Increased content of bombesin/GRP in human synovial fluid in early arthritis: Different pattern compared with substance P

T. Westermark¹, S. Rantapää-Dahlqvist², S. Wållberg-Jonsson², U. Kjörell¹, S. Forsgren¹

¹Department of Integrative Medical Biology, Section for Anatomy; ²Department of Rheumatology, Umeå University, Umeå, Sweden.

Abstract

Objective

Bombesin (BN) and the mammalian homologue gastrin-releasing peptide (GRP) are known trophic factors, neurotransmitters and paracrine hormones. BN/GRP has not previously been demonstrated in synovial fluid. In this study, the amounts of BN/GRP and substance P (SP) present in synovial fluid from the knee joints of patients with rheumatoid arthritis (RA) and of healthy controls were measured.

Methods

Synovial fluid from the knee joint was collected from patients with either longstanding RA (n = 32) or early arthritis (symptoms for < 12 months; n = 9) and from control subjects, i.e., individuals without known joint disease (n = 10). These samples were analyzed using radioimmunoassays.

Results

Levels of BN/GRP-like peptide were below the assay detection limits in synovial fluid from controls. Detectable levels of immunoreactive BN/GRP were present in the majority of patients with either longstanding RA or early arthritis. The levels were significantly higher in the synovial fluid from patients classified as having early arthritis compared with those with longstanding RA (p < 0.05). There was a strong correlation between BN/GRP levels and the number of leukocytes in the synovial fluid in the patients with early arthritis. The levels of SP-like peptide in the patients, whether with early arthritis or longstanding RA, were significantly elevated compared with controls. However, there was no difference in the levels between these two patient groups.

Conclusions

These observations show that BN/GRP-like peptide is present in the synovial fluid of joints affected by arthritis and that the pattern of BN/GRP increase differs from that of SP. It appears as if the presence of BN/GRP is particularly related to the early processes of joint involvement. These observations are of interest because BN/GRP has well-known trophic and paracrine effects and chondrocytes have recently been shown to produce neuropeptides such as BN/GRP.

Key words

Rheumatoid arthritis, synovial fluid, neuropeptides, BN/GRP, substance P, inflammation.

Introduction

Both clinical observations and experimental studies favour a contribution of the nervous system to the pathophysiology of arthritis, particularly rheumatoid arthritis (RA) (1-3). Thus, a neurogenic component of the inflammatory response in the pathogenesis and maintenance of arthritis, mediated mainly by the unmyelinated afferent fibres and small myelinated A - type fibres supplying the joint, has been proposed (2). Neuropeptides not only act as neurotransmitters but also as controllers of the micromilieu (4). During the past two decades a profusion of studies relating neuropeptides to joint damage have been performed, neuropeptides not least being believed to be involved in the neurogenic inflammation of the joint (2). Increased levels of neuropeptides such as substance P (SP), calcitonin gene-related peptide, vasoactive intestinal peptide (VIP), neurokinin A and neuropeptide Y in synovial fluid have been reported in several studies of arthritis in man (5-9); however, there are also studies contradicting these findings (10, 11).

The mammalian equivalents of bombesin (BN), a tetradecapeptide originally isolated from the skin of the frog Bombina bombina (12), are gastrin releasing peptide (GRP), neuromedin B and neuromedin C (13-15). BN/GRP-like peptides have potent effects on various systems in mammals, functioning both as neurotransmitters/neuromodulators and tissue-specific growth factors and as paracrine hormones. BN/GRP-like peptides affect functions and processes such as satiety, thermal regulation, nociception, activation of the sympatho-adrenomedullary outflow (16, 17), and cell proliferation in the endocrine pancreas (18). BN/GRP promotes the growth of, and stimulates both endocrine and exocrine secretions from, the pancreas (19). In addition, BN/GRP promotes the growth of gastrointestinal tissues (20), stimulates gastric and pancreatic secretion and influences intestinal motility (21, 22). BN/GRP immunoreactivity has been found in various tissues, including the brain (23), and in a recent study it was proposed that BN is also involved in the mediation of stress responses (24). Furthermore, BN/GRP-like peptides may be mediators of inflammatory reactions during pulmonary inflammation and fibrosis (25). Increased levels of BN/GRP have been found in experimental situations, such as in the laryngeal (26) and salivary (27) glands, as a result of the tissue remodeling that occurs in response to radiotherapy. The levels of BN/GRP in the spinal cord were furthermore increased in adrenalectomized rats substituted with a high dose of dexamethasone (28).

