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Vasoactive Intestinal Peptide Modulates Langerhans Cell Immune Function

Sreedevi Kodali,* Wanhong Ding,* Jing Huang,* Kristina Seiffert,* John A. Wagner,† and Richard D. Granstein2*

Epidermal nerves lie in close proximity to Langerhans cells (LC) and are capable of releasing peptides that regulate LC function, including calcitonin gene-related peptide and pituitary adenylate cyclase-activating polypeptide. The neuropeptide vasoactive intestinal peptide (VIP) has also been found in cutaneous nerves and mRNA, for the VIP receptor vasoactive intestinal peptide receptor type 1, and vasoactive intestinal peptide receptor type 2 have been found in murine LC and the LC-like cell line XS106. We examined the effects of VIP on LC function and cutaneous immunity. VIP inhibited elicitation of a delayed-type hypersensitivity response in previously immunized mice by epidermal cells enriched for LC content pulsed with Ag in vitro. VIP also inhibited the ability of unseparated epidermal cells to present Ag to a T cell clone and hybridoma and the ability of highly enriched LCs to present to the T cell clone. Inhibition of presentation to the hybridoma was observed with an antigenic peptide that does not require processing, suggesting that VIP is active at a step independent of Ag processing. To elucidate the mechanism(s) by which VIP may mediate these effects, we determined the effects of VIP on LC cytokine production using the XS106 cell line as a surrogate for LC. VIP augmented the production of the IL-10 in LPS-stimulated XS106 cells while down-regulating IL-12 and IFN-γ.

Previous evidence has shown that cutaneous nerves lie in close proximity to Langerhans cells (LC),3 potent dendritic APCs residing within the epidermis. Furthermore, it has been demonstrated that these nerves are capable of releasing peptides that regulate LC function (1). Several neuropeptides, including calcitonin gene-related peptide, α-melanocyte-stimulating hormone, and pituitary adenylate cyclase-activating polypeptide (PACAP), have been shown to inhibit LC immune function (1–6).

In vivo, both calcitonin gene-related peptide and PACAP inhibited the induction of contact hypersensitivity and elicitation of delayed-type hypersensitivity (DTH) (1, 3, 6). In vitro, they inhibited LC Ag presentation function and modulated LC cytokine production (1, 2, 6). α-Melanocyte-stimulating hormone inhibits the induction and elicitation of contact hypersensitivity and induces hapten-specific tolerance (5). Due to the inhibitory effects of these peptides, there may be great therapeutic potential for the use of these factors in inflammatory skin diseases. Understanding how neuropeptides regulate LC function should further understanding of dermatological disease and cutaneous immunity.

We studied the neuropeptide vasoactive intestinal peptide (VIP), a 28-aa peptide that is part of a larger family that includes PACAP, glucagon, secretin, and growth hormone-releasing hormone. In fact, VIP and PACAP 27 share 68% sequence homology and bind to an overlapping group of receptors. Two of these, vasoactive intestinal peptide receptor type 1 (VPAC1) and VPAC2, bind VIP and PACAP with equal affinity. They are both G protein-coupled receptors that activate adenylate cyclase with consequent stimulation of cAMP production (7, 8). Although first isolated in the porcine duodenum (hence its name) (9), VIP has since been found in multiple organs, including the central and peripheral nervous system, endocrine system, reproductive system, and immune system. VIP plays a variety of roles, including stimulation of exocrine secretions, hormone release, and muscle relaxation (8, 10). More recently, the role of VIP in immune regulation has been studied. Work by other groups has shown that VIP and PACAP have a predominantly immunosuppressive effect on peritoneal macrophages through regulation of cytokine production and cell surface maker expression (11, 12). When injected into mice, VIP and PACAP also play a protective role in a murine model of endotoxin-induced sepsis (13). In the larger picture, VIP and PACAP are thought to suppress Th1-mediated responses and drive the immune system toward Th2-mediated processes (14, 15).

