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Cutting Edge: Hemokinin Has Substance P-Like Function and Expression in Inflammation

Ahmed Metwali, Arthur M. Blum, David E. Elliott, Tommy Setiawan, and Joel V. Weinstock

Substance P (SP) belongs to the tachykinin family of molecules. SP, cleaved from preprotachykinin A, is a neuropeptide and a proinflammatory leukocyte product. SP engages neurokinin 1 receptor (NK-1R) to stimulate cells. Hemokinin (HK) is another tachykinin that binds NK-1R. HK comes from preprotachykinin C, which is distinct from preprotachykinin A. We determined whether HK functions like SP at inflammatory sites. Preprotachykinin C mRNA was in murine schistosome granulomas and intestinal lamina propria mononuclear cells. Granuloma T cells and macrophages expressed preprotachykinin C mRNA. HK bound granuloma T cell NK-1R with high affinity. SP and HK stimulated IFN-γ production with equal potency. NK-1R antagonist blocked the effect of SP and HK on IFN-γ secretion. Thus, both HK and SP are expressed at sites of chronic inflammation and share cell origin, receptor, and immunoregulatory function. Two distinct but functionally overlapping tachykinins govern inflammation through NK-1R at sites of chronic inflammation. The Journal of Immunology, 2004, 172: 6528–6532.

The tachykinin 1 gene (Tac1) encodes a protein called preprotachykinin A, which is enzymatically processed to produce substance P (SP). SP stimulates cells via a high-affinity seven-transmembrane, G protein-coupled receptor named neurokinin 1 receptor (NK-1R). Macrophages (1–3), dendritic cells (4), lymphocytes (5) (6), and/or other immune cell types (7) make SP locally in schistosome granulomas, at mucosal surfaces, and at other sites of inflammation.

Animal models of inflammation and human disease provide ample evidence that NK-1R, located on leukocytes and vascular endothelium, and presumably its natural ligand SP influence immune responses. For instance, *Trichinella spiralis* is a helminthic parasite that induces a Th2-type immune response in rat intestine. Intestinal colonization of rats with this organism induces a T cell-dependent increase in SP in the muscle-mesenteric plexus, and blocking NK-1R improves the intestinal inflammation (8). *Salmonella* is a bacterium that induces gastroenteritis. In a murine model of salmonellosis, treating mice with an NK-1R antagonist leaves them more susceptible to infection, and they display a diminished mucosal IFN-γ response (9). *Clostridium difficile* is a bacterium that can colonize the intestines and produce toxins, which induce colitis in humans. An animal model of this disease suggests that NK-1R helps mediate *C. difficile* toxin-induced mucosal injury (10).

Mice can harbor the human schistosome called *Schistosoma mansoni*. As in humans, ova lodge in host tissue inducing granulomatous inflammation. Although the inflammation of murine schistosomiasis is best characterized as a Th2 response, it also generates Th1-type cytokines like IFN-γ that are tightly constrained. NK-1R and possibly SP are important parts of this regulatory process (11). CD4+ T cells within the schistosome granulomas are the major producers of the IFN-γ (12). Mice with defective NK-1R expression develop schistosome granulomas with both impaired IFN-γ and IgG2a secretion (13). This observation attests to the importance of NK-1R in controlling IFN-γ circuitry. Additional in vitro and in vivo studies using highly selective NK-1R antagonists confirm the importance of NK-1R (14). SP regulates T cell IFN-γ production through interaction with the NK-1R expressed on these cells (15). Two T cell-selective, NK-1R expression models demonstrate that it is the T cell NK-1R which directly governs the IFN-γ response (15).

Humans can develop an immunologic disease associated with chronic, destructive inflammation of the intestines called inflammatory bowel disease (IBD). In human IBD, there is increased NK-1R mRNA expression in the tissue, which associates with mucosal T cells (16, 17).

Murine models of human IBD suggest that NK-1R helps regulate intestinal inflammation. Treatment with an NK-1R antagonist limits the inflammation in trinitro- or dinitrobenzene sulfonic acid-induced colitis (18, 19). The IL-10-deficient mouse spontaneously develops intestinal inflammation that...
worsens as the animal ages. NK-1R antagonists suppress intestinal IFN-γ production and inhibit the ongoing colitis (20). Another recently discovered molecule belonging to the tachykinin family of neuropeptides called hemokinin (HK) 1 derives from a protein named preprotachykinin (PPT) C (21–23). It is a product of a gene (Tac4) distinct from that of SP. Murine SP and HK are both 11 aa long and share 55% homology. HK has similar pharmacological properties as SP with regard to receptor interaction. There is PPT C mRNA, as determined by RT-PCR, in mouse pre-B cells obtained from bone marrow, and it may help control B lymphopoesis (22). We followed this lead to determine whether HK could have wider actions in the immune system. In this communication, we report that both HK and SP are expressed at sites of chronic inflammation. They share cell origin, receptor, and immune regulatory function. Thus, there are two distinct but functionally overlapping tachykinins that govern inflammation via high-affinity interaction with NK-1R.

