Parathyroid hormone-related peptide expression in rat collagen-induced arthritis

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Parathyroid hormone-related peptide (PTHrP) was initially described as a potent bone resorptive agent in humoral hypercalcemia of malignancy (HHM) and breast cancer where it was thought to promote osteoclastogenesis through induction of receptor activator of NF-κB ligand (RANKL) expression [1, 2]. The importance of PTHrP in bone remodelling has been further highlighted in PTHrP knockout mice, which are characterized by defects in the progenitors of osteoblasts and chondrocytes, early embryonic lethality and by PTHrP-null heterozygotes with juvenile osteoporosis [3–6]. The N-terminal portion of PTHrP [amino acids (aa) 1–34] is the major domain of the protein involved in enhancement of bone resorption through its effects on cells of osteoblastic lineage and subsequent osteoclast differentiation and activation [7, 8].

Previous studies have also implicated PTHrP in regulation of chondrocyte survival and proliferation [9]. In a model of antigen-induced arthritis (AIA), Gomez-Barrena et al. [10] observed an overall decrease in PTHrP-positive chondrocytes related to the cartilage damage in the advanced stages of the disease. This indicated involvement of PTHrP in cartilage maintenance in inflammatory arthritis [10].

In the setting of rheumatoid arthritis (RA), PTHrP has been identified as one of the local bone resorptive factors produced by the pannus tissue [11, 12]. It was also suggested to have a pro-inflammatory role, based on its strong associations with C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), interleukin-1 receptor (IL-1R) [12, 13] and IL-6 [14] in the RA synovial fluid. Furthermore, prostaglandin E2, tumour necrosis factor-α (TNF-α), IL-1α, IL-1β and IL-6, cytokines abundant in the rheumatoid synovium, induced PTHrP expression in fibroblasts from RA and osteoarthritis patients [11, 15, 16]. Conversely, treatment of the synoviocytes with PTHrP (aa 1–34) stimulated IL-6 secretion [12]. On that basis, it has been suggested that PTHrP may be an important cytokine in the pro-inflammatory cascade that could become an effective target in the treatment of inflammatory diseases such as RA [17].

However, in streptococcal cell wall (SWC)-induced arthritis, blockade of PTHrP had no effect on joint inflammation, although it partially reduced bone and cartilage erosion through its effect on osteoclast formation, and decreased granuloma formation at SWC deposition sites in liver and spleen, associated with PTH/PTHR receptor positive neutrophil influx [18].

In this study we have examined the patterns of PTHrP expression during progression of collagen-induced arthritis (CIA), a well-established animal model for RA [19, 20]. The changes in PTHrP mRNA and protein were examined in inguinal lymph nodes (ILNs), popliteal lymph nodes (PLNs) and distal interphalangeal (DIP) joints, to elucidate whether there are any associations between PTHrP expression and the pathology of CIA.

Materials and methods

Induction of CIA and tissue processing

CIA was induced in 10 week-old dark Agouti (DA) female rats by intradermal injections of native rat type II collagen (CII) emulsified in complete Freund’s adjuvant. CIA was induced in female dark agouti rats. Inguinal (ILNs) and popliteal (PLNs) lymph nodes and distal interphalangeal joints (DIP) were retrieved at different time points. Tissues were processed for detection of PTHrP and cell marker proteins by immunohistochemistry. Lymph node RNA was extracted, and PTHrP mRNA quantified using competitive reverse transcriptase polymerase chain reaction.

Results

Hyperplasia of ILNs was observed 2 days after injection, coinciding with the peak in PTHrP expression in ILNs (1240 ± 373 gene copies/ng RNA vs normal 339 ± 120, P < 0.05). Hyperplasia of PLNs was first seen at 1 day after onset of arthritis, coinciding with the peak in PTHrP expression in PLNs (2267 ± 697 vs normal 781 ± 136, P < 0.01). PTHrP expression in PLNs remained increased 5 days after onset (1361 ± 302 vs normal 781 ± 136, P < 0.05). In both PLNs and ILNs PTHrP protein was localized to high endothelial venules, lymphocytes and monocytes/macrophages. In DIP joint synovium PTHrP staining was first detected on day 10 after onset, and was most abundant at day 20 after onset, at sites of bone resorption and deposition, where it was localized to neutrophils, cells of monocyte lineage and osteoblasts.

