Lower density of synovial nerve fibres positive for calcitonin gene-related peptide relative to substance P in rheumatoid arthritis but not in osteoarthritis

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Objectives. Sensory nerve fibres (NFs) contain two major neuropeptides, substance P (SP) and calcitonin gene-related peptide (CGRP). The pro-inflammatory role of SP is known, while CGRP has anti-inflammatory activities by inhibiting T helper type 1 cytokines, TNF secretion and leucocyte proliferation. We demonstrated the increase of SP-positive NFs in RA as compared with OA. This study investigated the density of CGRP-positive NFs relative to SP-positive NFs or sympathetic NFs in synovial tissue of patients with RA and OA.

Methods. By immunofluorescent staining of synovial tissue of 25 patients with RA and 35 patients with OA, NFs positive for CGRP, SP and tyrosine hydroxylase (sympathetic NFs) were quantified.

Results. Density of CGRP-positive NFs was higher in OA than in RA, and density of SP-positive NFs tended to be higher in RA. In RA patients, comparison of CGRP-positive and SP-positive NFs in the same synovial tissue demonstrated less CGRP-positive than SP-positive NFs. The ratio of CGRP-positive NFs to SP-positive NFs was lower in RA as compared with OA. In OA, but not in RA, density of CGRP-positive NFs positively correlated with density of sympathetic NFs, which is much lower in RA patients.

Conclusion. The preponderance of SP-positive NFs over CGRP-positive NFs or sympathetic NFs most probably supports the pro-inflammatory process in patients with RA. The reasons for the loss of CGRP in sensory NFs are not known.

Key words: Rheumatoid arthritis, Calcitonin gene-related peptide, SP, Sensory nerve fibre, Sympathetic nerve fibre, Tyrosine hydroxylase.

Introduction

In arthritic inflammation, the importance of sensory nerve fibres (NFs) and their main neurotransmitter, substance P (SP), has been documented [1–4]. SP stimulates many pro-inflammatory aspects such as secretion of TNF, IL-1β, IL-6, IL-8, IL-12, IFN-γ, superoxide anion production and chemotaxis of different cell types [5–12]. Along with SP, calcitonin gene-related peptide (CGRP) is stored in and released from sensory nerve terminals in the periphery, e.g. in the synovium. In contrast to SP, CGRP exerts several anti-inflammatory effects such as inhibition of the oxidative burst in human monocytes [13], inhibition of antigen presentation by human macrophages and Langerhans cells and proliferation of human peripheral blood mononuclear cells at high concentrations [14–16] and inhibition of IL-1β from stimulated Langerhans cells [15]. TNF from murine macrophages [17] and IL-12 and IFN-γ from human monocytes [15, 18]. In addition, CGRP inhibits osteoclast activity and bone resorption [19, 20]. Thus, a possible preponderance of SP over CGRP will probably be a pro-inflammatory signal in arthritis.

In arthritic rats and in patients with chronic RA, SP-positive NFs are somewhat increased as a sign of sensory NF sprouting [21–23]. In contrast, sympathetic NFs are lost in synovial tissue of patients with chronic RA [22, 23]. With respect to CGRP-positive NFs, the situation is not as clear as with SP because either increased or decreased densities of these NFs have been reported by different groups in arthritic rats and patients with RA [3, 21, 24–26]. It might be that the time point of investigation in relation to the phase of arthritis plays an important role because neuronal degeneration and regeneration have been demonstrated for CGRP NFs during the course of inflammation [27, 28]. In these latter studies, a decrease of CGRP-positive NFs was observed in the early phase of inflammation but a regeneration appeared in later phases when the inflammatory process came to a halt [27, 28]. In addition, determination of SP and CGRP in the synovial fluid is most probably not a good indicator for the presence of these NFs because activated non-neuronal cells are able to produce both neuropeptides [29, 30].

