other critical immune functions like antigen presentation. The IFN-gamma-driven pattern of immune response is called Th1-type inflammation (8). IFN-gamma is extremely important since IFN-gamma γ deficiency leaves the host highly susceptible to bacterial and viral invasion. However, excess IFN-gamma secretion can promote disease. Thus, IFN-gamma synthesis must be carefully controlled.

Although the inflammation of murine schistosomiasis is best characterized as a Th2 response, it also generates Th1-type cytokines that are tightly constrained. SP and probably HK are important parts of this regulatory process (9). SP, made locally within the granulomas, helps govern IFN-gamma release (10, 11). Splenocytes or granuloma cells cultured *in vitro* with low doses of schistosome egg antigen will produce much more IFN-gamma if stimulated with even nanomolar concentrations of SP. HK also enhances IFN-gamma synthesis with potency comparable to SP. Neurokinin A and B are two more members of the tachykinin family of hormones. Neither induces IFN-gamma secretion unless used at exceeding high concentrations (10^{-6} M).

CD4+ T cells within the schistosome granulomas are the major producers of the IFN-gamma (12). Without the aid of other cellular intermediaries, SP regulates T cell IFN-gamma production through interaction with the SP receptor (NK-1) expressed on these cells (13). NK-1 receptor engagement results in selective intracellular signaling that only affects T cell IFN-gamma production without influencing IL-4 or IL-5 synthesis.

IL-12 and TGF-beta help regulate IFN-gamma production both at mucosal surfaces and in granulomas (14). IL-12 promotes Th1 cell develop and IFN-gamma secretion, while TGF-beta can down-modulate the process. IL12 is produced mostly by antigen presenting cells like macrophages and dendritic cells. TGF-beta comes from many cell sources. In some situations, SP can decrease TGF-beta production from cultured peritoneal macrophages (15) and stimulates IL-12 production (16). However, in murine schistosomiasis, SP does not modulate either IL-12 or TGF-beta synthesis. It governs T cell IFN-gamma secretion independently of these two cytokines.

The effect of SP on IFN-gamma production is substantial and biologically important since SP also stimulates IgG2a secretion. Murine B cells require IFN-gamma to switch to immunoglobulin subclass IgG2a. Modulation of IFN-gamma synthesis is the mechanism through which SP affects IgG2a production (17).

Mice with defective NK-1 receptor expression develop schistosome granulomas with both impaired IFN-gamma and IgG2a secretion (18). This observation attests to the importance of the SP/HK receptor (NK-1) in controlling IFN-gamma circuitry. Additional *in vitro* and *in vivo* studies using highly selective NK-1 receptor antagonists confirm the importance of the NK-1 receptor (17, 19). Macrophages, T cells and other cell types can display authentic NK-1 receptor. Two T cell-selective, NK-1 receptor expression models demonstrate that it is the T cell NK-1 receptor, which directly governs the IFN-gamma response (13).

Somatostatin is another small bioactive peptide produced by the brain, peripheral nerves, paracrine cells of the gut, macrophages (20) and dendritic cells. In murine schistosomiasis and in other inflammatory states, somatostatin inhibits IFN-gamma secretion. SP blocks somatostatin synthesis in macrophages (21) and dendritic cells. This regulation does not require the presence of T and B cells and is subject to blockade with NK-1 receptor antagonists. Thus, SP likely works directly through a macrophage/dendritic cell NK-1 receptor to limit

somatostatin expression. This is a particularly interesting regulatory circuit, since SP is a Th1-type cytokine, and somatostatin works to inhibit the Th1 pathway (22).

Acting through unknown mechanisms, IL-4 can prevent SP from inhibiting macrophage somatostatin synthesis (21). IL-4 has no effect on NK-1 receptor mRNA expression. Most likely, somatostatin synthesis persists in the SP-rich, Th2 environment of the schistosome granuloma because these granulomas make IL-4.

Since HK and SP bind the same receptor and both are produced in schistosome granulomas, it is assumed that they have similar and overlapping functions. Th1-type granulomas also express SP, but little is known regarding its importance for IFN-gamma production in Th1 granulomatous responses. It is unknown if Th1 granulomas make HK.