Conclusions

Changes in ILN and PLN PTHrP mRNA expression suggest that elevated levels of the cytokine are associated with aggravation of the inflammatory immune response. Changes in PTHrP in DIP joints indicate its involvement in late rather than early pathogenic events in CIA joints.

Key words: Collagen induced arthritis (CIA), Inflammatory arthritis, Leucocyte activation, PTHrP.
in incomplete Freund’s adjuvant (IFA) at the base of the tail [19]. Each animal received a total of 300 ml of CII/IFA at a concentration of 2 mg/ml or IFA alone, distributed between five or six sites at the base of the tail. All injections were performed under ether anaesthesia. Onset was classified upon initial visualization of inflammation in DIP joints of hind paws occurring 13.4 ± 1.17 days after injection. CIA groups included rats killed at day 2 after injection (D2, n = 8), day 10 after injection (D10, n = 7), onset (O, n = 7), 1 day after onset (O + 1, n = 8), 3 days after onset (O + 3, n = 6), 5 days after onset (O + 5, n = 8), 10 days after onset (O + 10, n = 7) and 20 days after onset (O + 20, n = 6). The controls included: five normal rats killed at day 0 (D0) and four IFA controls killed at subsequent time-points of D2, D10, O, O + 3, O + 5 and O + 20.

The ILNs draining the CII/IFA injection site were collected from five rats per group at D0 (normal controls), D2, D10, O, O + 1 and O + 5. PLNs draining the affected joints were collected at D0 (n = 4), D10 (n = 5), O + 1 (n = 6) and O + 5 (n = 6). DIP joints were collected from all rats. Two ILNs and/or PLNs were collected from each rat: one was frozen in liquid nitrogen for RNA extraction and the other was embedded in paraffin for haematoxylin–eosin (H&E) assessment and immunohistochemical analysis. The DIP joints from each animal were decalcified and embedded in paraffin for H&E assessment and immunohistochemical analysis. The process of decalcification was carried out with EDTA solution as previously described by Bakharevski et al. [19] and monitored using X-rays. This study was approved by the Alfred Hospital Animal Ethics Committee and conformed to NHMRC guidelines for animal experimentation.

Clinical assessment of disease severity
Rats were monitored daily. Their weights and the paw thickness, measured using a dial gauge calliper (Mitutoyo, Japan), were recorded on the day of CII/IFA or IFA injection and on the day of killing. The degree of arthritis was evaluated using a 0 to 16 scale with a maximum of 4 for each paw, as described by Bakharevski et al. [19]. No arthritis was ranked as 0, swelling and/or redness of one to two interphalangeal (IP) joints was considered as 1, involvement of three to four IP joints or one large joint was ranked as 2, involvement of more than four joints was considered as 3, and severe arthritis of the entire paw was classified as 4.

Histopathology
The investigators were blinded when evaluating the disease severity from the H&E sections of DIP joints. The following changes were graded from 0 to 4. Infiltrate: absent = 0, mild = 1, moderate = 2, severe = 3, severe (complete). Pannus defined as synovial tissue invading bone and/or cartilage: absent = 0, occupying less than 25% of the joint space (JS) = 1, 25–50% of JS = 2, 50–75% of JS = 3, over 75% of JS = 4. Joint space loss: none = 0, 75% or more of JS present = 1, 50–75% of JS present = 2, 25–50% of JS present = 3, 25% or less of JS present = 4. Fibrosis: absent = 0, mild = 1, moderate = 2, moderate/severe = 3, severe = 4. Joint cartilage loss: none = 0, superficial loss = 1, loss of under 1/3 = 2, loss of 1/3 to 2/3 = 3, loss of cartilage equal to or greater than 2/3 = 4. Epiphysial bone loss: none = 0, superficial lacunae associated with newly formed osteoclasts = 1, loss under 1/3 = 2, loss of 1/3 to 2/3 = 3, loss equal to or greater than 2/3 = 4.

Detection and quantification of gene expression
RNA extraction. Total RNA was extracted and purified from whole lymph nodes using the RNeasy extraction kit (Qiagen Inc., Hilden, Germany). The total RNA concentration was determined spectrophotometrically.