In recent years, we applied a laborious quantitative technique in order to measure the density of NFs in synovial tissue, which was adapted from an extensive histological study of RA synovium [31]. The aim of the present study was to apply this technique to the parallel measurement of CGRP-positive NFs together with SP-positive and sympathetic NFs in the material of the same patient. In order to validate our findings, we investigated a large cohort of patients with chronic RA compared with patients with chronic OA.

Patients and methods

Patients

Twenty-five patients with chronic RA and 35 patients with chronic OA, who underwent elective knee joint replacement surgery, were included without further selection. Diagnosis of RA was based on the established ACR criteria [32]. All patients were informed about the purpose of the study and gave written consent according to the Declaration of Helsinki. The study was approved by the Ethical Committee of the University of Regensburg. Clinical and laboratory data for the entire group are presented in Table 1. Variables such as rheumatoid factor, erythrocyte sedimentation rate and C-reactive protein were measured by standard techniques. Histological markers of inflammation (see below for techniques) were used in order to delineate the inflammatory situation in RA patients as compared with OA.
**Synovial tissue preparation, histology, immunohistochemistry and immunofluorescence**

Synovial tissue samples were obtained immediately after opening the knee joint capsule. The preparation of the tissue for histology was as previously described [22, 23]. Briefly, one piece of ~9 cm² of synovial tissue was dissected. Fat tissue and a large number of vessels were removed. Eight ~1 cm² pieces of the same synovial area were used for histology. The samples intended for haematoxylin–eosin (HE) and alkaline phosphatase determinations were immediately placed in protective freezing medium (Tissue-Tek, Sakura Finetek Europe, Zoeterwoude, The Netherlands) and then quick-frozen by floating on liquid nitrogen. The tissue samples used for the detection of NFs were fixed for 12–24 h in phosphate-buffered saline (PBS) containing 4% formaldehyde and then incubated in PBS with 20% sucrose for 12–24 h. The tissue was then bedded in Tissue Tek and quick-frozen. Each patient’s samples of synovial tissue were stored at −80°C.

**Histological evaluation of inflammation** has been described in earlier studies [22, 23]. Briefly, the frozen tissue samples were cut into 6–8 μm thick sections and cell density and lining layer thickness were evaluated using a standard HE staining of ~45 sections. At a magnification of ×400, the extent of the lining layer thickness was determined by averaging the number of cells in a lining layer cross section at nine different locations. The cell density in the synovial tissue was determined by counting all stained cells in 17 randomly selected high-power fields (×400) and expressed per square millimetre. In order to determine the number of T cells (CD3, Dako, Hamburg, Germany), macrophages (CD163, Dako) and capillary vessels (collagen IV, Dako) in the synovial tissue of each patient, eight cryosections were investigated using APAAP staining and the number of identified structures was averaged from 17 randomly selected high-power fields (×400) and expressed per square millimetre.

**Determination of synovial innervation** has been extensively described in earlier studies [22, 23]. Briefly, six to eight 7–9 μm thick cryosections were used for immunofluorescence staining with primary antibodies against tyrosine hydroxylase (TH, the key enzyme for noradrenaline production in sympathetic nerve endings, polyclonal, rabbit, Chemicon, Temecula, USA), SP (polyclonal, rabbit, Chemicon) and CGRP (polyclonal, rabbit, Chemicon). An Alexa Fluor 546-conjugated secondary antibody (monoclonal, mouse anti-rabbit, Molecular Probes, Invitrogen, Leiden, The Netherlands) was used to achieve immunofluorescent staining of these NFs. Figure 1 gives an example of CGRP-positive NFs in synovial tissue of two patients with RA and two patients with OA. The numbers of positive NFs per square millimetre were determined by averaging the number of stained NFs (minimum length 50 μm, determined through a micrometre eyepiece) in 17 randomly selected high-power fields of view (×400). We controlled the positive NF staining by incubating the tissue with respective control antibodies (rabbit immunoglobulin, Sigma, Deisenhofen, Germany), which always yielded a negative result.