Materials and Methods

**Mice and infection**

CBA/J and C57BL/6 wild-type (WT) mice (The Jackson Laboratory, Bar Harbor, ME) were used throughout this study. Also used were C57BL/6 IL-10−/− mice that were bred and maintained at the University of Iowa. Some mice were infected s.c. with 35 cercariae of the parasite S. mansoni (24). Mice were sacrificed at 8 wk of infection.

**Colitis induction**

To induce colitis in IL-10−/− mice, mice 4–5 wk of age were given piroxicam (Sigma-Aldrich, St. Louis, MO) mixed into their feed (National Institutes of Health-31M) for 2 wk. They received 60 mg of piroxicam/g of food in 50 g/mg streptomycin (complete medium; Sigma-Aldrich), anti-Thy 1.2-FITC (TS; Sigma-Aldrich), anti-CD19-FITC (BD Biosciences, Mountain View, CA).

**Isolation of T cells, macrophages, and B cells**

T cells and B cells were isolated from dispersed granuloma cells using paramagnetic beads coated with Thy 1.2 or E220 mAb (Dynabeads M-450; Dynal, Lake Success, NY) as suggested by the manufacturer.

Macrophage/monocyte populations were isolated from dispersed cells also using the Dynabead method. Dispersed cells were exposed to rat IgG anti-F4/80 mAb (MACP 497; Serotec, Raleigh, NC) at 1 µg/106 cells for 1 h on ice. The cells then were washed three times in complete medium, and magnetic beads (Dynal) coated with sheep anti-rat IgG were added for cell isolation. Flow analysis confirmed the adequacy of all separations (>95%).

**Cell lines**

D 1.1 (Th1 cell line) was from Dr. A. K. Abbas (Harvard University, Cambridge, MA). Also examined were Th2 cell line D10.G4.1, B cell lines 38C13 and CH12.LX, and macrophage cell lines RAW 264.7 and TIB192 (American Tissue Culture Collection, Manassas, VA).

**Cell culture**

Cells were cultured for 24 h in 96-well microtiter plates (Corning, Cambridge, MA) with 200 µl of medium (2 × 105 cells/well) at 37°C to measure IFN-γ secretion. The culture medium was RPMI 1640 containing 10% FCS, 25 mM HEPES buffer, 2 mM l-glutamine, 5 × 10−5 M 2-ME, 1 mM sodium pyruvate, 100 U/ml penicillin, 5 mg/ml gentamicin, and 100 mg/ml streptomycin (all Life Technologies). The cells were cultured alone or with soluble egg Ag (SEA) at 0.3 ng/ml. The SEA was made as described elsewhere (18). Also, some cultures contained SP (Sigma-Aldrich), HK (Bachem, Torrance, CA), and/or the NK-1R inhibitor SR 140333 (Sanofi Recherche, Montpellier, France).

**Cytokine assay**

IFN-γ was quantified using a two-sandwich ELISA. IFN-γ was captured with HB170 (American Type Culture Collection) and detected with a rabbit polyclonal anti-INF-γ antiserum (Dr. M. Wilson, University of Iowa, Iowa City, IA) followed by application of biotinylated goat anti-rabbit mAb (Accurate Chemical and Scientific, Westbury, NY). The sensitivity of the IFN-γ ELISA was 30 pg/ml. The ELISA used streptavidin-peroxidase conjugate and ABTS substrate (Zymed Laboratories, San Francisco, CA).

**Flow cytometric analysis**

Granulomas or LPMC were washed twice and adjusted to 107 cells/ml in FACS buffer (HBSS containing 20 µM HEPES, 10% FCS, and 0.02% sodium azide). The cell suspensions then were dispersed into microcentrifuge tubes each containing 106 cells in 100 µl of FACS buffer and stained with saturating amounts of conjugated mAb for 30 min at 4°C. Following staining, cells were washed twice and resuspended for analysis on a BD FACS 440 flow cytometer (BD Biosciences, Mountain View, CA).

**RNA extraction and RT-PCR assay**

The total cellular RNA was extracted using guanidinium/acid-phenol (25). The RNA was quantified spectrophotometrically and checked for intact 18S and 28S bands by gel electrophoresis. Also, samples were compared for content of hypoxanthine phosphoribosyltransferase (HPRT) to further confirm equivalent mRNA content and reverse transcription. Reverse transcriptase reactions were performed for 2 h at 42°C using 5 µg of RNA, 400 U of Moloney murine leukemia virus reverse transcriptase, and 0.5 µg of 18-mer oligo(dT) for random priming all in a total volume of 40 µl.

PCR was performed using a Robocycler 40 (Stratagene, Menlo Park, CA) in a total volume of 50 µl using 3 µl of TaqDNA polymerase and a primer pair specific for a 330-bp fragment of mouse PPT C mRNA that spanned over intron 1–3 of mouse Tac. The primers for HK1 PCR were: sense, 5'-AAGCTTGGTTGGCTAGACAG-3' and anti-sense, 5'-AGATGCTACAGCTGGTGGAGTG-3'. PCR was performed at 1.2 mM Mg2+.

