Differential metabolism of 25-hydroxyvitamin D₃ by cultured synovial fluid macrophages and fibroblast-like cells from patients with arthritis

M E Hayes, D Bayley, P Still, J Palit, J Denton, A J Freemont, R G Cooper, E B Mawer

Abstract
Differential metabolism of 25-hydroxyvitamin D₃ (25(OH)D₃) has been shown for macrophages and fibroblast-like cells (possibly synoviocytes) cultured for two to 50 days after isolation from the synovial fluid of 12 patients with various forms of inflammatory arthritis. Macrophages synthesised the active metabolite of vitamin D₃, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), the synthesis of which was increased by bacterial lipopolysaccharide, a known macrophage activating factor. In contrast, fibroblast-like cells formed 24, 25-dihydroxyvitamin D₃ (24,25(OH)₂D₃), synthesis of which was stimulated by 1,25(OH)₂D₃ and inhibited by lipopolysaccharide. The synthesis of 1,25(OH)₂D₃ and 24,25(OH)₂D₃ by macrophages and fibroblast-like cells respectively was inhibited by ketoconazole, indicating that both hydroxylases are dependent on cytochrome P-450.

Mean (SEM) synovial fluid and serum 25(OH)D₃ concentrations were 16.7 (1.7) and 22-2 (2-6) ng/ml and those of 1,25(OH)₂D₃ were 29.4 (4.8) and 43-3 (4-0) pg/ml respectively. In most cases concentrations were lower in synovial fluid than in paired serum samples, but in two patients 1,25(OH)₂D₃ concentrations were greater in synovial fluid than in serum, suggesting local synthesis within the affected joints.

Vitamin D₃ is hydroxylated by cytochrome P-450 dependent enzymes to 25-hydroxyvitamin D₃ (25(OH)D₃) in the liver and then to 24,25-dihydroxyvitamin D₃ (24,25(OH)₂D₃) or 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) in the kidney. Both 25(OH)D₃ and 24,25(OH)₂D₃ have limited biological effect on calcium homeostasis, whereas 1,25(OH)₂D₃ promotes intestinal calcium absorption, stimulates osteoclast mediated bone growth and mineralisation, increases osteoclastic bone resorption, inhibits renal 25(OH)D₃-1α-hydroxylase activity, and increases renal 25(OH)D₃-24-hydroxylase activity. All these actions seem to be induced after binding of 1,25(OH)₂D₃ to specific intracellular receptors and formation of a hormone-receptor complex that selectively activates or suppresses the transcription of various genes.

Studies on the extrarenal metabolism of 25(OH)D₃ have shown that 1,25(OH)₂D₃ is also synthesised in vitro by normal human macrophages activated with γ interferon, bacterial lipopolysaccharides, or tumour necrosis factor α, and by alveolar macrophages from patients with granulomatous conditions, such as sarcoidosis. In sarcoidosis this synthesis appears to be dependent on substrate and is not homero-statically controlled, in contrast with the well regulated renal synthesis of 1,25(OH)₂D₃ by parathyroid hormone, calcium, and 1,25(OH)₂D₃. Our own studies have shown that macrophages, activated by peritonitis, from the peritoneal cavity of patients with renal failure receiving continuous ambulatory peritoneal dialysis and macrophages cultured from synovial fluid of patients with arthritis also synthesise 1,25(OH)₂D₃.

Receptor binding studies on samples from patients with rheumatoid arthritis have shown that peripheral blood lymphocytes and fibroblasts derived from the synovial tissue express specific receptors for 1,25(OH)₂D₃ but the role of the metabolite in these cells is not known. Studies on the synthesis of 24,25(OH)₂D₃ in cultured cells have shown that this metabolite is occasionally formed by macrophages and is almost invariably synthesised by fibroblasts. In both cell types 24,25(OH)₂D₃ synthesis also seems to be increased by 1,25(OH)₂D₃.

As colonies of fibroblast-like cells may form in long term cultures of macrophages derived from synovial fluid we carried out this study to establish whether these two cell types express differential metabolism of 25(OH)D₃. We also measured 25(OH)D₃ and 1,25(OH)₂D₃ concentrations in paired serum and synovial fluid samples from the patients from whom cells were obtained.