**Presentation of data and statistical analysis**

The values are given as mean ± S.E.M. (Table 1). Frequencies in two different groups were compared by the chi-squared test using Yates’ continuity correction or Fisher’s exact test if possible (Table 1). Groups were compared by the Mann–Whitney U-test, and correlations were calculated by Spearman rank correlation analysis (SPSS/PC, V.12.0, SPSS Inc., Chicago, USA). In paired samples of the same patients, a difference was detected using the Wilcoxon signed rank test (SPSS). P < 0.05 was considered to be statistically significant.

**Results**

**Synovial inflammation in patients with RA and OA**

Patients with chronic RA demonstrated a thicker lining layer than did patients with chronic OA (Table 1). RA patients also presented an increased synovial cellular density, more synovial T cells and more synovial macrophages as compared with OA (Table 1). This indicates a more severe synovial inflammation in RA as compared with OA patients.

**Density of SP-positive and CGRP-positive NFs**

In patients with chronic RA, the density of SP-positive NFs tended to be higher as compared with OA, without reaching
the significance level (Fig. 2A). In contrast, density of CGRP-positive NFs was significantly lower in patients with chronic RA compared with chronic OA (Fig. 2B). In a direct comparison of the density of NFs in the same patient, the preponderance of SP-positive NFs over CGRP-positive NFs was obvious in RA patients but not in patients with OA (Fig. 2C and D). In order to delineate this preponderance of SP-positive NFs over CGRP-positive NFs, we calculated the ratio of the density of CGRP-positive NFs to SP-positive NFs (a ratio without a unit). It turned out that this ratio was lower in patients with chronic RA as compared with OA (Fig. 3A). This is due to the fact that CGRP-positive fibres were lower in relation to SP-positive fibres in RA compared with OA patients (Fig. 3A), given that SP-positive fibres did not differ significantly between groups. Since the median of this ratio was 1 in OA patients, this latter patient group demonstrates an even balance of SP-positive and CGRP-positive NFs (Fig. 3A). This was completely different in patients with RA, who presented a median of 0.06, which represents 16.7 NFs positive for SP vs one CGRP-positive NF (Fig. 3A).

Density of sympathetic NFs and CGRP-positive NFs

Neurotransmitters of sympathetic NFs have an anti-inflammatory effect on arthritic inflammation when concentrations of these neurotransmitters are high enough to bind to β2-adrenergic receptors (and A2 adenosine receptors or μ-opioid receptors) [33]. In the investigated cohort of patients with chronic RA, density of sympathetic NFs was markedly lower than in OA patients (Fig. 3B). However, the direct comparison of the density of CGRP-positive NFs and sympathetic NFs did not reveal a significant difference in OA (Fig. 3C) and RA patients (Fig. 3D).

In addition, NF density of CGRP-positive NFs correlated positively with density of sympathetic NFs in OA (Fig. 4A) but not in RA patients (Fig. 4B).

Density of CGRP-positive NFs and parameters of inflammation or medication in RA and OA

In RA patients and OA controls, inflammatory parameters such as lining layer thickness, cellular density, T-cell density, macrophage density, vascularity or daily prednisolone (mg/day) did not correlate with the density of CGRP-positive NFs or with the ratio of CGRP-positive NFs to SP-positive NFs (data not shown). Only in RA patients, erythrocyte sedimentation rate
negatively correlated with density of CGRP-positive NFs (Fig. 4C) and the ratio of CGRP-positive NFs/density of SP-positive NFs (Fig. 4D). Only in RA patients, a similar trend existed for the interrelation between serum C-reactive protein and density of CGRP-positive NFs ($R_{\text{rank}} = -0.406, P = 0.054$) and the mentioned ratio ($R_{\text{rank}} = -0.382, P = 0.072$).

However, treatment with NSAIDs, prednisolone, methotrexate, hydroxychloroquine, sulphasalazine, azathioprine, leflunomide or tramadol was not associated with a high or low density of CGRP-positive NFs or with a high or low ratio of CGRP-positive NFs to SP-positive NFs (data not shown).

