Osteoarthritis and Cartilage (2001) **9,** 423–431
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doi:10.1053/joca.2000.0408, available online at http://www.idealibrary.com on IDEAL®
1063–4584/01/050423+09 $35.00/0

Osteoarthritis and Cartilage
Journal of the OsteoArthritis Research Society International

Expression of vitamin D receptors and matrix metalloproteinases in osteoarthritic cartilage and human articular chondrocytes *in vitro*

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Summary

Objectives: To examine the *in situ* distributions of vitamin D receptors (VDR) and matrix metalloproteinases (MMPs) in osteoarthritic cartilage for comparison with non-arthritic, normal cartilage; and to assess the *in vitro* effects of 1α,25 dihydroxyvitaminD_3 (1α,25$(OH)_2D_3$) on MMPs-1, -3 and -9 and prostaglandin E_2 (PGE_2) production by cultures of human articular chondrocytes (HAC) shown to be VDR-positive.

Methods: Using immunohistochemistry VDR expression in different specimens of osteoarthritic cartilage (N=11) was compared to that in normal cartilage (N=6), along with the immunodetection of MMPs-1, -3 and -9. The effects of 1α25$(OH)_2D_3$ on MMP and PGE_2 production by HAC *in vitro*, with and without stimulation by TNFα or phorbol myristate acetate (PMA), was evaluated using ELISA methodology.

Results: VDR was demonstrated in HAC of all specimens of osteoarthritic cartilage, especially the superficial zone, whereas only two of five normal cartilage specimens were VDR^+ for a minor proportion of HAC. Immunolocalization of MMPs-1, -3 and -9 was often seen in areas where chondrocytes were VDR^+, and dual immunolocalization has demonstrated individual chondrocytes positive for both VDR and MMP-3 *in situ. In vitro*, 1α25$(OH)_2D_3$ alone had no effect on MMP-1, -9 and PGE_2 production by HAC, but MMP-3 production was up-regulated by 1α25$(OH)_2D_3$ either with or without stimulation with TNFα or PMA. By contrast the increased production of MMP-9 and PGE_2 induced by PMA was significantly suppressed by concomitant treatment with 1α25$(OH)_2D_3$.

Conclusions: The demonstration of VDR expression by HAC in osteoarthritic cartilage was often associated with sites where MMP expression was prevalent, observations in contrast to their virtual absence in normal age-matched cartilage. Together with HAC *in vitro* studies, the data suggests that 1α25$(OH)_2D_3$ contributes to the regulation of MMP and PGE_2 production by HAC in osteoarthritic cartilage.

Key words: Osteoarthritis, Vitamin D receptors, 1α,25dihydroxyvitaminD_3, Matrix metalloproteinases, Chondrocytes, Prostaglandin E_2.

Introduction

Osteoarthritis (OA) is the major cause of disability in the elderly yet little is known about its aetiopathogenesis[1]. Early changes in osteoarthritic cartilage include fraying of the superficial articular surface, the appearance of fibrillations and loss of cartilage proteoglycan (PG), each of which becomes more prominent as the disease progresses, along with the noticeable proliferation of chondrocytes into clusters or 'clones'[2]. Chondrolytic matrix metalloproteinases (MMPs) are known to be important in osteoarthritic cartilage degradation[3] where the focal expression of mRNA for different MMPs by chondrocytes has been reported[4]. Cytokines have been implicated in the complex regulation of MMP production by chondrocytes[5], and recent animal studies have demonstrated a contributory role for vitamin D3 in cartilage metabolism[6–8].

The biologically active metabolite of vitamin D, 1α,25dihydroxyvitaminD_3, (1α,25$(OH)_2D_3$), has a recognized role in calcium and phosphorus metabolism and in a variety of bone and mineral disorders such as osteoporosis and rickets[9]. 1α,25$(OH)_2D_3$ has other biological functions including the regulation of cell proliferation, differentiation and immune function[10] brought about by its binding to vitamin D receptors (VDR) that are expressed by a variety of cell types such as monocytes, fibroblasts, activated lymphocytes, various myeloid cells and also chondrocytes[10,11]. 1α,25$(OH)_2D_3$ is purported to modulate chondrocyte behavior within different zones of the growth plate cartilage[8,12] and has different effects on proteoglycan and collagen synthesis, plasminogen activator expression and the regulation of matrix metalloproteinase production[12,13]. However, most information to date relates to animal models or chondrocyte cultures derived from rat or rabbit cartilage.