Because VIP has been localized in cutaneous nerves (16, 17) and LC and the LC-like cell line XS106 express mRNA for the VIP receptors VPAC1 and VPAC2 (18), we hypothesized that VIP may modulate LC immune function. To our knowledge, the effects of VIP on LC have not been explored and only little is known of the effects of VIP on other dendritic cells. In this study, we demonstrate that VIP reduces the ability of LCs both to elicit an immune response and to present Ag to T cells. These effects may be...
caused, in part, by a reduction of IL-1β and IL-12 production and an increase in IL-10 production.

Materials and Methods
Reagents
VIP and VIP,10-28 (an inactive VIP fragment) (19, 20) were purchased from Peninsula Laboratories (San Carlos, CA). Recombinant murine IL-12 and IL-1β were obtained from BD Pharmaning (San Diego, CA), as was mononclonal anti-I-A, 

The VPAC1 agonist (Lys3,Arg6,Leu7) VIP (I–7)/growth hormone releasing factor (8–27), the VPAC1 antagonist Ac His1(D-Phc2,Lys4,Arg6,Leu7) VIP (3–7)/growth hormone releasing factor (8–27), and the VPAC2 antagonist PG 99–465 were the generous gifts of F. Gregoire (University of Brussels, Brussels, Belgium).

Mice
Six- to 12-wk-old female BALB/c (H-2b) and CAF 1 (H-2d/a) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were kept in the animal facility of Weill Medical College of Cornell University on a 12-h light/dark cycle.

Media and cell lines
Complete medium (CM) consisted of RPMI 1640 (Cellgro, Herndon, VA), 10% FCS (Gemini Bio-Products, Woodland, CA), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 mM nonessential amino acids, 0.1 mM essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, and 10 mM HEPES buffer (all CM components from Mediatech, Herndon, VA).

The XS106 cell line was the generous gift of A. Takashima (University of Texas, Southwestern Medical Center, Dallas, TX). It is a LC-like cell line derived from neonatal A/J epidermis. XS106 cells are dendritic in nature, capable of Ag presentation, and have many phenotypic characteristics of LC (21, 22). XS106 cells were grown in CM with the addition of 0.5 ng/ml murine rGM-CSF (Chemicon International, Temecula, CA), 10% NS cell supernatant (supernatant conditioned by a fibroblast-like cell line), and 5% of the growth of epidermal APC-derived cell lines (23), and 5 × 10−3 M 2-ME (Sigma-Aldrich, St. Louis, MO).

The HDK-1 cell line, a keyhole limpet hemocyanin (KLH)-specific, I-A, -restricted Th1 clone, was also the gift of A. Takashima (24). It was maintained in CM supplemented with 5 × 10−3 M 2-ME and 10 ng/ml murine IL-2 (Chemicon International).

The S1509a cell line, a methylcholanthrene-induced fibrosarcoma line derived from A/J mice, was the generous gift of M. Greene (University of Pennsylvania, Philadelphia, PA) (25). S1509a cells were grown in CM.

The DOll.1 cell line, a T cell hybridoma responsive to chicken OVA (cOVA), was the generous gift of P. Marrack (National Jewish Medical and Research Center, University of Colorado, Denver, CO) (26). These cells were grown in S-MEM (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin (Sigma-Aldrich), 0.1 mM nonessential amino acids, 0.1 mM essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES buffer, and 5 × 10−3 M 2-ME.

Preparation of epidermal cells (ECs), enriched ECs (eECs), and purified LCs (pLCs)
BALB/c ECs were prepared using a modification of a standard protocol (27, 28). The truncal skins were first shaved and chemically depilated. The s.c. fat and carnosus panniculus were removed by blunt dissection. The skins were then floated dermis side down for 45 min in Ca2+/H11001/100 mM 2-ME and 0.38% trypsin (Sigma-Aldrich). Epidermal sheets were collected in PBS containing 0.5 U of dispase/ml (Boehringer Mannheim, Indianapolis, IN) and 0.38% trypsin (Sigma-Aldrich). Epidermal sheets were collected by scraping and dissecting by continuous mild agitation for 20 min in HBSS (Mediatech) supplemented with 2% FCS. The ECs were then filtered through a 40-μm nylon gauge (BD Biosciences, Franklin Lakes, NJ) and washed in CM.

