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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.

Health depends on numerous regulatory interactions between the basic framework constituted by the three systems involved in homeostasis: the nervous, endocrine and immune systems. In chronic inflammatory diseases, disruption of neuroendocrine–immune interactions has been proposed [1, 2]. An important factor in these interactions is the existence in neural and immune cellular elements of common mediators, such as neuropeptides and their receptors, that can participate in the pathogenesis of chronic inflammation [3, 4]. Vasoactive intestinal peptide (VIP) is a neuropeptide that is detected in human serum in the picomolar range [5]. It is released by nerve fibres and lymphocytes in the lymphoid microenvironment and modulates innate and adaptive immunity through cellular signalling mediated by G protein-coupled receptors [6, 7]. In murine experimental disease models, modulation of this pathway has demonstrated a remarkable therapeutic effect in several inflammatory and autoimmune disorders, such as septic shock, TNBS (trinitrobenzene sulphonic acid)-induced colitis, and collagen-induced arthritis [8–10].

Chronic inflammatory rheumatic diseases, such as osteoarthritis (OA) and rheumatoid arthritis (RA), are characterized by the migration of leukocytes into the synovial tissue. Leukocytes and resident cells produce various inflammatory mediators, including cytokines and matrix-degrading enzymes, that perpetuate chronic inflammation and tissue damage. We have recently described the beneficial effects of VIP and its related peptide pituitary adenylate cyclase-activating polypeptide (PACAP) by inhibiting both the inflammatory and the autoimmune component in collagen-induced arthritis (CIA) [9, 11].

The local synthesis of proinflammatory cytokines by inflammatory cells, notably interleukin (IL)-1, IL-6 and tumour necrosis factor α (TNF-α), plays an important role in initiating and perpetuating inflammatory and destructive processes in the rheumatoid joint [12]. Chemokines released by resident synoviocytes are important effectors involved in the recruitment of neutrophils, monocytes and lymphocytes, and play an important role in inflammatory cell infiltration [13]. Although our previous data in murine CIA indicated that VIP inhibits both chemotactic and proinflammatory cytokines [9], its participation in human arthritis has not been elucidated and studies on the role of VIP as an anti-inflammatory agent in humans are scarce [14]. The aim of the present study was to evaluate *in vitro* the potential effects of VIP in the synthesis of chemotactic and

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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 (1×10^6 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 solution (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′-AGAAGGATTCCTATGT GGGCG- 3′, antisense 5′-CATGTCGTCCCAGTTGGTGAC-3′; human IL-6, sense 5′-GTGGCTGCAGGACATGACAA-3′, antisense 5′-TGAGGTGCCCATGCTACATTT-3′; human CCL2/MCP-1, sense 5′-ACTCTCGCCTCCAGCATGAA-3′, antisense 5′-TTGATTGCATCTGGCTGAGC-3′; human CXCL8/IL-8, sense 5′-AAGAGCCAGGAAGAAACCACC-3′, antisense 5′-CGTCAATCTTTTCTGGGAGTCC-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 $2^{-\Delta\Delta Ct}$, 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 $\Delta\Delta$Ct 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 total local 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 CXCL-8 in TNF-α-stimulated RA FLS.