Patients and methods
Samples of synovial fluid were obtained from 12 patients with knee joint effusions attending the rheumatology clinics of two local hospitals. Patients were assigned to well recognised diagnostic groups on the basis of clinical criteria and synovial fluid total and differential white cell counts (see table), supplemented by appropriate immunological, radiological, and crystallographic investigations. Only two patients with symmetrical polyarthritis or oligoarthropathies could not be assigned to a specific diagnostic group and were therefore classified as non-specific inflammatory arthritis.

Cells were harvested from synovial fluid (10-70 ml) by centrifugation at 2000 g for 15 minutes followed by resuspension in RPMI-1640 medium containing 10% fetal bovine serum, 2 mmol/l glutamine, and 50 μg/ml streptomycin (Flow Laboratories) to give about 10×10⁶ cells/ml. Cells were then plated at a density of 5×10⁵ cells per well (6-24 wells per well).
## Summary of patient data

<table>
<thead>
<tr>
<th>Sample</th>
<th>Diagnosis</th>
<th>Sex</th>
<th>Age</th>
<th>Synovial fluid value cell count (×10³/mL)</th>
<th>White cell differential (%)</th>
<th>25(OH)D₃ (pg/mL)</th>
<th>1,25(OH)₂D₃ (pg/mL)</th>
<th>Principal cell type present in culture</th>
<th>Day of culture on which cells were incubated (inc) with [1,H]25(OH)D₃</th>
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<tbody>
<tr>
<td>A</td>
<td>Reactive arthritis</td>
<td>M</td>
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<td>3-12</td>
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*Poly⁺=polymorph; lymph⁺=lymphocyte; macro⁺=macrophage; synov⁺=synoviocyte; SF⁺=synovial fluid.

A mobile phase of n-hexane: propan-2-ol: methanol, 110:6:4 by volume at 2 mL/min using a model 6000A HPLC solvent delivery pump (Waters Associates) and a Zorbox-Sil HPLC column (25 cm × 4.6 mm; Du Pont). For each analysis eluent fractions were collected by a model 202 fraction collector (Gilson) and the radioactivity associated with the substrates 24,25(OH)₂D₃ and 1,25(OH)₂D₃ estimated by a 1217 Rackbeta liquid scintillation counter (LKB Wallac). Standard 25(OH)₂D₃, 24,25 (OH)₂D₃ and 1,25(OH)₂D₃ and their tritiated forms were also chromatographed, and the retention times of these markers determined by ultraviolet absorption at 265 nm by a model 440 absorbance detector (Waters Associates) with an AR 25 linear chart recorder (Pye Unicam) or by liquid scintillation counting respectively.

For each experiment the cell numbers were determined with a haemocytometer after trypsinisation of parallel cultures, and the mean of four determinations from each well was used to normalise 25(OH)₂D₃-24-hydroxylase and 25(OH)₂-1α-hydroxylase activity for cell number. Cytocentrifuge preparations were also prepared for identification of cell lineage, which was determined by their morphology in Giemsa stained preparations and their reaction for non-specific esterase using a-naphthyl propionate as substrate.

Paired 3 mL serum and synovial fluid samples were also analysed for 25(OH)₃D₃ and 1,25(OH)₂D₃ as previously described in detail. Briefly, samples for assay were mixed with 3 mL of acetonitrile, applied to C18 silica Sep-paks (Waters Associates), and vitamin D metabolites were eluted in acetonitrile. Further separation of the metabolites was achieved by normal phase HPLC developed with hexane: isopropanol:methanol, 110:6:4 by volume at 2 mL/min using an automatic sample injector (Waters Associates), a programmable fraction model.
collector (LKB Superac), and a model 745 data module (Waters Associates). The 25(OH)D₃ fraction was reapplied to the automated HPLC system, developed with hexane:isopropanol, 98:2 by volume at 2 ml/min and the concentration determined from the height of the ultraviolet absorption peak recorded at 265 nm after calibration with a 25(OH)D₃ standard (detection limit 2 ng/ml). The fraction containing 1,25(OH)₂D₃ was assayed by radioimmunoassay with a detection limit of 3 pg/ml using an antiserum 02282.