Reverse transcription (RT). The samples were reverse transcribed using the Perkin-Elmer Reverse Transcription System (Perkin Elmer Inc., Norwalk, CT), as per the manufacturer’s instructions, with RNA concentrations in the range of 4.5 to 139.2 ng/ml of RNA.

Primer design. Primers were designed using the Oligo 5 Software for Windows (National Biosciences Inc., Plymouth, MN). One sense (354U) and two antisense (717L and 540L) primers and one composite primer that combined the two antisense primers were designed for the PTHrP mRNA sequence. The primer sequences are listed in Table 1.

Competitive PCR—quantification of PTHrP mRNA
Synthesis of the competitor. Native RNA was reverse transcribed and amplified with the sense 354U primer and the antisense 540L primer, and with the sense 354U primer and the antisense 717L primer, to confirm the amplification of the correct PCR products. Once this was verified, the composite primer was synthesized. The sense 354U and the composite primer were used to amplify the native cDNA to generate the competitor (deletion mutant) as previously described [21]. After electrophoresis, the competitor was purified using the Qiagen DNA gel purification kit, quantified spectrophotometrically and stored at −20°C.

Quantitative analysis of PTHrP gene expression. The 25 ml polymerase chain reactions (PCR) consisted of 1.5 mM MgCl₂, 2.25 ml of ×1 PCR buffer, 0.2 pmol/ml of combined fluoresceinated sense primer 354U and unlabelled antisense primer 717L, 0.05 units/ml of AmpliTaq Gold (Applied Biosystems), 2.5 ml of RT reaction mixture and 1 ml of varying dilutions of the competitor made up to 25 ml with ribonuclease (RNase) free water. 25 ml of oil was added to avoid sample evaporation. PCR cycling conditions consisted of 40 cycles at 55°C annealing temperature. The PCR products were run on a 2% agarose gel and scanned using the FluorImager 575 (Molecular Dynamics, Sunnyvale, CA). The native cDNA was amplified as a 402 bp band, while the competitor was amplified as a 223 bp band. Band densities were determined using ImageQuant software, and the ratio between competitor and target calculated. The log of the

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<th>Genes</th>
<th>Primers</th>
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<tr>
<td>Sense 354U</td>
<td>5′-fluoro-CCGTGGTGGTGGTACAGCAGAC-3′</td>
<td>223</td>
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<tr>
<td>Antisense 540L</td>
<td>5′-GGGGCTGGGGGTTCCTCAAG-3′</td>
<td>402</td>
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<tr>
<td>Antisense 717L</td>
<td>5′-TTCCTGGGGGAGACAGTTGTGAT-3′</td>
<td>247</td>
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<td>Composite primer</td>
<td>5′-TTCCTGGGGGAGACAGTTGTGATGGGCTGGGGTTCCTCAAG-3′</td>
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ratio was plotted versus the log of the copy number, and linear regression analysis was performed using the statistical software SPSS (11.5 for Windows). The gene copy number was determined at the point where \( y = 0 \) (ratio = 1). The copy number value was then standardized against the RNA concentration and expressed as gene copies per ng of RNA.

**Quantification of PTHrP mRNA in inguinal and popliteal lymph nodes.** PTHrP expression in ILNs was quantified in six groups of five rats each: D0 (normal rats), D2, D10, O, O + 1 and O + 5. PTHrP expression in PLNs was quantified in five groups of four to six rats each: D0 (normal rats), D10, O + 1 and O + 5. RT reactions from each sample were co-amplified with five competitor concentrations, and the number of PTHrP mRNA copies was determined. The gene expression was determined from two or more separate RT reactions per sample, and the mean value was used.

**Statistical analysis of gene expression**

Competitive PCR results were analysed using SPSS multiple independent samples Kruskal–Wallis one-way analysis of variance (ANOVA) and two-independent samples Mann–Whitney U-test. Changes were considered significant at \( P < 0.05 \).

**Immunohistochemistry—detection/localization of PTHrP protein expression**

*Four-layer technique.* Paraaffin embedded tissues were cut at 5 mm onto Super Frost Plus slides, and dewaxed. A four-layer immunoperoxidase technique was used to detect monocytic/macrophages as previously described [22]. The primary antibody applied was ED1 (anti-rat monocyte/macrophage, kindly provided by Dr Dijkstra, Free University, Amsterdam, The Netherlands) [23]. Non-specific staining was blocked by pre-incubation with 10% normal rabbit serum (NRS), 5% fetal calf serum (FCS) for 10 min at room temperature. The sections were then incubated overnight in a humid chamber with the ED1 antibody. A second layer of swine antirabbit peroxidase antibody for 30 min at RT. Metal-enhanced diaminobenzidine (DAB) substrate (Pierce) was applied to the sections for 10 to 20 min. Slides were then counterstained with Harris’ haematoxylin, and coverslipped.