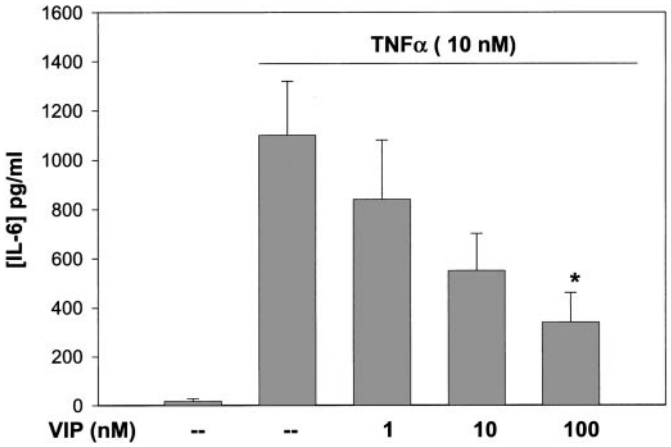

FIG. 1. Dose–response effect of different concentrations of VIP on TNF-α-stimulated IL-6 protein in FLS cultures from OA patients. The graph represents IL-6 values in basal and TNF-α-stimulated conditions. Each result is the mean ± S.E.M. of three separate experiments performed in triplicate. $*P < 0.05$,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

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.

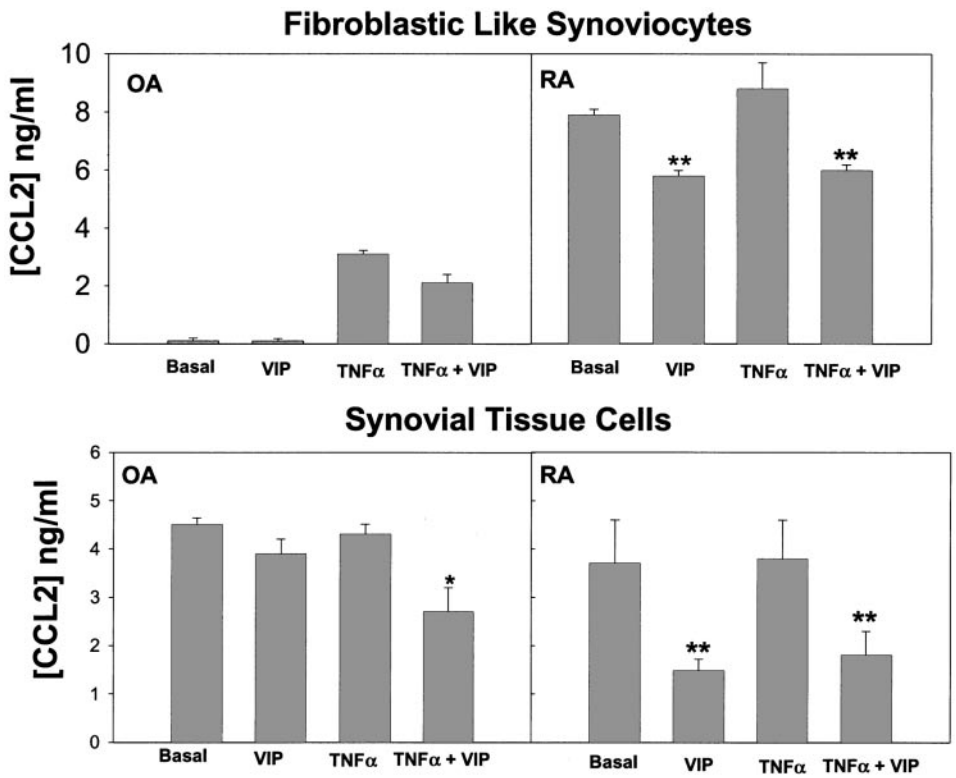

FIG. 2. Effect of 10 nM VIP on CCL2 production by FLS and STC cultures from OA and RA patients in the basal condition and after stimulation with 10 nM TNF-α. Each result is the mean ± S.E.M. of three separate experiments performed in triplicate. $*P < 0.05$, $**P < 0.01$, comparing basal values of cells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