Temperature conditions
were denaturing at 93°C for 60 s, annealing at 65°C for 85 s, and extension at 72°C for 72 s. Each tube contained 5 μl each of 2 mM dNTP, 1.5 mM Mg2+ in 10X PCR buffer, 1.5 U TaqDNA polymerase, and 10 pM of both primers in a total volume of 50 μl. The PCR sequence was 93°C for 60 s to denaturing, 60°C for 80 s to anneal, and 72°C for 80 s to extend. HPRT primers were: sense, 5’-GTTGGATACAGGCCAGACTTTGT TG-3’ and antisense, 5’-GAGGTTGAGGCTGGGCTATGGCT-3’. The PCR cycling sequence was 93°C for 60 s to denaturing, 60°C for 80 s to anneal, and 72°C for 80 s to extend. All PCR were repeated for 40 cycles.

Statistical analysis

Data are means ±SE of multiple determinations. Difference between two groups was compared using Student’s t test. Values of p < 0.05 were considered to be significant.

Results and Discussion

PPT C mRNA is expressed at sites of inflammation

PPT A mRNA encodes for SP, whereas PPT C encodes for HK. LPMC from the intestine of WT mice or LPMC from IL-10−/− animals with or without colitis express PPT A transcripts. Also, granulomas from mice with schistosomiasis contain PPT A mRNA (6). Experiments determined whether LPMC or granuloma cells expressed PPT C mRNA. Also studied were dispersed bone marrow cells, which are a known source of PPT C transcripts (22).

RNA was extracted from freshly dispersed LPMC, liver granulomas, and bone marrow cells. A PCR assay readily detected PPT C transcripts in LPMC RNA from either WT or IL-10−/− animals and in RNA from dispersed schistosome liver granulomas (Fig. 1A). The control bone marrow preparation was positive also. The authenticity of the PCR product derived from LPMC and granuloma was confirmed by sequencing (data not shown).

Granulomas are complex inflammatory responses composed of various inflammatory cell types. Previous studies showed that granuloma T cells and macrophages express PPT A mRNA. Therefore, T cells, B cells, and macrophages were isolated from dispersed granulomas to localize the source of the granuloma PPT C transcripts. The cellular preparations were >98% pure as determined by flow analysis. PPT C mRNA was detected in both the T cells and macrophages. B cells were negative. All RNA preparations had comparable quantities of the HPRT housekeeping gene, suggesting that the mature B cells expressed little or no PPT C transcripts compared with the other cell types (Fig. 1B). Several well-characterized murine T cell and macrophage cell lines readily expressed PPT C mRNA (Fig. 2). Both the D1.1 Th1 cell line and the D10 Th2 cell line tested positive. Immature B cell lines also contained PPT C transcripts. Isolated splenic B cells tested positive also.

HK binds to the granuloma T cell NK-1R

SP binds to granuloma T cells via engagement of NK-1R (15). Cells that express NK-1R can be identified by flow cytometry using fluorescently labeled SM-SP (Alexa-SM-SP). Flow analysis of dispersed granuloma cells showed that ~10% of both the CD4+ and CD8+ T cells bound Alexa-SM-SP (Fig. 3). SR 140333 is a potent and highly selective inhibitor of NK-1R. This inhibitor displaced Alexa-SM-SP from the T cells showing that the binding was specific for NK-1R (Fig. 3). HK used at equimolar concentration to that of the inhibitor also displaced Alexa-SM-SP with comparable efficiency. This suggested that HK, like SP, binds to the granuloma NK-1R with high affinity.

SP and HK induce IFN-γ production

Soluble Ags from the schistosome egg (SEA) stimulate splenic T cells to produce IFN-γ. SP enhances T cell IFN-γ production.
In the bone marrow, expression was localized to B cells that were in their early stage of development (22). We detected HK in two immature B cell lines (38C-13 and CH12.LX) and splenic B cells, but not in granuloma B cells. Other studies suggested that HK was an autocrine or paracrine factor, which supports survival and proliferation of early B cells. However, NK-1R \(^{-/-}\) mice are not lymphopenic (29), which suggests that HK is not essential for B cell growth. Granuloma B cells are mature and end-stage differentiated. This may explain why HK was not detected in these cells.

In summary, we have examined HK expression and function at a site of chronic inflammation in murine schistosomiasis. Our results show that HK and SP likely have similar origins and functions in the granulomatous response. This expands the immunoregulatory function of HK from a pre-B cell growth factor to a cytokine involved in the control of chronic inflammation. The presence of HK mRNA in intestinal LPMC from healthy and colitic mice suggests that HK also has immune regulatory functions in other inflammatory states.

References


15. Zhang et al. (22) first described murine PPT C and HK (22). Human PPT C encodes for a peptide with partial homology to murine HK and also for a truncated version (4–11, 26). PCR analysis detected PPT C transcripts in murine bone marrow (22) and in other tissues (i.e., skin, heart) (26), suggesting that it may have several functions in humans and rodents.