To prepare eECs, ECs were incubated in a 1/2000 dilution of Thy-1.2 Ab (Sigma-Aldrich) for 30 min at 4°C. The cells were then washed twice and incubated in a 1/40 dilution of low toxicity H-rabbit complement (Cedarlane Laboratories, Hornby, Canada) for 30 min at 37°C. The cells were then washed twice in PBS and incubated in PBS with 80 μg/ml DNase I (Sigma-Aldrich) and 0.05% trypsin for 4 min at room temperature. The cells were finally washed in CM. This procedure enriches for LC content by selectively removing epidermal T cells and some keratinocytes. FACS analysis has shown that the resulting population consists of ~12% LC.

To prepare pLCs, eECs were incubated with anti I-A, Ab (BD Pharmingen) at a 1/50 dilution for 30 min at 4°C. They were then incubated with goat anti-mouse IgG conjugated to magnetic microspheres (Dynabeads M-450, Dynal Biotech, Lake Success, NY) for 10 min with continuous gentle agitation. The cells were then washed repeatedly (up to five times) to purify for LC, keeping only the cells that attached to the beads. By FACS analysis (using anti-I-A, mAbs), this procedure yields a cell population of 90–98% LC.

Immunization of mice and elicitation of DTH
In preparation for the experiment, a soluble tumor-associated Ag solution (TAA) was prepared by suspending S1509a cells at 1 × 105 cells/ml in CM. The cells were then freeze thawed (~30°C) four times, and the supernatant was spun twice at 3000 rpm, with the supernatant collected after each spin.

Naïve CAF1 mice were immunized three times at 1-wk intervals with 2 × 106 dead S1509a cells via s.c. injection to the flank. In the fourth week, eECs were prepared and incubated in CM containing 100 nM VIP or CM alone for 3 h. The cells were then washed and incubated with TAA (5 × 105 eEC/ml TAA) or CM with/without 100 nM VIP for 3 h. The cells were then washed again and resuspended in PBS. DTH was elicited by injecting 7.5 × 105 eECs into the left hind footpad of the previously immunized mice. Footpads were measured before injection and then 24 and 48 h after injection using an engineer’s micrometer. The difference before and after injection was used as an indicator of DTH response.

In vitro Ag presentation to T cell clones
For the assay using HDK-1 cells as responders, BALB/c ECs or pLCs were prepared and plated in 96-well plates at 5 × 105 cells/well (ECs) or 1 × 106 cells/well (pLCs) in a total volume of 200 μl. They were then incubated with varying concentrations of VIP for 3 h. After 3 h, the cells were cocultured with KLH (Sigma-Aldrich) at a concentration of 50 μg/ml, still in the presence of VIP. After an additional 3 h, the cells were washed three times with CM to remove soluble VIP and KLH and then cocultured with HDK-1 cells (5 × 105 per well). Supernatants were collected after 72 h and analyzed for IFN-γ production. In experiments examining the possible role of IL-12 or IL-1β in the VIP effect, either IL-12 (500 pg/ml) or IL-1β (10 ng/ml) was added to each culture well at the time of coculture of ECs and HDK-1 cells.

Similarly, for the assay using DO11.1 cells as responders, ECs were plated in 96-well plates at 1 × 105 cells/well. The cells were then treated with VIP for 3 h, after which 0.66 μM cOVA (Pepitides International, Louisville, KY) was added. After an additional 3 h, the cells were washed three times to remove the Ag and VIP. The ECs were then cocultured with 1 × 105 DO11.1 cells/well. Supernatants were collected after 24 h, and IL-2 concentration was measured using an IL-2 ELISA kit (BD Pharmingen).

Cytokine analysis
IFN-γ production by HDK-1 cells was analyzed by sandwich ELISA using purified rat anti-mouse IFN-γ mAb (4 μg/ml), biotinylated rat anti-mouse IFN-γ mAb (1 μg/ml), avidin-HRP (1/1000 dilution), and ABTS substrate read at a wavelength of 405 nm (all reagents BD Pharmingen).