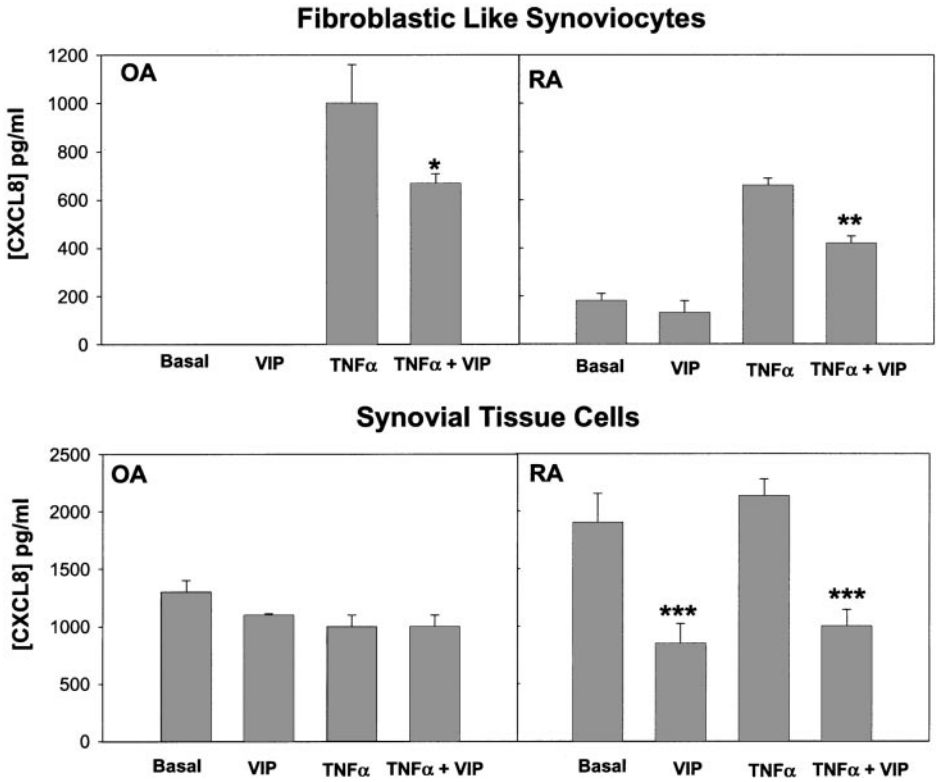

FIG. 3. Effect of 10 nM VIP on CXCL8 production by FLS and STC cultures from OA and RA patients in the basal condition and after stimulation with 10 nM TNF-α. Each result is the mean $\pm$ S.E.M. of three separate experiments performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$,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