The presence of BN/GRP in synovial fluid has not yet been investigated nor is it known whether BN/GRP is involved in the arthritic process. Since BN/GRP has a multitude of effects in different organ systems, the levels of BN/GRP in human synovial fluid of arthritic patients with early or long-standing RA and of healthy controls were investigated using radioimmunoassay (RIA). For comparison, we also analysed the levels of SP in the synovial fluid.

Materials and methods

Patients

Forty-one (31 female and 10 male) patients with RA according to ACC criteria (29), and having active inflammation of one of the knee joints, were consecutively included in the study. The patients were divided into two categories depending on their disease duration: patients with a disease duration of less than 12 months were categorized as having “early arthritis” (n = 9) and patients with a longer disease duration (mean ± SD, 13.3 ± 8.1 years) as having “longstanding RA” (n = 32). Synovial fluid was aspirated from the affected knee joint. Three and 16 of the patients from each group respectively were on oral corticosteroids and 5 and 22, respectively, were on disease modifying anti-rheumatic drugs such as sulphasalazine, intramuscular or oral gold, methotrexate, cyclosporine and azathioprine. Three and 4 patients from each patient group, respectively, were not on any of these medications. The control material consisted of knee synovial fluid from healthy volunteers without any joint disease (n = 10).

The fluid was aspirated according to the method of Dixon and Emery (30).
After counting the number of leukocytes, the aspirates were centrifuged, and the leukocytes were removed. The samples were then frozen and stored at -80 °C until the time of the assay. Blood samples were collected from the patients in parallel for determination of the erythrocyte sedimentation rate (ESR, mm/h). The age and gender distribution of patients and controls are presented in Table I. The protocols had been approved by the ethical committee of Umeå University (dnr 96-229).

The radioimmunoassay
Briefly, for the RIA of BN/GRP the synovial fluid samples were thawed, and 200-300 μl aliquots were freeze dried and individually reconstituted to their original volume with buffer supplied with the kit used. Samples were then vortexed with a spatula for 30 s and centrifuged (8000 rpm for 15 min) before 100 μl of each sample was finally collected for assay. The concentrations of BN-like material were determined using an 125I-RIA kit (Peninsula Laboratories Inc., San Carlos, CA, USA) according to the manufacturer’s instructions. The anti-BN antibody used was directed against synthetic BN/GRP and, according to the manufacturer, displayed 100% cross-reactivity with bombesin, (Lys3)-bombesin and (Tyr3)-bombesin, 50% cross-reactivity with porcine GRP, and 0.01% cross-reactivity with SP and VIP. The detection limit in this particular assay was cited as 10 pg/ml.

For the SP assay a 125I-RIA kit (Euro-Diagnostica, Malmö, Sweden) was employed. Aliquots (200-300 μl) of synovial fluid were thawed and 100 μl of each sample was used. The anti-SP antibody was directed against synthetic SP, shown by the manufacturer to have 0.05% cross-reactivity with BN, and 0.001% cross-reactivity with neurokinin A and B, eleodoisin and neuromedin B. The sensitivity of the assay was 2.5 pg/ml.

Counting of leukocytes
Immediately after aspiration and prior to centrifugation, the number of leukocytes in each sample of synovial fluid from the patients was counted in a light microscope using a Bürger chamber. The aspirate volumes of synovial fluid from the controls were insufficient to allow both a leukocyte count and RIA analysis.

