Vasoactive intestinal peptide modulates proinflammatory mediator synthesis in osteoarthritic and rheumatoid synovial cells

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Objective. Vasoactive intestinal peptide (VIP) has demonstrated beneficial effects in several murine models of immune-mediated inflammation by inhibiting both the inflammatory and the autoimmune components of the disease. We investigate its potential to modulate the release of proinflammatory cytokines and chemokines by human synovial cells from patients with rheumatoid arthritis (RA).

Methods. Fresh suspensions of synovial tissue cells (STC) or cultured fibroblast-like synoviocytes (FLS) were obtained from patients with RA or osteoarthritis (OA). The effects of VIP on basal or tumour necrosis factor α (TNF-α)-stimulated production of CCL2 (MCP-1, monocyte chemotactic protein 1), CXCL8 (interleukin (IL)-8), IL-6 and TNF-α were studied by specific ELISAs (enzyme-linked immunosorbent assays). The mRNAs for CCL2, CXCL8 and IL-6 in FLS were analysed by real-time reverse transcription–polymerase chain reaction.

Results. VIP at 10 nM down-regulated chemokine production by STC and FLS from RA and OA patients. VIP also down-regulated the expression of mRNAs for CCL2, CXCL8 and IL-6. The effects of VIP were more clearly detected in RA samples and after stimulation with TNF-α.

Conclusion. Our observations confirm that the proposed anti-inflammatory actions of VIP in murine models also apply to human synovial cells ex vivo. Further studies are encouraged to evaluate the use of VIP as a potential therapy for chronic inflammatory joint diseases.

Key words: VIP, Rheumatoid arthritis, CXCL8, CCL2, TNF-α, IL-6, Synoviocytes.
proinflammatory cytokines by synovial cells from OA and RA patients.

Materials and methods

Patients and tissue samples
Synovial tissue was obtained from six patients with RA and three patients with osteoarthritis (OA) at the time of knee prosthetic replacement surgery. All RA patients fulfilled the American College of Rheumatology 1997 criteria for the diagnosis of RA [15]. The study was performed according to the ethics recommendations of the Declaration of Helsinki and was approved by the Ethics Committee of Clinical Investigation of the Hospital 12 de Octubre. Fibroblast-like synoviocyte (FLS) cultures were established from homogenized synovium in 10% FCS-DMEM (fetal calf serum–Dulbecco’s Modified Eagle Medium) [16]. FLS cultures were used between passages 3 and 6. Freshly obtained total synovial tissue cells (STC) were also prepared from three patients with RA and three patients with OA by collagenase digestion of synovial tissue, and were maintained in RPMI medium (Life Technologies, Paisley, UK) [9]. FLS or STC were stimulated with 10 nM TNF-α (Genzyme, Cambridge, MA, UK) in the presence or absence of different concentrations of VIP (Neosystem, Strasbourg, France) for 24 h. Culture supernatants were harvested and stored at −20 °C for determination by enzyme-linked immunosorbent assay (ELISA). RNA was also obtained from FLS cultures (1 × 106 cells/ml) by using the Ultraspec RNA Isolation System (Biotecx Laboratories, Houston, TX, USA) according to the manufacturer’s instructions. RNA was resuspended in DEPC (diethyl pyrocarbonate)-treated water and quantitated spectrophotometrically at 260/280 nm.

Cytokine determination: ELISA assay
The amounts of IL-6, TNF-α, CCL2 (MCP-1, monocyte chemotactic protein 1) and CXCL8 (IL-8) in the supernatants of cell cultures were determined with a human capture ELISA assay. Briefly, a capture monoclonal anti-human IL-6, TNF-α, CCL2 or CXCL8 antibody (Pharmingen, Becton Dickinson Co, San Diego, CA, USA) was used to coat microtitre plates (ELISA plates; Corning Inc., New York, USA) at 2 μg/ml at 4°C for 16 h. After washing and blocking with phosphate-buffered saline (PBS) containing 3% bovine serum albumin, culture supernatants were added to each well for 12 h at 4°C. Unbound material was washed off and a biotinylated monoclonal anti-human IL-6, TNF-α, CCL2 or CXCL8 antibody (Pharmingen) was added at 2 μg/ml for 45 min. Bound antibody was detected by addition of avidin–peroxidase for 30 min followed by addition of the ABTS substrate (Sigma Chemicals Co., St Louis, MO, USA). Absorbances at 405 nm were taken 20 min after the addition of substrate. A standard curve was constructed using various dilutions of human recombinant rIL-6, rTNF-α, rCCL2 or rCXCL8 in PBS containing 10% fetal bovine serum. The amounts of cytokines in the culture supernatants were determined by extrapolation of absorbances to the standard curve. The intra-assay and inter-assay variability for cytokine determination was < 5%.