To assess IL-10, IL-12, and IL-1β production, XS106 cells were plated in 12-well plates (1 × 106 cells/ml XS medium). The next day, the medium was changed to CM and the cells were preincubated in varying concentrations of VIP for 3 h, after which they were stimulated with 0.05 μg/ml LPS (IL-10) or 1.0 μg/ml LPS (IL-12, IL-1β) for 24 h. Supernatants were analyzed for cytokine expression using an ELISA kit (BD Pharmingen).

Statistical analysis
The significance of differences among groups in each assay system was measured using the Student’s two-tailed t test for unpaired samples (Excel software; Microsoft, Redmond, WA).

Results
VIP inhibits in vitro Ag presentation to T cell clones
To determine whether VIP affects LC Ag presentation function, murine ECs were preincubated with or without VIP and then treated with Ag KLH. The VIP and KLH were then carefully washed out, and the ECs were cocultured with the KLH-responsive T cell clone HDK-1 for 72 h. IFN-γ production by the T cells was used as a marker of T cell stimulation (29). ECs not treated with KLH induced IFN-γ production in the HDK-1 cells. Addition of KLH stimulated a significant increase in IFN-γ production;
were cocultured with the T cell hybridoma DO11.1. The negative control cOVA323–339 was added. VIP and cOVA323–339 were removed, and the ECs were cocultured with the fragment cOVA323–339, which is presented on the LC MHC receptor without processing (26). As above, murine ECs were pretreated with VIP before addition of cOVA323–339. The ECs were then washed and cocultured with DO11.1 cells, and IL-2 production by the T cells was used as a marker of T cell stimulation (31). When murine ECs were pretreated with VIP before cOVA323–339 addition, there was a dose-dependent decrease in IL-2 production (Fig. 1, lower panel). Thus, VIP inhibits Ag presentation at a step independent of Ag processing.

To determine which receptor(s) was involved in VIP suppression of Ag presentation, we used specific peptide inhibitors of VPAC1 and VPAC2. Murine ECs were preincubated with each peptide for 2 h, followed by addition of VIP for an additional 3 h. Then, the cells were treated with the Ag KLH. The VIP and KLH were then carefully washed out, and the ECs were cocultured with HDK-1 cells for 72 h and measurement of IFN-γ production using ELISA. Mean difference (±SD) between groups is shown. The supernatants were collected after 24 h and analyzed for IL-2 production using ELISA. Mean difference (±SD) between the groups is shown. The data shown are representative of three separate experiments (*, p < 0.05 for N vs O, P, Q, and R; **, p < 0.01 for B vs A, C, D, E, and F; H vs G, I, and J; and N vs M and S).

To test whether VIP inhibits LC Ag processing, we used the cOVA-responsive T cell hybridoma, DO11.1. These cells respond to the fragment cOVA323–339, which is presented on the LC MHC receptor without processing (26). As above, murine ECs were preincubated with VIP before addition of cOVA323–339. The ECs were then washed and cocultured with DO11.1 cells, and IL-2 production by the T cells was used as a marker of T cell stimulation (31). When murine ECs were pretreated with VIP before cOVA323–339 addition, there was a dose-dependent decrease in IL-2 production (Fig. 1, lower panel). Thus, VIP inhibits Ag presentation at a step independent of Ag processing.

Because there are many cell types within the epidermis, we wanted to ensure that VIP was directly affecting LC. Thus, the above experimental model was repeated using pLCs, which contain ~95–98% LC by FACS analysis (data not shown). With (M) was cultured with DO11.1 cells without VIP or cova323–339. Supernatants were collected after 24 h and analyzed for IL-2 production using ELISA. Mean difference (±SD) between the groups is shown. The data shown are representative of three separate experiments (*, p < 0.05 for N vs O, P, Q, and R; **, p < 0.01 for B vs A, C, D, E, and F; H vs G, I, and J; and N vs M and S).
pLCs, we again saw the same dose-dependent inhibition of IFN-γ production with VIP from 0.1 to 1000 nM, also with a plateau in response after 10 nM (Fig. 2). Thus, VIP directly inhibits the ability of LC to present Ag to elicit a T cell response.