Statistics
Differences between the continuous data for BN/GRP and SP levels for the subject groups were assessed using Kruskal-Wallis one-way analysis of variance and, when significant, were further analysed using the Mann-Whitney U test. Correlation analyses were performed with the Spearman rank-order test. P-values < 0.05 were considered significant.

Results
Bombesin/gastrin releasing peptide
The levels of immunoreactive BN/GRP in the synovial fluid from all of the control subjects were below the detection limit, whilst there were clearly detectable levels of BN/GRP immunoreactivity in the majority of patients with either longstanding RA or early arthritis (Table II). Furthermore, the levels in the synovial fluid from patients with early arthritis were significantly higher than from patients with longstanding RA (p < 0.05).

Leukocytes and ESR
The number of leukocytes in the synovial fluid from patients with early arthritis and longstanding RA was 7.6 x10⁹/L (6.8-13.1) (median and interquartile range Q1-Q3) and 8.9 x10⁹/L (6.3-14.0), respectively. We found a strong correlation (r = 0.862, p < 0.05) between the BN/GRP-like levels and the number of leukocytes in the patients with early arthritis, but no such correlation was found for the other

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Male</th>
<th>Female</th>
<th>Mean age ± S.D. years (male / female)</th>
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</thead>
<tbody>
<tr>
<td>Longstanding RA</td>
<td>8</td>
<td>24</td>
<td>57.1 ± 4.1 / 59.7 ± 2.0</td>
</tr>
<tr>
<td>Early RA</td>
<td>2</td>
<td>7</td>
<td>60.0 ± 17.0 / 45.4 ± 7.2</td>
</tr>
<tr>
<td>Controls</td>
<td>6</td>
<td>5</td>
<td>30.8 ± 4.1 / 36.8 ± 6.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substance P (pg/ml)</th>
<th>Longstanding RA (n = 32)</th>
<th>Early RA (n = 9)</th>
<th>Controls (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BN/GRP (pg/ml)</td>
<td>17.7***</td>
<td>30.7***†</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>(7.3 - 24.1)</td>
<td>(17.9 - 37.7)</td>
<td>(5.0 - 5.0)</td>
</tr>
<tr>
<td>Substance P (pg/ml)</td>
<td>56.2***</td>
<td>48.4***</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td>(39.8 - 76.7)</td>
<td>(29.9 - 84.8)</td>
<td>(1.25 - 1.25)</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>34.5</td>
<td>22.0</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>(20 - 47)</td>
<td>(9 - 31.3)</td>
<td></td>
</tr>
<tr>
<td>Leukocytes/SF (x10⁹/L)</td>
<td>8.9</td>
<td>7.6</td>
<td>0.1†</td>
</tr>
<tr>
<td></td>
<td>(6.3 - 14.0)</td>
<td>(6.8 - 13.1)</td>
<td></td>
</tr>
</tbody>
</table>

***p < 0.001 with respect to the controls, †p < 0.05 with respect to longstanding RA.
nd = not done, †n = 2.

Table I. Age and sex distribution among patients and controls.

Table II. Peptide levels, ESR and number of leukocytes in the synovial fluid (SF) in different groups, presented as medians and interquartile ranges within parenthesis (Q1-Q3). The BN/GRP levels in all of the controls and the SP levels in all but one of the controls were below the detection limit. For the statistical calculations, those values below the detection limit were set to 50% of the detection limit, i.e. 5.00 pg/ml for BN/GRP and 1.25 pg/ml for SP.

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patient group or between SP and leukocyte numbers. The mean ESR was 34.5 mm/h for patients with longstanding RA and 22.0 mm/h for early arthritis. There was no correlation between ESR and the levels of neuropeptides.

