Vitamin D receptors in the rheumatoid lesion: expression by chondrocytes, macrophages, and synoviocytes

Lynne C Tetlow, Susan J Smith, E Barbara Mawer, David E Woolley

Abstract

Objectives—The active form of vitamin D_3, $1\alpha,25$ dihydroxyvitamin D_3 ($1,25D_3$), through its interaction with vitamin D receptors (VDR), is reported to effect a variety of anabolic and catabolic events, especially in bone and cartilage tissues. As cartilage degradation and tissue remodelling are characteristic features of the rheumatoid lesion, the distribution and expression of VDR at sites of cartilage erosion was examined.

Methods—Immunolocalisation techniques using a rat monoclonal antibody to VDR and an alkaline phosphatase conjugated avidin/biotin detection system were used to examine VDR in 18 specimens of cartilage-pannus junction, 10 specimens of rheumatoid synovium or cartilage tissue, and four primary cultures of adherent rheumatoid synovial cells (RSC). For comparison, VDR expression was examined in 10 specimens of normal, healthy age matched articular cartilage.

Results—VDR was demonstrated in 15 of 18 cartilage-pannus junctions either at the interface (8 of 18), within the pannus tissue (12 of 18), and by chondrocytes often close to the erosive lesion (10 of 18). All the rheumatoid synovial tissue and 5 of 10 cartilage specimens showed cells with positive staining, but the extent of this was variable. Negligible VDR staining was observed for normal cartilage. Primary cultures of RSC also showed variability in both the numbers and proportions of macrophages or synovial fibroblasts stained for VDR (range 10–50%), this being more common in cultures with a high proportion of macrophages.

Conclusions—VDR expression has been demonstrated by most specimens of cartilage-pannus junction; was associated with various cell types, including chondrocytes, but not exclusively with CD68$^+$ macrophages. The focal nature of VDR expression within the rheumatoid lesion suggests a contributory role for $1\alpha,25D_3$ in the pathophysiological processes of rheumatoid arthritis.

(*Ann Rheum Dis* 1999;**58**:118–121)

University Department of Medicine, Manchester Royal Infirmary, Oxford Road, Manchester, M13 9WL

Correspondence to:
Dr D E Woolley.

The biologically active metabolite of vitamin D, $1\alpha,25$dihydroxyvitaminD_3 ($1,25D_3$), has a recognised role in a variety of bone and mineral disorders such as Rickets and osteoporosis, has a pivotal role in calcium and phosphorous metabolism, and has been shown to have other biological roles such as the regulation of cell proliferation, differentiation and immune function.[1] $1,25D_3$ functions by binding to specific intracellular vitamin D receptors (VDR) that are expressed by a variety of cell types. These include monocytes, fibroblasts, activated lymphocytes, and various myeloid cells.[1,2] Recent studies have examined the roles of $1,25D_3$ and VDR expression in chondrocyte cultures and their contribution to the behaviour of different zones of growth plate cartilage.[3,4] Such studies have shown that vitamin D metabolites are capable of regulating the transcription of a large number of genes relating to both anabolic and catabolic events of chondrocytes in culture.[1,3,5] Their effects on chondrocyte phenotype are shown to be dependent upon the cartilage source and the differentiated state.[6,7] For example, vitamin D metabolites are reported to modulate chondrocyte functions such as proteoglycan and collagen synthesis,[5,7] and to regulate specific matrix metalloproteinase expression by chondrocytes[8,9] as well as human mononuclear phagocytes.[10]

Human blood derived macrophages are reported to express mRNA for VDR[11] and functional VDR have been detected in pulmonary alveolar macrophages,[12] but to date no reports of VDR at sites of cartilage erosion in rheumatoid arthritis (RA) have been published. Here we demonstrate, by immunolocalisation, that different types of cell in the rheumatoid lesion express VDR, suggesting the possibility that vitamin D metabolites could be implicated in the pathophysiological processes associated with rheumatoid disease.