3.2. Trichinella spiralis

Trichinella spiralis is a helminthic parasite that induces a strong Th2-type immune response in the rat intestine. Intestinal colonization of rats with this organism induces a T cell-dependent increase in SP in the muscle-myenteric plexus. Also, administration of a NK-1 receptor antagonist (23) or blocking SP anti-serum (24) protects rats from the intestinal inflammation induced by *Trichinella spiralis*. The mechanism of action remains unknown.

Neutral endopeptidase is an enzyme on cell surfaces that can quickly degrade the SP released into surrounding tissue. Compared to wild-type controls, healthy neutral endopeptidase knockout mice have more SP in the colon and more readily extravasate fluid into the intestines (25). *Trichinella spiralis* colonization of the intestine down-regulates neutral endopeptidase activity in the ileum and decreases the rate of SP degradation in the gut (26). It is postulated that *Trichinella spiralis* promotes inflammation partly through this mechanism.

3.3. Salmonellosis

Salmonella is a bacterium that inhabits the gut and induces gastroenteritis. It usually is acquired through consumption of contaminated water or food. In a murine model of salmonellosis, treating mice with an NK-1 receptor antagonist leaves them more susceptible to infection, and they display a diminished mucosal IFN-gamma response (27). Following exposure to intestinal *Salmonella*, there is a rapid increase in preprotachykinin mRNA in the spleen and regional mucosal lymphs. This suggests that the inflammation is inducing expression of this hormone.

IL-12 and IFN-gamma secretion, and macrophage activation are part of the initial response to *Salmonella* that helps limit growth and dissemination of this bacterium. Treatment with NK-1 receptor antagonist decreases *Salmonella*-induced expression of IL12 and IFN-gamma mRNA in the regional lymph nodes. The activated macrophages produce IL-12, TGF-beta and other molecules that help control IFN-gamma synthesis. Murine macrophages cultured *in vitro* express NK-1 receptors in response to *Salmonella* (27). SP can decrease LPS-induced TGF-beta production from cultured peritoneal macrophages (15) and stimulate IL-12 secretion (16). Thus, it is possible that SP or HK can modulate production of these critical cytokines to effect IFN-gamma synthesis and *Salmonella* infection.

3.4. *Clostridium difficile* toxin-induced intestinal injury

Clostridium difficile is a bacterium that can colonize the intestines and produce toxins, which can induce colitis in humans. An animal model suggests that NK-1 receptors help mediate *C. difficile* toxin-induced mucosal injury (28). The rat model of this disease involves injecting toxin into surgically created, blind ileal loops. The toxin induces acute epithelial cell necrosis leading to neutrophil infiltration. Mice given SP receptor antagonists (29) or mice lacking the NK-1 receptor are protected from toxin-induced enteritis (28). Lamina propria mononuclear cells isolated from these acutely injured intestines produce SP (30), but the role of leukocyte-derived SP in the disease process is not defined. The acute nature of the injury and inflammatory response suggest that SP or perhaps HK is affecting the inflammation through innate pathways. The protective effect of NK-1 blockade does not necessarily implicate a direct link between SP or HK and leukocytes as the mechanism of action. Various parenchyma cell types (eg. muscle, endothelial and epithelial cells) can express NK-1 receptors. For instance, the toxin enhances NK-1 receptor display on intestinal epithelial cells (31). Thus, SP, and by inference perhaps HK, may be affecting the function of the vascular endothelium, intestinal muscle and/or intestinal epithelium leading to disease susceptibility.

3.5. Inflammatory bowel disease

Immunohistochemistry and *in situ* hybridization showed that the NK-1 receptor is expressed on normal human intestinal lamina propria mononuclear cells, lymphoid follicles, vascular endothelium, epithelial cells and myenteric plexus (32, 33). Isolated human lamina propria mononuclear cells from healthy regions of colon consistently express NK-1 receptor mRNA and protein, whereas peripheral blood mononuclear cells are negative for this receptor. Flow analysis revealed that CD4+ T cells express most (> 60%) of the lamina propria mononuclear cell NK-1 receptor, although other leukocyte subsets express this receptor also (33). NK-1 receptor also is expressed on murine lamina propria T cells, but not on resting splenocytes (34).