**Discussion**

In a large cohort of patients with chronic RA and OA, this study of synovial tissue demonstrated a preponderance of SP-positive NFs relative to CGRP-positive NFs in RA patients but not in OA. We investigated all NF types in the same patient in order to compare their density in a paired manner. The results most probably indicate a pro-inflammatory situation because the immunostimulating role of SP and the immunoinhibitory role of CGRP and sympathetic neurotransmitters have been demonstrated [5–18, 33]. In addition, a favourable influence of CGRP on bone resorption has been delineated due to inhibition of osteoclast activation [19, 20].

This study further shows that an even balance exists between SP-positive and CGRP-positive NFs in RA, but that a largely shifted balance away from CGRP to SP is present in RA (Fig. 3A). Indeed, in RA patients, ~7 SP-positive NFs match one CGRP-positive NF, whereas in OA patients, this ratio is 1:1 (Fig. 3A). This uneven balance in RA patients surprisingly exceeds the recently described preponderance of SP-positive NFs over sympathetic NFs, which appeared as a ratio of $\sim 8:1$ [23]. Thus, local concentrations of important anti-inflammatory neurotransmitters such as CGRP from sensory NFs, and noradrenalin, adenosine and endogenous opioids from sympathetic NFs are inadequately low in relation to the pro-inflammatory SP from sensory NFs. These statements apply for patients with longer-lasting disease since we do not know the situation in early RA vs early OA. However, since in animal models the inflammation-related change of NFs occurred early in the disease process [28], similar changes might be expected in earlier phases of RA and OA.

These results suggest the existence of a differential regulation of CGRP and SP in sensory NFs, which probably depends on local and/or systemic inflammation (see the relation to erythrocyte sedimentation rate and fibre density as given in Fig. 4C and D). A differential release into the circulation of CGRP and SP has been demonstrated under activation of the sympathetic nervous system during exercise [34]. In this study, exercise induced a fast rise of plasma CGRP but not of plasma SP, which was interpreted as a vasodilation compensation for sympathetically induced vasoconstriction [34]. Another important study demonstrated that primary vagal sensory neurons produce more SP than CGRP in the presence of nerve growth factor and the opposite effect without nerve growth factor [35]. Although another study did not confirm the differential effect of nerve growth factor [36], additional circumstances together with nerve growth factor might be an important stimulus for the dysbalance of the two sensory neuropeptides. Since nerve growth factor is abundantly expressed in inflamed synovium [37–40], the observed preponderance of SP over CGRP might depend on the presence of this and other pro-inflammatory molecules.

Finally, we observed a positive correlation between the density of CGRP-positive and sympathetic NFs in OA, which we interpret as an anti-inflammatory signal due to the aforementioned effects of respective neurotransmitters. Such a positive correlation was not observed in synovial tissue of patients with RA, which demonstrates a markedly reduced presence of sympathetic NFs ($y$-axes in Fig. 4). Thus, in the less-inflamed tissue of OA patients, coupling of the two anti-inflammatory pathways might dampen the inflammatory process, a situation that is lost in the tissue of RA patients, indicating another example of neuroendocrine uncoupling.

In conclusion, the observed preponderance of SP-positive NFs over CGRP-positive and sympathetic NFs can be regarded as a pro-inflammatory signal, although the exact reasons for the differential behaviour of the two sensory neuropeptides SP and CGRP is presently not understood. A detailed analysis of the differential expression of SP and CGRP must include nerve growth factor together with typical pro-inflammatory cytokines such as TNF, and might unravel a new therapeutic approach in order to re-install the even balance of SP and CGRP in peripheral sensory NFs in RA.

**Rheumatology key messages**

- RA patients have a lower density of CGRP-positive NFs in relation to SP-positive NFs, which is not observed in OA patients.
- Density of CGRP-positive NFs correlates with density of sympathetic NFs in OA but not in RA patients.

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