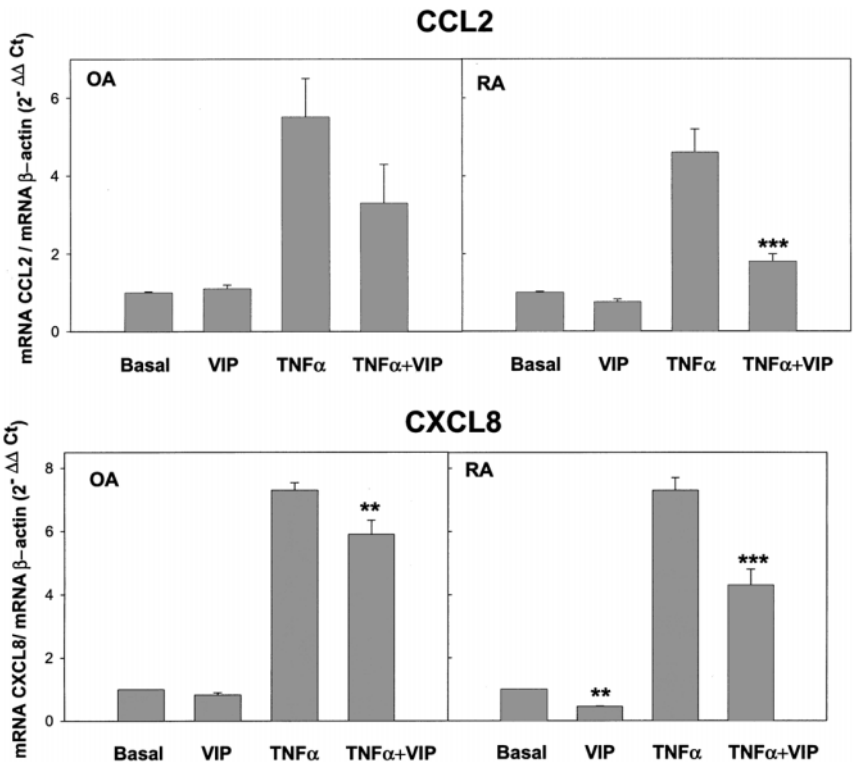

FIG. 4. Effect of 10 nM VIP on mRNA expression for CCL2 and CXCL8 chemokines in FSL cultures from OA and RA patients in the basal condition and after stimulation with 10 nM TNF-α. The mRNA expression for these chemokines was measured by quantitative real-time RT-PCR and corrected for mRNA expression for β-actin for each sample (see Materials and methods). Each result is the mean $\pm$ 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.

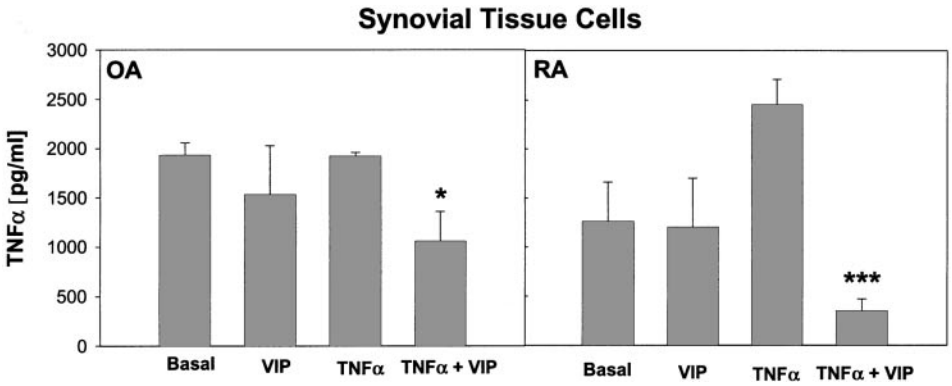

FIG. 5. Effect of 10 nM VIP on TNF-α production by STC cultures from OA and RA patients in the basal condition and after stimulation with 10 nM TNF-α. Each result is the mean $\pm$ S.E.M. of three separate experiments performed in triplicate. $^{*}P < 0.05$, $^{***}P < 0.001$, comparing basal values of cells grown in the absence or presence of VIP, and the same comparison for TNF-α-stimulated values.