Statistical analysis was carried out by Student’s t test and, where appropriate, p<0.05 or p<0.01 has been indicated as * or ** respectively.

Results
The table gives details of the samples from 12 patients with arthritis (six with rheumatoid arthritis; two with reactive arthritis; one with juvenile chronic arthritis; two with non-specific inflammatory arthritis; two with osteoarthritis—one secondary to rheumatoid arthritis). Mean (SEM) concentrations of vitamin D₃ metabolites in serum and synovial fluid respectively were 22.2 ± 2.6 and 16.7 ± 1.7 ng/ml for 25(OH)D₃ and 43.3 ± 4.0 and 29.4 ± 4.8 pg/ml (p<0.05) for 1,25(OH)₂D₃. In all patients synovial fluid 25(OH)D₃ was lower than in paired serum samples and only one serum sample had a low value (<5 ng/ml). In two patients (one with rheumatoid arthritis, one with hypertrophic osteoarthritis) serum 1,25(OH)₂D₃ was above the normal reference range (18–56 pg/ml, mean 37 (2SD)) and in two patients (one with juvenile chronic arthritis, one with reactive arthritis) synovial fluid 1,25(OH)₂D₃ concentrations were higher than in serum. The table also lists details of the cell types cultured from each synovial fluid sample and the number of days after isolation that incubations with [³H]25(OH)D₃ were carried out.

The macrophage cultures were characterised by non-proliferating cells which initially had the morphology of mononucleated monocytes and immature macrophages but after several days in culture had the appearance of larger more mature macrophages, many of which were multinucleated (three to 25 nuclei). These cells also stained for non-specific esterase, an enzyme characteristically expressed by monocytes and macrophages. In contrast, proliferating fibroblast-like cells could not readily be identified in early cultures; however, after a few days, rapidly growing colonies of these cells could be seen. It is most likely that the fibroblast-like cells developed from synoviocytes, which were identified in only five (A,B,C,D, and J) of the original 12 samples, four of which developed fibroblast colonies. Fibroblast-like cells also developed in samples F and G, of which sample G was from an osteoarthritic patient with a synovial fluid white cell count too low for a routine differential cell count to be carried out. However, osteoarthritic synovial fluid usually contains significant numbers of synoviocytes in addition to macrophages.

Figure 1a shows the HPLC analyses of [³H]25(OH)D₃ metabolism by cells cultured for 15 days after isolation from the synovial fluid of a patient with reactive arthritis (Reiter’s disease; sample A). These cells were predominantly macrophages (many multinucleated) which formed 1,25(OH)₂D₃, though some fibroblast-like cells were also present, accounting for some synthesis of 24,25(OH)₂D₃. Figure 1b shows the synthesis of 24,25(OH)₂D₃ by a 15 day culture of fibroblast-like cell colonies from the same sample. These cultures contained different cell types probably because the macrophages had been seeded from clumps of aggregated cells, whereas the fibroblast-like cells had developed from a suspension of single cells.