Discussion

Our observations show that BN/GRP-like and SP-like peptides are detectable in the synovial fluid from knee joints of subjects suffering from early arthritis or longstanding RA. The levels of both peptides were below the detection limit in virtually all of the samples from control subjects, only one control having measurable levels of SP-like peptide. In most studies on the level of neuropeptides in the knee synovial fluid, patients with RA have been compared with patients with osteoarthritis or traumatic knee injury, i.e. with joints undergoing low grade inflammatory and/or tissue repair processes. In this study we examined synovial fluid from healthy controls. That we were unable to detect immunoreactive BN/GRP nor, in principle, SP in these controls suggests that the presence of BN/GRP and SP-like peptides in the synovial fluid is part of the inflammatory and destructive process and not a phenomenon of the normal knee joint. This is consistent with the finding of a correlation between SP and the pro-inflammatory cytokine interleukin-6 in joint inflammation (31). Furthermore, our detection of SP in arthritic synovial fluid supports the finding of higher SP levels in synovial fluid from the joints of patients with RA than in the joints of patients with osteoarthritis or traumatic injury (7,11,32). Thus, it is likely that the damage in RA is related to an increased release of SP-like peptide, as well as of BN/GRP-like peptide, into the joint cavity. The observations of a decreased SP-innervation of the synovium of arthritic joints are also interpreted to be the result of a local release of SP into the joint fluid, leading to a depletion of SP (33-35). The decrease in the number of SP-containing nerve fibres may be related to a loss of nerve fibres (35) due to synovial tissue hyperplasia without the proliferation of new fibres. Alternatively, the decrease in synovial innervation seen in longstanding arthritis may result from a destruction of neuropeptide-containing nerves by locally produced proteolytic enzymes and reactive oxygen species from the nearby inflammatory cells (36). Contrary to these findings, a large number of SP-immunoreactive nerve fibres occur in the synovium of knee joints with osteoarthritis (37).

Of greatest interest was the observation of a difference in the patterns of BN/GRP- and SP increase between the two patient groups investigated here. The BN/GRP levels were higher in synovial fluid from patients with early arthritis compared with patients with longstanding RA, a distinction that was not apparent when considering the levels of SP. Therefore, it seems that BN/GRP is particularly related to the early processes of joint involvement. Another interesting observation was the finding of a strong correlation between BN/GRP levels and the number of leukocytes in the patients with early arthritis, further indicating a role for the BN/GRP-like peptides in the early stages of joint inflammation. This correlation was not seen in the longstanding RA group nor when considering SP in either of the two patient groups. There was no correlation seen between peptide levels and disease activity as measured by ESR. The origin of the BN/GRP-like peptide found in the synovial fluid is uncertain. Previously, BN/GRP-like peptide has been detected in the superficial layers of the spinal cord in both rat (28,38,39) and man (40), and in dorsal root ganglion cells (38) using immunohistochemical methods, indicating that BN/GRP may be of sensory origin. However, due to a certain sequence homology between BN/GRP and SP in the C-terminal ending of the peptides, observations on BN/GRP immunoreactivity in sensory innervation should always be interpreted with caution, as cross-reactions with SP may occur. In any case, immunoreactive BN/GRP detected after the pre-absorption of BN/GRP antiserum with SP is found to a small extent in the dorsal horn of the rat (28) and is present in the parasympathetic innervation of salivary (27) and laryngeal (26) glands. Whether BN/GRP-like peptides can be found in the nerve tissue supplying joints is unknown. In a study on joint innervation, BN/GRP could not be detected in either normal nor pathological synovial tissue samples from human knees (35). Interestingly, Lapadula et al. (41) and Iannone & Lapadula (42) found BN/GRP-like peptide, as well as SP and enkephalin and their neuropeptidases, in human chondrocytes. The levels of the peptides in chondrocytes from osteoarthritic joints were significantly raised in the cartilage containing more osteoarthritic lesions (41,42). The increase of intra-cellular levels of neuropeptides seen in chondrocytes in osteoarthritis was suggested to be associated with a reduction in cell surface neuropeptidases, an imbalance which might drive the chondrocytes towards an excessive activation of synthetic processes (42). The possibility that peptides like BN/GRP are secreted from other cells relevant to an inflamed joint, such as immunologically active cells or synoviocytes, cannot be excluded. Thus, synovial cells themselves may produce neuropeptides (43) and may also express neuropeptidases on their surface. Furthermore, Hashimoto et al. found preproatkin mRNA within macrophage-like cells in the synovium (44). The findings of a correlation between the number of leukocytes and the level of BN/GRP in early arthritis might suggest an involvement of the leukocytes in BN/GRP production.