*Three-layer technique.* A three-layer immunoperoxidase technique used to detect PTHrP protein in ILNs, PLNs and DIP joints, was a modification of the procedure described by Stein-Oakley et al. [22]. The expression was examined in four to six rats per CIA group in: ILNs between day 2 after injection and 5 days after onset, PLNs from rats between day 10 after injection and 5 days after onset and DIP joints from rats between onset and 20 days after onset. Controls included ILNs, PLNs and DIP joints from untreated rats. Non-specific staining was blocked by pre-incubation with 20% normal swine serum (NSS), 5% FCS for a minimum of 2 h at RT. The sections were then incubated overnight with the anti-PTHrP (aa 1–14) immune rabbit serum diluted 1:800 at RT in a humid chamber. Endogenous peroxidase activity was blocked as described above. Slides were then incubated with a second layer of swine antirabbit peroxidase antibody for 30 min at RT, and a third layer of rabbit peroxidase antiperoxidase antibody for 30 min at RT. Colour was developed with DAB. The slides were then counterstained with Harris’ haematoxylin, and coverslipped.

The primary antibody used for detection of PTHrP was a polyclonal antiserum raised in rabbits against human N-terminal PTHrP (aa 1–14) (kindly provided by Dr Jane Moseley, St Vincent’s Hospital, Melbourne, Australia). Its specificity was evaluated and confirmed by enzyme-linked immunosorbent assay (ELISA) [24]. The serum does not cross-react with PTH. The antihuman PTHrP (aa 1–14) whole antiserum was successfully used to detect rat PTHrP protein. For each sample the negative control included substitution of the anti-PTHrP (aa 1–14) antiserum with whole non-immune rabbit serum at the same dilution as the antiserum. Normal rat skin was used as a positive control.

**Results**

**Clinical signs of arthritis**

None of the IFA injected rats developed arthritis, whereas there was 100% incidence of the disease in CIA/IFA injected rats. In contrast to the IFA controls there was no body weight gain in the CIA/IFA injected rats prior to onset (Fig. 1A). At onset, signs of arthritis included swelling and redness over the joints, and rats developed a limp but were still able to move around freely. From onset until day 5 after onset, there was a marked increase in arthritic score and paw thickness, which was associated with decrease in body weight below baseline (Fig. 1A, B, C). At this stage rats had general signs of illness/distress. At later stages of the disease (O + 10 and O + 20), marked decrease in paw thickness and increase in body weight gain were observed, accompanied by an overall improvement in rat well-being.

**Histopathological signs of arthritis**

Synovial infiltrate scores mirrored paw thickness measurements throughout the disease (Fig. 1D). Both measurements indicated the degree of the inflammatory response, which subsided at later stages of the disease (O + 10 and O + 20). In contrast, pannus formation, cartilage, bone and joint space loss scores continuously increased from onset until day 20 after onset (Fig. 1E, G, H, I). A marked increase in fibrosis scores was also observed between day 5 and day 20 after onset (Fig. 1F).

**Lymph node hyperplasia and PTHrP mRNA**

Hyperplasia initially occurred at day 2 after CIA/IFA injection in ILNs and at 1 day after onset in PLNs. ILNs and PLNs remained hyperplastic throughout the progression of the disease as shown in Fig. 2. The relative levels of PTHrP expression were evaluated in these tissues using competitive RT-PCR as described in Materials and methods. The expression of PTHrP in isolated ILN RNA is shown in Fig. 3. PTHrP expression was significantly elevated at day 2 after CIA induction compared with normal ILNs (\( P < 0.05 \)). This peak coincided with the initial enlargement of ILNs. The level of expression decreased to normal at day 10 after injection. The expression of PTHrP in isolated PLN RNA is shown in Fig. 4. It was normal at day 10 after CIA injection, then significantly increased 1 day after onset (\( P < 0.05 \) compared with day 10 after injection, and \( P < 0.01 \) compared with normals), also coinciding with the initial enlargement of PLNs. At 5 days after onset there was a significant decrease in PTHrP expression compared with 1 day after onset (\( P < 0.01 \)); however, it was still significantly higher than the baseline (\( P < 0.05 \)).
FIG. 1. Clinical parameters and scoring of H&E sections from DIP joints at different stages of CIA and corresponding age matched IFA controls: (A) weight gain; (B) arthritic score; (C) paw thickness; (D) synovial infiltrate; (E) pannus formation; (F) degree of fibrosis; (G) cartilage loss; (H) bone loss; (I) joint space loss. This figure is available in colour as supplementary data at Rheumatology Online.