Methods

Samples of rheumatoid synovial tissue (n=10), cartilage tissue (n=10), and cartilage-pannus junction (n=18) were obtained from arthroplasty specimens from patients with classic late stage RA. Normal articular cartilage samples (n=10) were obtained from lower limb amputations. Tissue samples were fixed in Carnoy's fixative at 20°C for 1–2 hours, embedded in paraffin wax and 5 μm sections cut. Tissue sections were dewaxed, rehydrated, and examined for the presence of VDR; in some cases consecutive sections were also examined for the presence of macrophages. In addition rheumatoid synovial tissue (n=4) was enzymically digested as previously described,[13] and the resulting adherent cell cultures were grown on glass coverslips in Dulbecco's modified

Eagle's medium with 10% fetal calf serum supplement for 24 hours before fixation in 70% ethanol and subsequent immunostaining.[13]

IMMUNOLOCALISATION OF VDR

Tissue sections were pretreated with 2N HCl at 37°C for 30 minutes, this being the antigen retrieval procedure recommended by the supplier. Non-immune rabbit serum at 10% (v/v) in TRIS buffered saline (TBS) was applied to the sections for 20 minutes at 20°C before incubation with the primary antibody. Rat monoclonal antibody to chick VDR (known to cross react with human VDR), (Biogenex, San Ramon, USA) was applied to the sections for two hours at 20°C after dilution 1:40 in TBS. After 3 × 10 minute washing in TBS, biotinylated rabbit antirat IgG (DAKO, Glostrup, Denmark) diluted 1:200 in TBS was applied to the sections for 45 minutes at 20°C. After further washing in TBS, alkaline phosphatase conjugated ABC (Avidin-Biotin system, DAKO) was applied to the sections for 45 minutes at 20°C, diluted as instructed by the supplier. After further washes in TBS the alkaline phosphatase was developed using New Fuchsin substrate to give a red colour. Adherent primary cell cultures were rehydrated in TBS immediately after fixation and immunostained for VDR as described above.

IMMUNOLOCALISATION OF MACROPHAGES

Consecutive tissue sections to those immunostained for the VDR were immunostained for macrophages using mouse monoclonal antibody (CD68/KP1, DAKO) diluted 1:100 in TBS. Biotinylated rabbit antimouse IgG secondary antibody (diluted 1:300, DAKO) was used with the procedure as described above.

CONTROL TISSUE SECTIONS

Controls consisted of substituting the primary antibodies with non-immune rat or mouse IgG at similar concentrations and proceeding as described above. All consistently gave negative results.

DUAL IMMUNOLOCALISATION OF VDR AND MACROPHAGES IN PRIMARY CELL CULTURES

Cells were rehydrated in TBS and pretreated with 10% (v/v) goat serum and 10% (v/v) rabbit serum in TBS for 10 minutes at 20°C. Rat monoclonal antibody to VDR and mouse monoclonal antibody to macrophages (CD68/

KP1) were diluted in TBS (1:40 and 1:100, respectively) and applied to the coverslips for two hours at 20°C. After washing three times in TBS, biotinylated rabbit antirat IgG (DAKO) diluted 1:200 and fluoroisothiocyanate (FITC) conjugated goat antimouse IgG (DAKO) diluted 1:20 were applied for one hour at 20°C. Both secondary antibodies were preabsorbed to eliminate cross species reactivity. After washing three times, alkaline phosphatase conjugated ABC (DAKO) was applied to the sections for 45 minutes at 20°C and subsequently developed using Fast Red, which fluoresces under conventional filter sets for rhodamine or Texas red. Coverslips were washed and mounted in Immumount (Shandon). Fluorescence was viewed with filter sets for FITC or FR.

All tissue sections were lightly counterstained in either Harris's haematoxylin or acidified Toluidine blue, dehydrated, cleared and subsequently mounted in Histomount (Mensura Technology, Wigan, UK). Tissue sections and cell cultures were examined and photographed using a Zeiss Photomicroscope III and Ektachrome 160 tungsten film.

Results

VDR EXPRESSION AT CARTILAGE–PANNUS JUNCTIONS (CPJS)

Most of the 18 specimens of CPJ examined for VDR expression showed positively stained cells (table 1). Twelve specimens showed significant staining of synoviocytes within the pannus tissue, 10 demonstrated staining of chondrocytes within the cartilage matrix, and eight specimens showed VDR expression by cells at the cartilage-pannus interface (fig 1a and b). In all cases the cellular staining was restricted to a variable proportion of the total cells within each tissue. This was particularly evident with the cartilage where chondrocytes located close to the cartilage:pannus interface were more likely to demonstrate VDR staining. Indeed, 8 of the 18 CPJ specimens showed prominent staining of cells in immediate contact with the cartilage matrix (fig 1b); such observations were restricted to microfoci, and never extended along the whole length of any CPJ specimen. By contrast, nearly all of the 10 specimens of normal articular cartilage showed no evidence of VDR expression (fig 1c; table 1).