Humans can develop an immunologic disease associated with chronic, destructive inflammation of the intestines. Inflammatory bowel disease usually is divided into two types called Crohn's disease and ulcerative colitis. Human inflammatory bowel disease probably results from a dysregulated mucosal immune response to normal intestinal bacteria. In human inflammatory bowel disease, there is increased NK-1 receptor mRNA expression (35) in the tissue. Goode, *et al.* (36) showed, using quantitative RT-PCR, a 7-fold increase in NK-1 receptor transcripts in Crohn's disease colon and a somewhat more modest increase in ulcerative colitis. Compared to normal control colon, however, immunohistochemistry revealed no ectopic sites of NK-1 receptor expression.

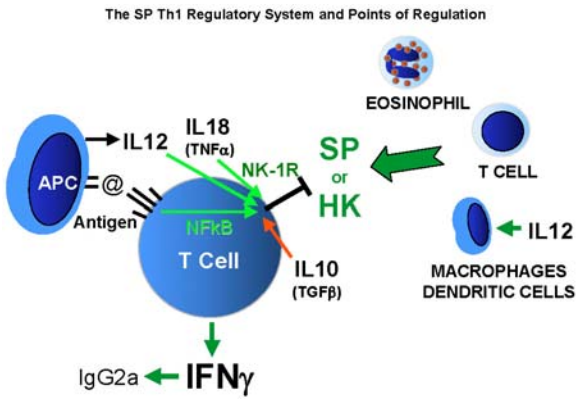

Figure 1. SP and/or HK promote antigen-driven IFN-gamma production through engagement of an authentic T cell NK-1 receptor. T cells, macrophages, dendritic cells and eosinophils can make SP and probably HK at mucosal surfaces and other sites of inflammation. IL-12 induces macrophages and dendritic cells to secrete SP. Antigen presentation to the T cell receptor, IL-12 and IL-18 induce NFkB signaling, which drives NK-1 receptor expression on T cells. TNF-alpha induces the NK-1 receptor weakly. IL-10, and to a lesser extent TGF-beta strongly inhibit of NK-1 receptor expression.

While SP is abundant in the intestines, there are somewhat conflicting data regarding the concentration of SP in the intestinal mucosa of patients with inflammatory bowel disease (37, 38, 39). Some studies report high levels that correlate with activity of the disease (38, 39, 40). Others also report high levels of SP in intestinal nerve fibers (41, 42, 43). Nothing is known regarding HK.

Although the intestine is one of the major sites for SP production, the importance of leukocyte NK-1 receptor expression in the mucosa remains unclear. The expression of NK-1 receptors on intestinal leukocytes infers an important role for SP and/or HK in regulation of mucosal inflammation. The disproportionate over-representation of NK-1 receptors on human mucosal CD4+ T cells, however, suggests that expression of NK-1 receptors on T cells is of particular importance.

Murine models of human inflammatory bowel disease suggest that SP and HK help regulate intestinal inflammation. Rectal instillation of the hapten trinitrobenzene sulfonic acid (TNBS) into rats or mice induces a severe Th1-type colitis with some immunological features suggestive of Crohn's disease (44). Mononuclear cells infiltrate the lamina propria, and CD4+ T cells secrete high levels of IL-12, IFN-gamma and TNF-alpha (45). These cytokines help to initiate and sustain this inflammation. Treatment with an NK-1 receptor antagonist limits the inflammation in TNBS-induced colitis (46).

The IL-10 deficient mouse spontaneously develops a lymphocyte-rich intestinal inflammation that gradually worsens as the animal ages. The colitis results from an aberrant immune response to elements of normal intestinal flora (47). The inflammation is a Th1 response producing IFN-gamma and IL-12, and little IL-4 or IL-5. As in TNBS colitis, Th1 cytokines are important for persistence of the inflammation. In many respects, the inflammation mimics human inflammatory bowel disease. This animal model also suggests that the cytokine IL-10 is critical for maintenance of mucosal immune homeostasis.