FIG. 2. H&E sections cut through a pale stained inner medulla continuous with the hilum of ILNs and PLNs at different stages of CIA: (A) ILN extracted from an untreated rat; (B) hyperplasia in ILN at day 2 after CII injection; (C) hyperplasia in ILN at day 10 after onset; (D) PLN extracted from an untreated rat; (E) hyperplasia in PLN at 1 day after onset; (F) hyperplasia in PLN at day 10 after onset. This figure is available in colour as supplementary data at Rheumatology Online.
PTHrP protein in lymph nodes

PTHrP protein was constitutively detected in ILNs and PLNs, throughout cortical, subcapsular, paracortical and medullary sinuses (Fig. 5A, B). It was localized to irregular shaped cells with large pale nuclei (Fig. 5D), that also stained positive for ED1 on sequential sections (Fig. 5E). In both ILNs and PLNs PTHrP staining was also observed on high endothelial venules (HEVs) and a small percentage of lymphocytes (Fig. 5G, H). However, there were no apparent differences in PTHrP expression patterns in ILNs or PLNs at different stages of the disease.

PTHrP and ED1 staining in DIP joints

ED1-positive cell counts in the synovium of DIP joints significantly increased at onset of the disease as compared with the controls, and remained elevated throughout progression of the disease (Fig. 6). However, the synovial cells were negative for PTHrP in rats between onset and day 5 after onset (Fig. 7C). In contrast, cartilage chondrocytes were positive for PTHrP throughout the course of the disease and in normal controls. At day 10 after onset, PTHrP staining was first detected in the synovium and/or periosteum in all rats; in four rats it was localized to the synovium.

**Fig. 3.** Quantification of PTHrP mRNA in CIA ILNs. The PTHrP gene copy number was significantly elevated at day 2 after injection of CII/IFA compared with normals, with the expression returning to baseline at later stages of the disease (#P < 0.05 vs normal, *P < 0.05 vs day 2 after injection). This figure is available in colour as supplementary data at Rheumatology Online.

**Fig. 4.** Quantification of PTHrP mRNA in CIA PLNs. The PTHrP gene copy number was significantly elevated at 1 day after onset and 5 days after onset compared to normals, and at 1 day after onset compared with day 10 after injection and 5 days after onset (*P < 0.05 vs normal, #P < 0.05 vs 1 day after onset, ##P < 0.01 vs 1 day after onset). This figure is available in colour as supplementary data at Rheumatology Online.
FIG. 5. PTHrP staining in ILNs and PLNs. (A) PTHrP staining throughout paracortical and medullary sinuses of ILNs. (B) PTHrP staining throughout subcapsular, cortical and paracortical sinuses of PLNs. (C) Negative control for PTHrP staining in lymphoid sinuses. (D) PTHrP staining localized to irregular shaped cells with large pale nuclei that also stained positive for ED1 on sequential sections (E). (F) Negative control for PTHrP, stained with non-immune rabbit serum on sequential sections. (G) PTHrP staining on high endothelial venules (HEVs) and a small percentage of lymphocytes (labelled L) in ILNs. (H) PTHrP staining on high endothelial venules (HEVs) in PLNs. (I) Negative control for PTHrP staining of HEVs, stained with non-immune rabbit serum. (Magnification: A, B, C ×100; D, E, F, G, H, I ×400.)