VDR EXPRESSION IN RHEUMATOID SYNOVIAL TISSUE OR CARTILAGE

All of the 10 synovial specimens examined contained some cells stained for VDR, but the extent of this staining was variable, ranging from very few to up to 50% of total cells in one specimen (table 1). Immunostaining for macrophages using the CD68 marker showed that these cells were often associated with VDR expression, as judged by consecutive sections stained for both antigens. However, VDR expression within synovial tissue was not exclusive to macrophages; a minor proportion of other cells negative for CD68 such as fibroblastic synoviocytes, lymphocytic infiltra-

Table 1   Frequency of VDR positive cells in rheumatoid tissues

	Assessment of positive staining				
VDR⁺ specimens/total	++++	+++	++	+	−
1 Rheumatoid synovium* (n=10)					
10/10	1	4	3	2	0
2 "Rheumatoid" cartilage (n=10)					
5/10	0	0	0	5	5
3 CPJs (n=18)					
chondrocytes 10/18	0	0	3	7	8
pannus 12/18	0	0	3	9	6
interface 8/18	0	0	2	6	10
4 Normal cartilage (n=10)					
1/10	0	0	0	1	9

+, ++, +++, and ++++ represent gradings of <5, 5–10, 10–20 and up to 60% of total cells, respectively, as determined by two independent assessors; −, no cells stained for VDR. *Synovial tissue remote from cartilage interface.

tions containing a high proportion of B cells and endothelial cells were also positively stained for VDR (fig 1e and f).

Cartilage specimens from RA joints also showed a proportion of chondrocytes immunostained for VDR (5 of 10), especially in the
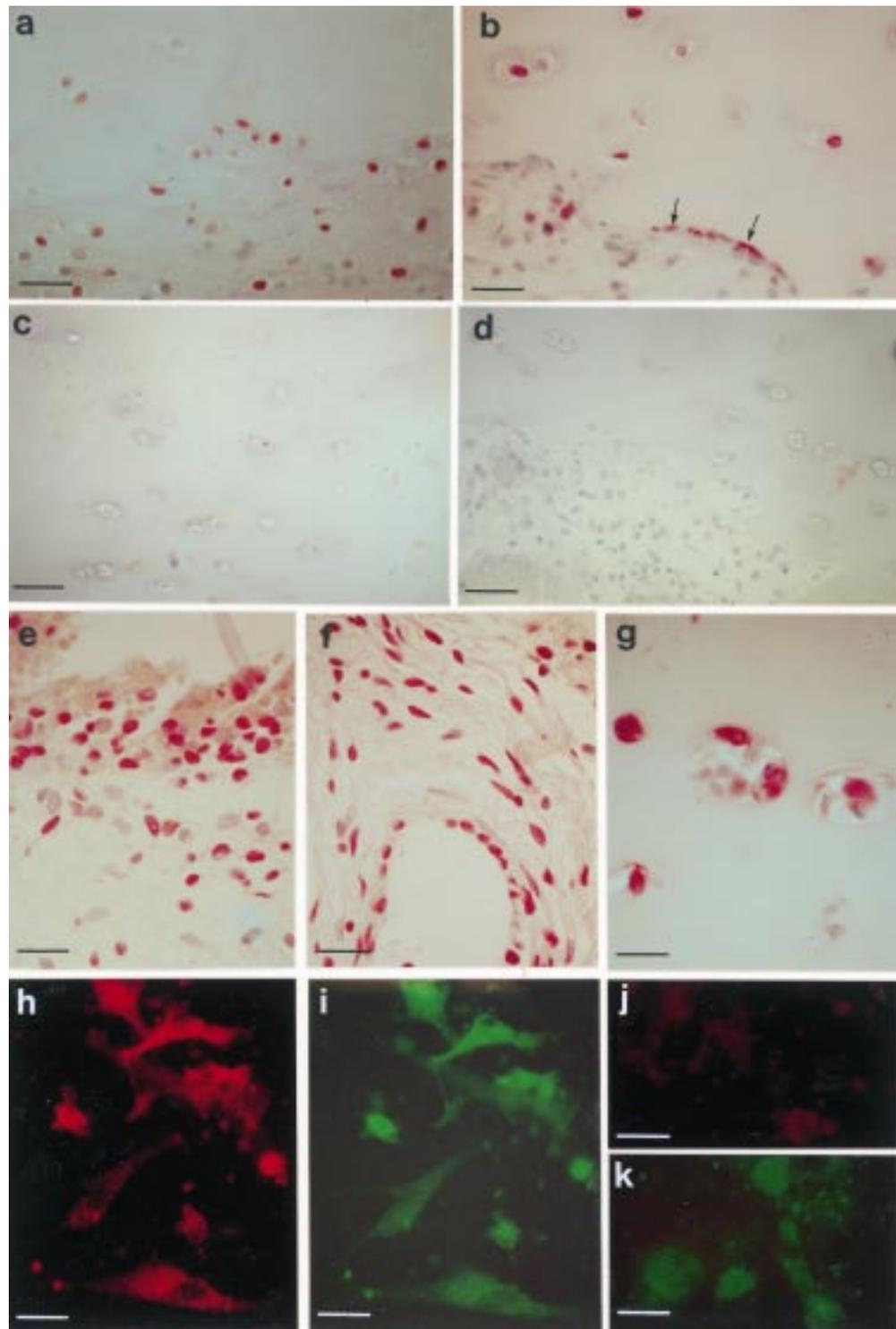
Figure 1   Immunolocalisation of VDR in rheumatoid tissues, normal cartilage, and primary synovial cell cultures. (a) VDR production (red stain) by a proportion of synovial cells close to the CPJ. (b) VDR expression by cells at the cartilage interface (arrows) and by some chondrocytes at the CPJ. (c) Note absence of VDR expression by chondrocytes of normal articular cartilage. (d) Negative control: adjacent tissue section to (b) in which primary rat antibody was substituted with normal rat IgG. (e) VDR expression by cells of the synovial lining layer and a proportion of sub-lining synoviocytes. (f) VDR production by endothelial cells and a proportion of the rheumatoid synoviocytes. (g) VDR expression by chondrocytes close to the CPJ. (h) Primary culture of RSC stained for VDR with Fast Red (i) same field as (h) stained with macrophage marker, CD68 (green). (j) and (k) Negative controls for the staining procedures used in (h) and (i) respectively. Note that all the VDR staining is intracellular; and that all micrographs also illustrate negatively stained cells. (a) and (b) Bar = 35 µm, (c) and (d) bar = 50 µm, (e) and (f) bar = 25 µm, (g), (h), (i), (j) and (k) bar = 20 µm.