Young IL-10 deficient animals rapidly develop severe inflammation of the colon and terminal ileum when given normally safe doses of a non-steroidal anti-inflammatory medication. The disease persists following withdrawal of the medication (48). In the non-steroidal anti-inflammatory medication-induced IL-10 knockout murine model of inflammatory bowel disease, NK-1 receptor antagonists suppress intestinal IFN-gamma production and inhibit the ongoing colitis. This suggests that SP and/or HK have an important roles in the disease process (49).

Neutral endopeptidase is expressed on the surface of many cell types including leukocytes, neurons, smooth muscle cells and epithelial cells. It hydrolyzes SP at the Gln^6-Phe^7, Phe^7-Phe^8 and Gly^9-Leu^{10} bonds terminating its biological activity (50).

Dinitrobenzene sulfonic acid given orally to rodents induces colitis. Neutral endopeptidase knockout mice develop markedly worse intestinal inflammation and injury in response to dinitrobenzene sulfonic acid compared to wild-type controls. Administration of recombinant neutral endopeptidase or NK-1 receptor antagonist prevents the exacerbated inflammation (25). This suggests that loss of neutral endopeptidase function leads to over-expression of SP and/or HK, which worsens the inflammation.

4. NK-1 RECEPTOR REGULATION

Lymphocytes and macrophages can express the SP/HK (NK-1) receptor in both human and other mammalian species (51, 16, 52, 53, 54). It also is present on several macrophage and T cell lines. In murine schistosome granulomas, granuloma T cells and macrophages display NK-1 receptor (21, 18). The granuloma cell composition is about 10% CD4 and 5% CD8. As revealed by flow analysis using Alexa-labeled SP analog, at least 10% of each of these T cell subsets express NK-1 receptor strongly. This also is the case in the intestinal infiltrate of the IL-10 knockout murine model of inflammatory bowel disease.

The NK-1 receptor is an important target for immune regulation. Immune regulatory circuits that control NK-1 receptor expression are evident in the spleen and granulomas of mice with schistosomiasis and in the lamina propria T cells of mice with IL-10 knockout colitis (Figure 1). Resting T cells do not display NK-1 receptors. However, T cell receptor engagement, IL-12, IL-18 (55) or TNF-alpha can stimulate T cells to express the NK-1 receptor. This regulation is mostly limited to the

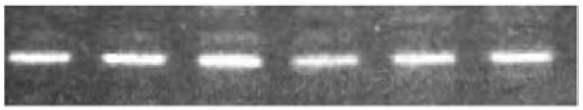

Figure 2. Preprotachykinin C (hemokinin) mRNA is expressed in intestinal lamina propria mononuclear cells (LP), granuloma cells (Grn) and bone marrow. RNA was extracted from dispersed lamina propria mononuclear cells isolated from the terminal ileum of normal wild type mice, from dispersed granulomas isolated from the liver of schistosome-infected mice or from normal murine bone marrow. RT-PCR used gene-specific, intron-spanning primers to profile preprotachykinin C expression. Shown are the results from duplicate experiments.

CD4+ T cell subset. IL-12, IL-18 and TNF-alpha use the NFkB intracellular signaling pathway to mediate receptor induction (56). IL-10 blocks the action of IL12 and IL18 preventing NK-1 receptor display (49). IL-10 also strongly down-modulates ongoing NK-1 receptor expression. TGF-beta is inhibitory also, but to a lesser degree. SP enhances production of the Th1 cytokine IFN-gamma. IL-12 and IL-18 are the major cytokines that drive development of IFN-gamma producing Th1 cells. IL-10 inhibits IL-12 production, and IL-10 along with TGF-beta are important modulators of the Th1 response. Thus, the NK-1 receptor is an important component of Th1-type inflammation that is regulated by these important cytokines. Loss of IL-10 with subsequent failure to prevent or down-modulate T cell NK-1 receptor expression could be one of the factors leading to Th1-type colitis in IL-10 deficient mice.