FIG. 6. ED1-positive cell numbers in the synovium throughout the course of CIA as compared to normal and IFA controls (*P<0.05 vs controls). This figure is available in colour as supplementary data at Rheumatology Online.
at sites of bone resorption (Figs 7D, E and 8A) and to the periosteum at sites of bone deposition (Fig. 6F, I). In one rat it was localized to the synovium only. Staining of consecutive sections revealed that some of the mononuclear cells and multinuclear giant cells in the synovium positive for PTHrP were also positive for ED1 (Fig. 7). The most abundant staining for PTHrP was found at day 20 after onset. It was localized to synovium and periosteum in all rats examined (Fig. 6G, H), with the same pattern of expression as observed at day 10 after onset.

**Discussion**

This study examined changes in PTHrP expression in ILNs, PLNs and DIP joints throughout the progression of CIA. This model was characterized by enlargement of ILNs 2 days after CII/IFA injection. The initial follicular and sinus hyperplasia is associated with expansion of cortex and medulla, and a significant increase in the number of B cells, T cells and IL-2 receptor-expressing cells compared with normal rat lymph nodes (not shown). Since ILNs drain the injection site, their initial enlargement may be representative of the initiation of pre-arthritis immunological events. Overt clinical disease manifested 12 to 15 days after injection. One day after onset, at the time of acute inflammation of DIP joints [19], PLNs became enlarged due to drainage of the affected sites.

In these settings, PTHrP protein and mRNA were detected constitutively in draining lymph nodes. However, PTHrP mRNA levels markedly increased during initial hyperplasia at day 2 after CII/IFA injection in ILNs and at 1 day after onset in PLNs. In ILNs PTHrP expression then decreased to baseline. In PLNs PTHrP mRNA also decreased, but was still significantly above the normal levels at 5 days after onset. The pattern of PTHrP expression in ILNs and PLNs suggests that increased PTHrP mRNA is associated with initiation of lymph node hyperplasia. Significantly higher levels of PTHrP expression in PLNs 5 days after onset compared with normal rats also indicate that elevated expression of PTHrP may be a feature in the perpetuation of the immune response in the draining lymph nodes.

A small proportion of lymphocytes expressed PTHrP protein in these lymph nodes, which is consistent with findings in equine sarcoidosis [25], systemic lupus erythematosus [26] and RA synovium [12], where it may regulate lymphocyte activation and

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**Fig. 7**. Staining for PTHrP (aa 1–14) in the periosteum/bone and synovium/pannus of DIP joints at different stages of CIA. (A) Synovium of normal controls. (B) Periosteum of normal controls. (C) Synovium and periosteum/bone at 5 days after onset. (D) Synovium at 10 days after onset, PTHrP on mononuclear irregular shaped cells. (E) Synovium at 10 days after onset, PTHrP on neutrophils. (F) Periosteum at sites of bone remodelling, 10 days after onset. (G) Synovium and pannus at 20 days after onset. (H) Periosteum at sites of bone remodelling, 20 days after onset. (I) Osteoblasts in the periosteum at sites of bone remodelling, 10 days after onset. B, bone; PA, pannus; S, synovium; Obl, osteoblasts; PO, periosteum. (Magnification: A, B, C ×50; D, E ×400; F, G, H ×100; I ×290.)
proliferation, as demonstrated in vitro [27, 28]. We also localized PTHrP to lymph node HEVs, where PTHrP may facilitate leucocyte trafficking and activation, as it has been previously shown to induce IL-8 expression in prostate carcinoma cells [29], and nitric oxide (NO) production in endothelial cells [30]. NF-κB and monocyte chemoattractant protein (MCP-1) up-regulation in vascular smooth muscle cells (VSMCs) of atherosclerotic plaques [31], and neutrophil chemotaxis [18]. Moderate to marked cytoplasmic PTHrP staining was also found in monocytes/macrophages (ED1-positive cells) in both ILNs and PLNs. This is consistent with previous studies that demonstrated PTHrP expression on tissue-infiltrating macrophages [32] and on over-activated sarcoid macrophages in lymph nodes [24, 25]. It was suggested that PTHrP expression in monocytes/macrophages is dependent on cell activation [32]. Although in this study changes in lymph node mRNA suggest a correlation between PTHrP expression and the immune response, this could not be demonstrated for PTHrP protein. PTHrP staining patterns in PLNs and ILNs did not change at different stages of the disease.