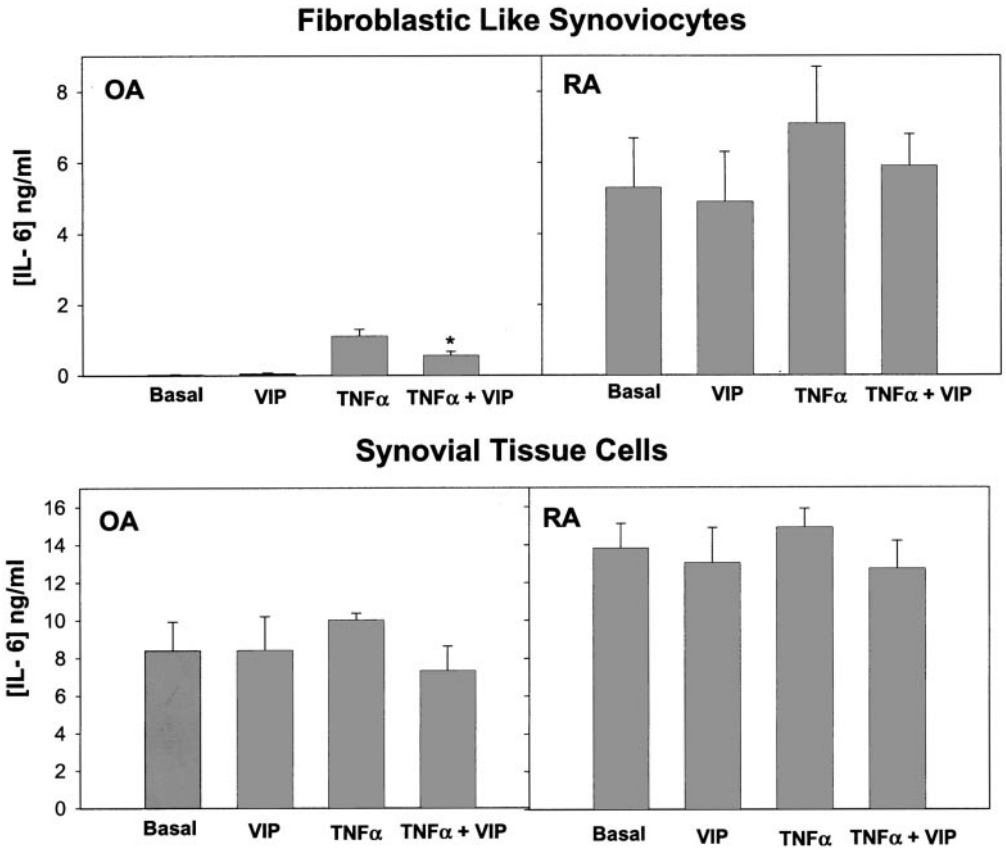

FIG. 6. Effect of 10 nM VIP on IL-6 production by FLS and STC cultures from OA and RA patients in the basal condition and after stimulation with 10 nM TNF-α. Each result is the mean $\pm$ S.E.M. of three separate experiments performed in triplicate. $^{*}P < 0.05$, comparing basal values of cells grown 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

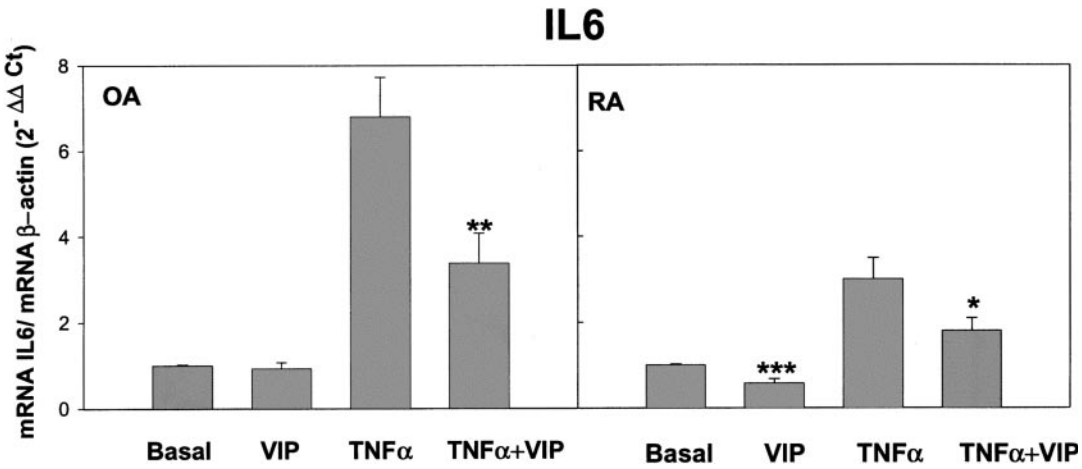

FIG. 7. Effect of 10 nM VIP on mRNA expression for IL-6 in FLS cultures from OA and RA patients in the basal condition and after stimulation with 10 nM TNF-α. The mRNA expression for this proinflammatory cytokine was measured by quantitative real-time RT-PCR and corrected for mRNA expression for β-actin for each sample. Each result is the mean $\pm$ S.E.M. of three separate experiments performed in duplicate. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ comparing basal values of cells grown in the absence or presence of VIP, and the same comparison for TNF-α-stimulated values.