Real-time quantitative reverse transcriptase–polymerase chain reaction
Quantitative polymerase chain reaction (PCR) analysis was performed using the SYBR® Green PCR Master Mix and reverse transcription (RT)-PCR kit (Applied Biosystems, Foster City, CA, USA). Reactions were performed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Reactions were performed in 20 μl with 50 ng RNA, 10 μl 2 × SYBR Green PCR Master Mix, 6.25 U MultiScribe reverse transcriptase, 10 U RNase inhibitor and 1 μM primers. The following sequences of primers were used: human β-actin, sense 5’-AGAAGGATCTCTATGTGGGCCG-3’, antisense 5’-CATGTCGCTCCAGTTTGTA-3’; human IL-6, sense 5’-GTGGCTGACAGCATGACCA-3’, antisense 5’-TGAGTGCCCATGCTACATT-3’; human CCL2/MCP-1, sense 5’-ACTCTGGCCTCCAGCAT-3’, antisense 5’-TTGATTTGATCTGCTAGGC-3’; human CXCL8/IL-8, sense 5’-AAGAGCGAGAAGAACCCACC-3’, antisense 5’-CTCACAATTTTCTGAGGAGTC-3’. The GenBank accession numbers and numbers for the 5’ and 3’ ends of the nucleotides for the PCR products are as follows: β-actin, E00829, 218–298; IL-6, NM00600, 614–714; CCL2/MCP-1 NM002982, 57–157; CXCL8, NM000584, 53–153. The amplification conditions were an initial 30 s at 48°C, 10 min at 95°C, 40 cycles of denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min.

For relative quantitation we used a method which compared the amount of target normalized to an endogenous reference. The formula used was 2−ΔΔCt, representing the n-fold differential expression of a specific gene in a treated sample compared with the control sample, where Ct is the mean of threshold cycle (cycle at which the amplification of the PCR product is initially detected). ΔCt was the difference in the Ct values for the target gene and the reference gene, β-actin (in each sample assayed), and ΔΔCt represents the difference between the ΔCt from the control and each datum. Before using this method, we performed a validation experiment comparing the standard curve of the reference and the target to demonstrate that efficiencies were approximately equal [17]. The correct size of the amplified products was checked by electrophoresis.

Statistical analysis
All data were expressed as mean ± S.E.M. Multiple-sample comparison (analysis of variance) was used to test differences between groups for significance. A value of P < 0.05 was considered to be significant. The program Statgraphics plus 5.0 (Statistical Graphics, Rockville, ND, USA) was used for all statistical calculations.

Results
In this study we used two different kind of samples: STC from homogenized synovium, which contains a heterogeneous population of cells (T cells, macrophages and fibroblasts) to study the local total effect of VIP in the joint, and FLS to elucidate the contribution of joint fibroblasts in the therapeutic effect of VIP.

VIP dose–response effects
In preliminary experiments, and in order to establish the optimal conditions for the study, we measured the VIP and TNF-α concentrations required to modify IL-6 release in FLS supernatant cultures from OA patients. Several concentrations and times of TNF-α stimulation were tested, and the results showed optimal induction of IL-6 after 18 h of treatment with 10 nM TNF-α. Under these conditions, the IL-6 level increased from 17 pg/ml (basal value) to 1100 pg/ml (TNF-α-stimulated value). FLS were also treated simultaneously with several concentrations of VIP. As shown in Fig. 1, VIP decreased the stimulated level of IL-6 in a dose-dependent manner between 1 and 100 nM. Similar data were observed in STC cultures from OA patients (data not shown). We selected 10 nM VIP for the rest of the studies as a concentration more relevant to the potential human pharmacological use of VIP [18].
Effect of VIP on the production and expression of chemokines

Synovial tissue cells from OA and RA patients displayed similar levels of the chemokines CCL2 and CXCL8, whereas RA FLS supernatants contained greater amounts of both chemokines. The effects of TNF-α on chemokine release in samples from OA patients showed pronounced differences between FLS and STC, the former having low basal release and high TNF-α inducibility, and the latter having high basal release and no significant inducibility. In samples from RA patients, these differences between FLS and STC were not apparent (Figs 2 and 3). VIP significantly decreased the basal and TNF-α-stimulated CCL2 levels in RA FLS or STC cultures, whereas in OA a statistically significant decrease was only observed for TNF-α-stimulated STC.