Although various studies have indicated involvement of PTHrP in the general immune response [11, 14, 33], and we have demonstrated its association with lymph node hyperplasia in CIA, in our setting this association was not present in the diseased joints. By 5 days after onset there was a ‘full blown’ inflammatory response as indicated by increased paw thickness, synovial infiltrate and ED1-positive cell influx, but PTHrP expression in CIA at this time point was the same as in controls, where it was only detected on cartilage chondrocytes.

Moderate PTHrP staining in the synovium was first observed at day 10 after onset in all CIA rat joints. This time point marked the change from the inflammatory phase to the progressive cartilage/bone remodelling phase of the disease, characterized by a persistent increase in fibrosis, and loss of cartilage, bone and joint space. This is consistent with previous CIA studies reporting cartilage destruction 11 days after onset [34] and significant decreases in bone mass of CIA animals [detected by histomorphometry and dual-energy X-ray absorptiometry (DEXA)] at 14 days after onset [35, 36].

At the later stages of the disease (days 10 and 20 after onset), PTHrP was found at sites of both bone resorption in the synovium and deposition in the periosteum, suggesting that PTHrP may participate in both processes in the same joint. In the synovium, cytoplasmic PTHrP was detected on isolated fibroblast-like cells and ED1-positive mononuclear and multinuclear cells particularly at the pannus/subchondral bone junctions. In neutrophils PTHrP staining was localized to the nucleus, where it may interact with RNA [37]. PTHrP translocation to the nucleus defines a distinct pathway from the classic PTH/PTHrP receptor activation cascade that has been associated with modulation of ribosome biosynthesis [38], prolonged cell survival in chondrocytes [39] and increased cellular proliferation in VSMCs [40, 41].

Previous studies have also reported that PTHrP can stimulate both bone resorption by osteoclasts [1, 2, 42] and deposition by osteoblasts and chondrocytes [43–46], but it is unclear as to how the switch between the two processes occurs. Thus, further investigation of involvement of PTHrP in bone resorption and deposition at late stages of CIA would be of significant interest. Factors involved in regulation of PTHrP expression as well as balance between bone resorption and deposition processes, such as 1,25-dihydroxyvitamin D₃, parathyroid hormone (PTH), prostaglandin E₂ (PGE₂), IL-11, IL-1 and IL-6 [8, 47] are likely to be involved in the late CIA events. Of these, the actions of PTH are the most relevant to those of PTHrP in our model [36], acting through a common PTH/PTHrP receptor in many cells including leucocytes [28, 48–51] and osteoblasts [8, 52]. The observation that PTH application in CIA had no effect on the inflammatory aspects of the disease [36], is consistent with our findings suggesting that PTHrP is involved in bone remodelling rather than inflammatory events in CIA joints, and with a previous study by Funk et al. [18], where blockade of PTHrP had no effect on joint inflammation.

However, there is also contrasting evidence from studies by Fukata et al. [36] and Funk et al. [18] regarding the potential role of PTHrP in bone remodelling in inflammatory arthritis. Blockade of PTHrP and, therefore, decreased PTH/PTHrP receptor stimulation during disease induction and throughout the course of SWC-induced arthritis [18], ameliorated bone resorption. Whereas stimulation of the PTH/PTHrP receptor by PTH at late/bone remodelling stages of CIA had no effect on bone resorption [as indicated by serum tartrate-resistant acid phosphatase (TRAP) values], while it induced bone formation [36]. These differences may be due to the involvement of PTH/PTHrP receptor in regulation of bone resorption prior to its facilitation of bone deposition at late stages of the disease. Therefore, in future studies investigating PTHrP as a potential therapeutic target, its application or blockade should be considered in relation to the time frame of the inflammatory arthritis.

In summary, the changes in ILN and PLN PTHrP mRNA expression and its localization to HEVs, lymphocytes and...
monocytes/macrophages suggest that elevated levels of the cytokine are associated with initiation and/or propagation of the inflammatory immune response in lymph nodes. Changes in expression of PTHrP in DIP joints on monocytes/macrophages, neutrophils and osteoblasts amongst many others at sites of bone remodelling indicate involvement of PTHrP in late rather than early pathogenic events of CIA.

### Key messages

**Rheumatology**

- Elevated PTHrP mRNA is associated with lymph node hyperplasia in CIA.
- PTHrP is likely to be involved in late rather than early pathogenic events in CIA joints.

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