Figure 2 shows that exposure to lipopolysaccharide (0.5–100 µg/ml) for three to four days increased 1,25(OH)₂D₃ synthesis in five cultures of macrophages (samples C, D, E, H, I) and three macrophage cultures that also contained significant numbers of fibroblast-like cells (samples B, F, G). For all samples, except sample E, this increase was significant (p<0.01). An experiment carried out using fibroblast-free macrophage cultures from both knees of a patient with reactive arthritis (sample L) showed that lipopolysaccharide (20 µg/ml; days 3–14) maintained high levels of 1,25(OH)₂D₃ synthesis compared with cells cultured in the absence of lipopolysaccharide for 14 days (fig 3).
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Figure 4 compares the effect of 1,25(OH)₂D₃ (10⁻¹⁰⁻¹⁰⁻⁷ mol/l) or lipopolysaccharide (20 µg/ml) included in the culture medium for three days on 1,25(OH)₂D₃ synthesis by synovial fluid cells from two patients with rheumatoid arthritis (one with secondary osteoarthritis). In cells from sample B (mixed culture) increasing concentrations of 1,25(OH)₂D₃ in the medium decreased the formation of 1,25(OH)₂D₃ compared with control cells cultured in the absence of the hormone (p<0.05), where lipopolysaccharide greatly increased 1,25(OH)₂D₃ synthesis (p<0.01). In macrophages (fibroblast-free) cultured from sample E neither 1,25(OH)₂D₃ nor lipopolysaccharide altered 1,25(OH)₂D₃ synthesis; in addition, neither factor induced 24,25(OH)₂D₃ synthesis. In contrast, 1,25(OH)₂D₃ was a potent stimulator of 24,25(OH)₂D₃ synthesis by fibroblast-like cells in three samples which included sample B (mixed culture) and samples J and G, which were macrophage-free (fig 5). In two of these

![Graph](image-url)
samples (B and G) lipopolysaccharide reduced 24,25(OH)₂D₃ synthesis.

Figure 6 shows the inhibition of 1,25(OH)₂D₃ synthesis by macrophages (sample K) and of 24,25(OH)₂D₃ and 1,25(OH)₂D₃ synthesis by a mixed culture (sample F) by the drug ketoconazole, a cytochrome P-450 enzyme inhibitor. Similarly, figs 7 and 8 show ketoconazole inhibition of 1,25(OH)₂D₃ potentiated 24,25(OH)₂D₃ synthesis by fibroblasts and lipopolysaccharide induced synthesis of 1,25(OH)₂D₃ by macrophages (sample D) respectively.

Discussion

We examined the metabolism of 25(OH)D₃ by cultured macrophages and fibroblast-like cells and confirmed our original observation that synovial fluid derived macrophages synthesise the active vitamin D metabolite, 1,25(OH)₂D₃, in vitro. In addition, we showed that 1,25(OH)₂D₃ is formed from physiological concentrations (25 ng/ml) of the substrate. We also showed for the first time that synovial fluid derived synoviocyte/fibroblast-like cells can synthesise 24,25(OH)₂D₃, though previous studies in vitro have shown synthesis of 24,25(OH)₂D₃ by articular cartilage and by cultured chondrocytes. We showed that both 24,25(OH)₂D₃ and 1,25(OH)₂D₃ are formed in mixed cultures of synovial fluid cells containing macrophages and fibroblast-like cells, with the predominant metabolite relating to the cell type present. Cultures containing only fibroblast-like
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cells, however, did not synthesise 1,25(OH)2D3, and cultures containing only macrophages did not synthesise 24,25(OH)2D3. In contrast, other studies have shown that macrophages from a patient with alveolar rhabdomyosarcoma and macrophages derived from normal human blood and bone marrow can synthesise 24,25(OH)2D3.12 In the latter study, however, the cells were cultured with γ-interferon and initially formed 1,25(OH)2D3 before synthesising 24,25(OH)2D3 after prolonged exposure.12 Previous studies have also shown that lipopolysaccharide stimulates macrophages to form 1,25(OH)2D3 and that 1,25(OH)2D3 stimulates fibroblasts to form 24,25(OH)2D3, but synovial fluid cells have not previously been investigated. We have now shown similar effects for lipopolysaccharide and 1,25(OH)2D3 on 25(OH)D3 metabolism in synovial fluid macrophages and fibroblast-like cells respectively. In our study it was also apparent that lipopolysaccharide reduced fibroblast 24,25(OH)2D3 synthesis, but the effect of 1,25(OH)2D3 on its own synthesis by macrophages was less clear as synthesis was inhibited in one sample and unchanged in another. We also showed that synthesis of both 24,25(OH)2D3 and 1,25(OH)2D3 was inhibited in a dose dependent manner by ketonozole, cytochrome P-450 inhibitor, indicating that the 25(OH)2D3-24-hydroxylase and the 25(OH)2D3-1a-hydroxylase are both enzymes dependent on cytochrome P-450.