To examine whether VIP inhibits the Ag-presenting capability of mature LCs, ECs were cultured for 48 h in the presence of 10 ng/ml GM-CSF, a regimen known to induce maturation of LCs (33), and then tested for the ability of VIP to inhibit their presentation of KLH to HDK-1 cells. VIP indeed inhibited Ag presentation by these cells (Fig. 3), suggesting that VIP can also modulate the function of more mature LCs.

VIP inhibits the elicitation of DTH

Because VIP inhibited the ability of LC to present Ag in vitro, we examined whether a similar inhibition occurred when eliciting an in vivo immune response. Naïve mice were immunized against the S1509a tumor at weekly intervals (see Materials and Methods). eECs (∼12% LC) were prepared and incubated with or without VIP, followed by treatment with TAA, soluble S1509a tumor Ag (see Materials and Methods). The eECs were then washed to remove VIP and TAA and then injected into the hind footpad of mice previously immunized to the S1509a tumor. Footpad swelling compared with baseline was used as a marker of immune response. Mice injected with eECs treated with TAA had a significant swelling compared with mice injected with eECs not treated with TAA. However, eECs preincubated with VIP before TAA addition had a significantly decreased response, ~50% less than the positive control, showing VIP’s inhibition of the elicitation of DTH (Fig. 4). To rule out an effect of VIP on the nonspecific, irritant component of the response, eECs were incubated with VIP, but not treated with TAA. As expected, this group had a baseline level of swelling similar to the negative control described above (Fig. 4). Thus, VIP reduces the elicitation of an immune response in vivo.

FIGURE 2. VIP inhibits the ability of pLCs to present Ag to the Th1 clone HDK-1. Murine pLCs were cultured in CM (B) or CM containing VIP (C–G), followed by addition of the Ag KLH. The VIP and KLH were then removed, and the pLCs were cocultured with the KLH-specific Th1 clone HDK-1. Negative controls (A) were cocultured with HDK-1 cells without treatment with VIP or KLH. Supernatants were analyzed for 72-h IFN-γ production using ELISA. Mean difference (±SD) between the groups is shown. The data shown are representative of three separate experiments (*, p < 0.05 for B vs C; and **, p < 0.01 for B vs A, D, E, F, and G).

FIGURE 3. VIP inhibits the ability of mature LCs to present Ag. Murine ECs were incubated in 10 ng/ml GM-CSF for 48 h. They were then washed and incubated in CM (A and B) or CM containing VIP (C–F) and then exposed to the Ag KLH. Neuropeptide and KLH were then removed, and the ECs were cocultured with the Th1, KLH-specific clone HDK-1. The negative control (A) was not treated with neuropeptide or KLH before coculture with HDK-1 cells. Supernatants were analyzed for 72-h IFN-γ production using ELISA. Mean difference (±SD) between the groups is shown. The data shown are representative of three separate experiments (*, p < 0.05 for B vs A, D, E, and F).

VIP modulates cytokine production in XS106 cells

Clearly, VIP inhibits the ability of LC to effectively elicit a T cell-mediated immune response. To elucidate a mechanism contributing to this phenomenon on a molecular level, we determined the effects of VIP on cytokine production in the LC-like cell line XS106. We chose to focus on IL-1β, IL-12, and IL-10 because all three have been shown to play important roles in LC function and cutaneous immunity. IL-1β and IL-12 are proinflammatory, up-regulating T cell-mediated immune responses, while IL-10 has been shown to be anti-inflammatory in its effects (34–37).

When XS106 cells were treated with LPS, there was a significant increase in IL-1β, IL-12, and IL-10 compared with cells treated with medium alone; however, pretreatment with VIP before and during the period of LPS stimulation inhibited the induction of IL-1β and IL-12 in a dose-dependent manner (Fig. 5). In contrast, VIP augmented the LPS-induced stimulation of IL-10 production (Fig. 5). These results cannot be accounted for by VIP-induced cell death, as trypan blue staining indicated no increase in cell death with VIP. In addition, cell death would be expected to either increase or decrease all levels of cytokines, not specifically increase one while decreasing others. Thus, VIP modulates cytokine production in XS106 cells.