joints [5, 19, 20]. Recently, we have demonstrated that VIP and its structurally related peptide PACAP have important therapeutic effects in murine CIA by down-regulating both the inflammatory and autoimmune components of the disease. In addition, it has been reported that the VIP concentration is elevated in synovial fluid of patients with RA, similarly to other inflammatory diseases, and this phenomenon has been interpreted as a natural down-regulatory mechanism of innate and adaptive immunity [21]. In the joint, VIP can be produced by either sympathetic innervation [22] or lymphoid cells [6]. As innervation is destroyed during pannus formation [22], the elevation of VIP in RA joint should be attributed to lymphoid cells. Previous data from Takeba *et al.* [14] suggest that neuropeptides may have opposite roles, increasing or decreasing the synthesis of cytokines or metalloproteases in RA FLS.

In the present study, we studied for the first time the effect of VIP on STC suspensions in humans. We confirm that VIP can down-regulate the expression and synthesis of chemokines in both STC and FLS from RA and OA patients. In general, the effects of VIP are more clearly detected in RA samples and after stimulation with TNF-α. VIP significantly inhibits the production of CCL2 and CXCL8, both at the basal level and after TNF-α stimulation, in fresh STC suspensions. This effect of VIP on chemokine production could therefore inhibit the entry of neutrophils, monocytes and lymphocytes to the inflamed synovium.

With regard to proinflammatory cytokines, our data demonstrate that, in RA, VIP inhibits the production of TNF-α only after stimulation. RA STC are complex mixtures of T cells, macrophages and fibroblasts that secrete inflammatory cytokines, including TNF-α [23–25]. Interestingly, induction of TNF-α was not detected in OA, suggesting fewer target macrophages in OA synovium samples [26]. In RA, inhibition by VIP of induced TNF-α production may interrupt this feedback circuit, which has been confirmed as a relevant therapeutic target by the clinical use of anti-TNF-α agents. Regarding IL-6, TNF-α induced an increase in its production in both OA and RA FLS, and VIP reduced IL-6 expression in basal conditions in RA and after TNF-α stimulation in OA at the mRNA level. At the protein level, Takeba *et al.* [14] also showed that VIP reduces the TNF-α-induced production of IL-6 by FLS, though this difference can probably be explained by the different protocols used. No significant differences in IL-6 production were detected in STC, which suggests complex regulation of this cytokine in this setting. IL-6 is a multifaceted cytokine that is involved not only in the RA cytokine cascade but also in actions such as the promotion of expansion and activation of T cells, the differentiation of B cells, the regulation of acute-phase protein genes and the regulation of chemokine production [27, 28]. Previous studies in rodents in our laboratory have demonstrated a dual and complex regulation of VIP-mediated IL-6 production [29–31]. Further studies are needed to clarify the effect of VIP on the production of this cytokine in human arthritis.

Collectively, these findings suggest that VIP modulates the production of proinflammatory factors in human synovial cells, and encourage further research on its therapeutic potential in inflammatory joint diseases. VIP seems to exert a more specific effect on RA samples that is, in some cases, reproduced in OA samples pretreated with TNF-α. The relevance of TNF-α in the pathogenesis of RA and the potential ability of VIP to partially neutralize TNF-α effector mechanisms make this peptide particularly attractive as a potential therapy for RA. VIP may exert both local anti-inflammatory and systemic immunoregulatory effects. Therefore, further studies to determine which target is more appropriate in RA are relevant to the choice of VIP as a local or systemic agent. As VIP is a natural protein with well-known physiological effects and its pharmacological application is feasible, properly designed clinical trials may ultimately demonstrate its potential value in the treatment of chronic inflammatory diseases in humans.

Acknowledgements

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Rheumatology	Key Messages
	VIP inhibits the production of proinflammatory products in synovial cells from OA and RA.

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