The assays of 25(OH)D3 concentrations were lower in all synovial fluid samples than in paired serum samples, with mean (SEM) values of 16.7 (1.7) 22-2 (2.6) ng/ml respectively. This observation has previously been reported in other studies,21 22 one of which also showed that both 24,25(OH)2D3 and the vitamin D binding protein were present in synovial fluid at about half serum concentrations.21 The lower concentration of vitamin D binding protein in synovial fluid is the most likely cause of the lower 25(OH)D3 concentrations in synovial fluid than in serum. 1,25(OH)2D3 concentrations were also lower in most synovial fluid samples, with mean (SEM) values of 29.4 (4.8) and 43.3 (4.0) pg/ml (p<0.05) for synovial fluid and serum respectively. The exceptions were one patient with juvenile chronic arthritis, in whom synovial fluid and serum concentrations were similar, and a patient with Reiter’s disease, in whom the synovial fluid of both left and right knees had greater 1,25(OH)2D3 concentrations than in serum. Reiter’s disease is a reactive arthritis, previously shown to be the most likely form to have synovial fluid macrophages capable of forming 1,25(OH)2D3, and thus the raised concentrations of 1,25(OH)2D3 were probably synthesised locally by activated macrophages present in the synovial fluid. In two other patients (one with rheumatoid arthritis, one with hypertrophic osteoarthritis) serum but not synovial fluid 1,25(OH)2D3 concentrations were above the normal reference range (18–56 pg/ml). In these patients the high serum values were probably not related to the ability of cells within synovial fluid to form 1,25(OH)2D3 but to the more mature activated macrophages likely to be found within synovial tissue itself. Overall, the determinants of serum and synovial fluid 1,25(OH)2D3 concentrations may be different. 

Further evidence that extrarenal synthesis of 1,25(OH)2D3 may occur in patients with rheumatoid arthritis is provided by a study in which the serum 1,25(OH)2D3 response to a challenge dose of 25(OH)D3 was significantly greater in patients than in healthy controls.23 In the latter study two patients also had initial serum 1,25(OH)2D3 concentrations above normal and two patients had synovial fluid 1,25(OH)2D3 concentrations greater than those in paired serum samples. In the few studies published to date, however, there is no evidence that patients with rheumatoid arthritis as a group have higher than normal circulating concentrations of 1,25(OH)2D3 as a result of net renal and extrarenal synthesis.23 24 This may in part result from feedback regulation of the renal 1,25(OH)2D3 synthesis by that formed outside the kidney. Synthesis of 1,25(OH)2D3 by macrophages, which is poorly regulated and seems to depend on the substrate concentration, may only result in raised serum concentrations when the 25(OH)D3 concentrations are abnormally high or the mass of tissue affected is large, as in some cases of sarcoidosis24 and malignant disease.25

The presence of specific receptors for 1,25(OH)2D3 in normal human monocytes and activated lymphocytes26 27 and in peripheral blood lymphocytes and synovial tissue derived fibroblasts28 from patients with rheumatoid arthritis indicate an important local role for 1,25(OH)2D3 in joint disease. Many of the reported receptor mediated effects of 1,25(OH)2D3 may be relevant to arthritis; for example, this metabolite seems to inhibit proliferation of both B and T activated lymphocytes and to reduce interleukin 2 production by activated T lymphocytes in vitro.28 29 1,25(OH)2D3 has also been shown to promote differentiation of monocytes into macrophages and the fusion of macrophages to multinucleated giant cells with bone resorbing activity.30 31 This is in addition to the known ability of 1,25(OH)2D3 to inhibit collagen synthesis by osteoblasts and to induce bone resorption by mature multinucleated osteoclasts, which unlike osteoblasts do not seem to have receptors for 1,25(OH)2D3.33 Thus within the synovial fluid and synovium 1,25(OH)2D3 may have complex paracrine and immunoregulatory functions which may be highly relevant to the joint disease and to the localised periarticular osteoporosis associated with arthritis.34 35 Control of its synthesis and
elucidation of its action in the arthritic joint clearly warrant further study.

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