Although not yet studied in murine schistosome granulomas or in gut inflammatory states, it also appears that the macrophage NK-1 receptor is regulated. LPS up-regulates rat macrophage NK-1 receptor mRNA expression (57). IL-4 or IFN-gamma elicits increases in NK-1 receptor protein and mRNA in murine peritoneal macrophages (58). IL-1 can induce human macrophages to express NK-1 receptor via activation of the NFkB intracellular signaling pathway (59). It therefore is likely that granuloma and lamina propria macrophage NK-1 receptors are subject to similar regulatory processes.

5. ORIGINS OF SP AND HK AT SITES OF CHRONIC INFLAMMATION

SP is produced at sites of inflammation, and its synthesis is subject to immunoregulation (20, 60, 61). Nerves secrete SP, and nerves frequently have a distribution in tissue favorable for immune regulation. However, human, mouse and rat leukocytes make SP also. It can come from T cells (62), macrophages (58, 52, 59), dendritic cells (63) or eosinophils (64). Leukocyte-derived SP may be the most important source of this neuropeptide in chronic inflammations.

Murine schistosome granulomas express preprotachykinin A mRNA. Preprotachykinin A mRNA can encode several distinct tachykinins of which only SP is made in the granuloma. The granulomas contain no nerves (65) suggesting that granuloma SP is completely of leukocyte origin.

Preprotachykinin A mRNA is expressed widely among the various cell types comprising schistosome granulomas being a product of eosinophils (64), macrophages and T cells. T lymphocytes and macrophages make SP both in Th1 and Th2-type granulomas. IL-12 signals through the STAT4 pathway to strongly stimulate macrophages and perhaps dendritic cells to make SP (unpublished observation). Thus, SP production is subject to immune regulation.

Macrophages in the lamina propria of IL-10 deficient and wild type mice make SP (unpublished observation). IL-12 also drives this expression.

The amino acid sequence of murine and human HK shares 45% homology with SP (2). SP and HK come from distinctly different genes and gene products. HK is a product of a protein called preprotachykinin C. HK originally was thought to come from immature B cells and to affect B cell development in the bone marrow (2). Granuloma inflammatory cells as well as lamina propria mononuclear cells of wild-type (Figure 2) and IL-10 mutant mice express preprotachykinin C mRNA transcripts (unpublished observation). In the schistosome granuloma, T cells and probably macrophages and dendritic cells all contain large numbers of preprotachykinin C mRNA transcripts and presumably produce HK. HK binds to NK-1 receptors with an affinity equal to that of SP (3, 66). This also is true for NK-1 receptors expressed on murine intestinal lamina propria leukocytes and on schistosome granuloma cells (unpublished observation). Moreover, like SP, HK drives IFN-gamma production, and highly selective NK-1 receptor antagonists completely block this IFN-gamma stimulation. Thus, there are two distinct molecules, SP and HK, that are produced at sites of inflammation and which regulate inflammation via the same NK-1 receptor.

6. PERSPECTIVES

SP and HK are tachykinins produced in granulomas and in the lamina propria of the intestine. They are products derived from granuloma and intestinal leukocytes like macrophages and T cells. Inflammatory mediators induce their expression. Both SP and HK influence immune responses through interactions with the NK-1 receptor. NK-1 receptors are displayed on T cells, macrophages and other cell types. NK-1 receptor expression is regulated by various proinflammatory and immunomodulatory cytokines. In granulomas and in the gut, SP and HK engage T cell NK-1 receptors to govern IFN-gamma secretion and the Th1 response (Figure 1). Many other cell types at these sites of inflammation display NK-1 receptors suggesting that SP influences granulomatous responses and intestinal inflammation through additional unknown mechanisms.

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Abbreviations: Hemokinin (HK), Neutral endopeptidase (NEP), Substance P (SP), Trinitrobenzene sulfonic acid (TNBS)

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