Whether BN/GRP-like peptides have a role in the inflammatory and/or destructive process is not known. It is frequently reported that BN/GRP-like peptides may have trophic and growth-promoting effects in various parts of the body. For example, BN/GRP-like peptides may have trophic effects on the salivary glands after irradiation (27), para- and autocrine trophic effects in certain cancers, such as small cell lung cancer and colon cancer (45-47), and be mitogenic in Swiss 3T3 cell cultures (48). Further to the observations presented in this study, it is of interest to note that GRP promotes the proliferation of ovine foetal chondrocytes (49). It has also been shown that BN/GRP significantly stimulates the antibody-dependent cellular cytotoxicity and natural killer activities of mouse leuocytes in vitro (50). Interestingly, in a previous study it was shown that BN/GRP-like peptides have a depressant effect on neurones located superficially in the dorsal horn and particularly on those receiving a nociceptive input (51). Hence, it was concluded that BN/GRP-
like peptides interact with the release of transmitters from nociceptive primary afferents (51). The recent finding that the expression of BN/GRP-like peptides in the rat spinal cord is influenced by glucocorticoids (28) may be of additional importance in the understanding of the functional role of BN/GRP. Further research is necessary before any definite conclusions can be made regarding the functional role of BN/GRP in joints.

Substance P has been extensively examined in relation to arthritis and is known to have pro-inflammatory properties. In one study it was shown that rat joints developing a severe adjuvant-induced arthritis were more densely innervated with SP-containing primary sensory neurones than those developing a moderate arthritis (1). In that study, the effects of intra-articular injections of SP were also examined and it was found that such a treatment increased the severity of the arthritis, both in terms of the degree of the inflammation and of joint destruction (1). Substance P is known to facilitate the proliferation of rheumatoid synoviocytes, and the production and release of pro-inflammatory mediators such as prostaglandin E2 and destructive enzymes such as collagenase (52). More recently, Walsh et al. (53), have shown that SP promotes angiogenesis in the synovial membrane, a fact which was considered to promote chronic inflammation. Other studies have shown a decrease in inflammatory activity in the rat knee using SP antagonists (54, 55), and that the injection of capsaicin into the knee joint of the rat prior to SP infusion abolishes the inflammatory response (56). It has been suggested that increased levels of SP in the synovial fluid in arthritis may lead to increased production of collagenase by the synoviocytes (57). As synoviocytes themselves possibly contain SP and express mRNA for NK1 and NK3 tachykinin receptors, preprotachykinins and neutral endopeptidases (58), it is possible that resident articular cells may work independently from the peripheral nervous system, possessing the autocrine and paracrine peptideergic systems (9).

This suggestion is further strengthened by the recent findings that synovial cells show the expression of neuropeptide receptors, as confirmed by RT-PCR and radioreceptor assays (59). The importance of the peptidases involved in the degradation of active peptides in synovial fluid has been studied in both patients with rheumatoid arthritis and those with osteoarthritis (41, 60). The peptidases examined included neutral endopeptidase (NEP). It has been suggested that the increase in NEP levels seen in the synovial fluid of patients with RA might be directly dependent on the inflammatory process, there being a continuous release of SP and a reactive production of NEP in the articular space (60). Based on studies on the effects of NEP in an articular model, it was also suggested that endogenous NEP-24.11 is unlikely to play an acute role in modulating peptide-induced inflammatory responses when competing with co-localizing peptide receptors expressed in high density (61). On the other hand, the administration of soluble recombinant NEP-24.11 might overcome this problem (61).

In conclusion, we have shown the occurrence of a specific increase of BN/GRP-like peptide in the arthritic joint and that these higher levels are associated with early disease rather than longstanding RA. Such a distinction was not found with respect to the levels of SP. The meaning and functional importance of this specific increase should be examined in the future.

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