VIP significantly decreased CXCL8 levels in OA and RA FLS stimulated by TNF-α but not in unstimulated FLS (Fig. 3). A strong and significant effect was also observed in RA but not OA STC.

We next tested the expression of CCL2 and CXCL8 mRNA by real-time RT-PCR in FLS from OA and RA patients. Constitutive CCL2 and CXCL8 mRNA expression was similarly detected in OA and RA FLS cultures; in both cases the expression was strongly induced by stimulation with TNF-α (Fig. 4). VIP significantly decreased TNF-α-induced CCL2 and CXCL8 mRNA expression in RA. In OA, a similar effect, but of smaller magnitude, was detected. Basal expression was minimally modified in all cases, only reaching statistical significance for CXCL8 in TNF-α-stimulated RA FLS.

Effect of VIP on the production and expression of proinflammatory cytokines

Next, we studied IL-6 and TNF-α as proinflammatory cytokines. IL-6 was similarly studied in all OA and RA cell samples, but FLS cultures did not produce TNF-α (data not shown), so that we determined its expression only in STC. Exogenous TNF-α was not detected at 24 h, when supernatants were collected for analysis in FLS, which did not release TNF-α in either non-stimulated or TNF-α-stimulated conditions.

FIG. 1. Dose–response effect of different concentrations of VIP on TNF-α-stimulated IL-6 protein in FLS cultures from OA patients. The graph represents IL-6 values in basal and TNF-α-stimulated conditions. Each result is the mean ± S.E.M. of three separate experiments performed in triplicate. *P < 0.05, comparing TNF-α-stimulated values of cells grown in the absence or presence of increasing concentrations of VIP.

FIG. 2. Effect of 10 nM VIP on CCL2 production by FLS and STC cultures from OA and RA patients in the basal condition and after stimulation with 10 nM TNF-α. Each result is the mean ± S.E.M. of three separate experiments performed in triplicate. *P < 0.05, **P < 0.01, comparing basal values of cells grown in the absence or presence of VIP, and the same comparison for TNF-α-stimulated values.
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**Fig. 3.** Effect of 10 nM VIP on CXCL8 production by FLS and STC cultures from OA and RA patients in the basal condition and after stimulation with 10 nM TNF-α. Each result is the mean ± S.E.M. of three separate experiments performed in triplicate. *P < 0.05, **P < 0.01, ***P < 0.001, comparing basal values of cells grown in the absence or presence of VIP, and the same comparison for TNF-α-stimulated values.

**Fig. 4.** Effect of 10 nM VIP on mRNA expression for CCL2 and CXCL8 chemokines in FSL cultures from OA and RA patients in the basal condition and after stimulation with 10 nM TNF-α. The mRNA expression for these chemokines was measured by quantitative real-time RT-PCR and corrected for mRNA expression for β-actin for each sample (see Materials and methods). Each result is the mean ± S.E.M. of three separate experiments performed in duplicate. **P < 0.01, ***P < 0.001, comparing basal values of cells grown in the absence or presence of VIP, and the same comparison for TNF-α-stimulated values.
Induced increase of TNF-α was observed only in RA samples, and VIP significantly decreased induced but not basal levels of this cytokine (Fig. 5). In OA cells, although no clear induction was detected, VIP decreased TNF-α levels only in TNF-α treated cells.

IL-6 protein production was minimally modified by TNF-α treatment. A trend towards less production was observed in VIP-treated OA and RA samples, but it did not reach statistical significance in most cases (Fig. 6). However, IL-6 mRNA was readily induced by TNF-α treatment and VIP treatment decreased OA-induced and RA basal IL-6 mRNA levels in FLS (Fig. 7).

**Discussion**

Cytokine and chemokine networks have been shown to play a key role in the maintenance of chronic synovial inflammation in RA and to a lesser degree in OA [2]. Cytokine-targeted therapies have demonstrated a remarkable impact in the course of several forms of chronic arthritis, which confirms the relevance of these factors. Endogenous neuropeptides and hormones, such as calcitonin gene-related peptide, melanocyte-stimulating hormone, endorphin and enkephalin, which are released in the immune microenvironment, have long been suspected to play a role in inflammatory disorders of
VIP modulates proinflammatory mediators in OA and RA


**Key Messages**

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References