superficial rather than deeper zones of the cartilage (fig 1g, table 1).

VDR EXPRESSION BY PRIMARY CULTURES OF RSC
Adherent RSC are usually restricted to cells of the fibroblastic and macrophagic morphologies, the proportions of these cell types varying both with individual specimens and with time in culture.[13] Each of four cultures examined showed some positive staining for VDR, but this ranged from approximately 10% in one culture to approximately 60% in another. CD68 staining of each RSC culture indicated that the VDR was more prominent in those cultures containing more macrophages, but VDR was not exclusive to these cells (fig 1h and i)

Discussion

$1,25D_3$ has a recognised role in a variety of bone and mineral disorders, and has been shown to modulate cell proliferation, differentiation, and immune functions.[1] Of special interest were reports that $1,25D_3$ caused an increase in matrix metalloproteinase (MMP) production by chondrocytes in vitro,[8 9] and that it regulates the levels of interleukin 1 in growth plate cartilage.[3] Moreover, $1,25D_3$ has been shown to modulate specific MMP production by macrophages, especially MMP-1 and MMP-9.[10]

This paper provides evidence for the in vivo expression of VDR by various cells of the rheumatoid lesion, including macrophages, chondrocytes, and synoviocytes. By contrast, normal articular cartilage was devoid of VDR expression with negligible staining being observed in only 1 of 10 specimens. Such observations suggest that VDR expression is significantly upregulated in RA tissues.Our earlier studies have used dual immunolocalisation techniques to demonstrate the co-distributions of the proinflammatory cytokines IL1β, TNFα, together with MMP-1 and MMP-3 within microfocal sites of the rheumatoid lesion.[14] At present it is not known whether VDR expression is present within such tissue sites. However, the recent reports that macrophages from synovial fluids can synthesise $1,25D_3$[15 16] and the increased concentrations of vitamin D metabolites reported in human arthritic synovial fluids,[17] lend support to the concept that VDR and $1,25D_3$ could well participate in the pathophysiological processes of the rheumatoid lesion.

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