These findings suggest that changes in cytokine expression induced by VIP in dendritic cells might be responsible, at least in part, for the VIP effect on Ag presentation. To examine this possibility, IL-12 or IL-1β was added to tissue culture wells of VIP-treated ECs used to present KLH to HDK-1 cells. Addition of IL-12 (500 pg/ml) fully restored the response of HDK-1 cells to VIP-treated ECs (Fig. 6). Addition of IL-1β (10 ng/ml) had no effect at the dose used (data not shown).

Discussion

We have described a novel role for VIP as an inhibitor of LC immune function. We demonstrated that in vitro, VIP inhibits the ability of murine ECs and pLCs to present Ag to elicit a Th1-mediated immune response. A similar effect was also shown in vivo, in which VIP inhibited the ability of murine eECs to elicit a
DTH response in previously immunized mice. We believe that VIP may mediate its effects, at least in part, through its modulation of cytokine production. In the XS106 LC-like line, VIP suppressed the LPS-stimulated induction of IL-12 and IL-1β while augmenting IL-10 production. Stimulation of XS106 cells by LPS was used as a surrogate for activation of LCs in vivo, and, of course, correlation of our results with cellular and molecular events in vivo must be inferred with caution.

A recent study reported that VIP enhances the Ag-presenting capacity of immature bone marrow-derived dendritic cells, while inhibiting the Ag-presenting capability of mature bone marrow-derived dendritic cells (32). Our findings demonstrate that VIP suppresses the Ag-presenting capacity of freshly obtained LCs, usually thought of as immature. These discordant findings may relate to intrinsic differences between LCs and bone marrow-derived dendritic cells. Alternatively, freshly obtained LCs may be more mature than the cells used in that study.

LC are the only known source of cutaneous IL-1β (38). IL-1β has been shown to be necessary for LC migration to regional lymph nodes to present Ag to T cells (39). Furthermore, IL-1β increases the expression of I-A, B7-2, CD40, and ICAM-1 in LC, all of which are crucial in stimulating T cells (40). At the T cell level, IL-1β promotes T cell proliferation by up-regulating T cell production of IL-2 and IL-2R (41). It is unclear whether LC production of IL-1β acts in an autocrine fashion on the LC, on the T cells, or both. By down-regulating IL-1β production in LC, VIP may contribute to an anti-inflammatory milieu within the skin. However, at the concentrations examined, addition of IL-1β failed to restore the ability of VIP-treated ECs to present KLH to HDK-1 cells.

The cytokine IL-12 is an important positive regulator of Th1 immune responses and augments IFN-γ production by T cells and induces proliferation of activated T cells (42). IL-12 is composed of two subunits, p35 and p40, which together make the biologi-
Periferal T lymphocytes were analyzed for 72-h IFN-γ-treated with neuropeptide or KLH before coculture with HDK-1 cells. Supernatants from the HDK-1 were then removed, and the ECs were cocultured with the Th1, KLH-specific clone HDK-1. At the time of coculture, IL-12 (500 pg/ml) was added to wells of B, E, and F. The negative controls (A and D) were not treated with neuropeptide or KLH before coculture with HDK-1 cells. Supernatants were analyzed for 72-h IFN-γ production using ELISA. Mean difference (±SD) between the groups is shown. The data shown are representative of three separate experiments (*, p < 0.01 for C vs A, B, and D; and not significant for C vs E and F).

**FIGURE 6.** IL-12 restores the Ag-presenting capability of VIP-treated EC. Murine ECs were incubated in CM (A, B, C, and F) or CM containing VIP (D and E) and then exposed to the Ag KLH. Neuropeptide and KLH were then removed, and the ECs were cocultured with the Th1, KLH-specific clone HDK-1. At the time of coculture, IL-12 (500 pg/ml) was added to wells of B, E, and F. The negative controls (A and B) were not treated with neuropeptide or KLH before coculture with HDK-1 cells. Supernatants were analyzed for 72-h IFN-γ production using ELISA. Mean difference (±SD) between the groups is shown. The data shown are representative of three separate experiments (*, p < 0.01 for C vs A, B, and D; and not significant for C vs E and F).

Cytokine production and inhibit Th1 cytokine production in Ag-stimulated macrophages and macrophage cell lines.

References