The recent demonstration of 1α,25$(OH)_2D_3$ synthesis by macrophages derived from the synovial fluid of patients with inflammatory arthritis[14,15], and the report that chondrocytes express VDR[11] have suggested a contributory role for 1α,25$(OH)_2D_3$ in the aberrant behavior of chondrocytes in OA. The present study has assessed the nature and extent of VDR expression in osteoarthritic cartilage together with the distributions of MMPs-1, -3 and -9 *in situ*. In addition, we have examined the effects of 1α,25$(OH)_2D_3$ on MMP production by human articular chondrocytes *in vitro*, especially after activation by tumor necrosis factor-α (TNF-α) and phorbol myristate acetate (PMA). Further, since prostaglandin E_2 (PGE_2) is known to contribute to bone remodeling/resorption and may modulate MMP expression[16,17], the effects of 1α,25$(OH)_2D_3$ on PGE_2 production by stimulated or 'activated' HAC has also been examined.

Received 21 July 2000; revision requested 14 September 2000; revision received 3 November 2000; accepted 28 November 2000.
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Table I
Frequency and distribution of vitamin D receptors (VDR) and matrix metalloproteinases (MMP)-1, -3 and -9 in osteoarthritic cartilage of different disease grades

Specimen	Grade	Superficial zone				Deep zone			
		VDR	MMP-1	MMP-3	MMP-9	VDR	MMP-1	MMP-3	MMP-9
698/2	3	+++	++	++	−	++	+	+	−
771/1	3	+++	+	++	+	++	+	−	−
707/3	3	++++	+++	++	+	+++	++	++	++
805/2	3	++++	++++	+++	+	++	++	++	−
697/3	2	+++	++	+++	+++	++	+	++	++
699/3	2	++	+++	++	++	+	++	−	−
792/5	2	++	+	+	−	+	−	−	−
794/2	2	++	+	−	+	+	−	−	−
667/1	1	+++	++	+	−	+	−	−	−
692/9	1	+++	+	+	−	+	+	+	−
804/2	1	+++	++	++	+	++	+	+	−
Normals									
NC/98/1	0	−	−	−	−	−	−	−	−
NC/98/3	0	−	+	−/+	−	−	−	−	−
NC/98/6	0	+	−	−	−	+	−	−	−
NC/98/8	0	−	−	−/+	−	−	−	−	nd
NC/99/2	0	+	−	−	−	−	−	−	−

Key: Histological Grades are described as follows: Grade 0, normal matrix and chondrocyte morphology; Grade 1, uneven cartilage surface with loss of metachromasia around enlarged chondrocytes of superficial zone, no fibrillations or chondrocyte clusters; Grade 2, some surface erosion, fibrillations and small chondrocyte clusters in superficial zone, decreased metachromasia extending to deep zone; Grade 3, deeper surface erosions with fibrillations extending into deep zone, large number of chondrocyte clusters containing several cells, major degenerative changes and loss of metachromasia in cartilage matrix.

Percentage of total chondrocytes in each zone positively stained for antigen: −, none; +, <10%; ++, 10–40%; +++, 40–80%; ++++, >80%. nd=not done.

Statistical analyses (Student's *t*-test) between osteoarthritic and normal specimens showed significant differences for the immunodetection of VDR and the three MMPs, viz: VDR, $P=0.0002$*; MMP-1, $P=0.003$*; MMP-3, $P=0.01$*; MMP-9, $P=0.05$*. In addition , for the VDR+ and MMP+ distributions between the superficial and deep zones of osteoarthritic cartilage, *t*-test *P* values were as follows: VDR, $P=0.00003$*; MMP-1, $P=0.0004$*; MMP-3, $P=0.002$*; MMP-9, $P=0.005$*. *=statistically significant.

Methods

TISSUE SPECIMENS

Osteoarthritic knee tissues, from 30 different patients, were removed at joint replacement surgery and transferred to the laboratory in Hanks balanced salt solution (HBSS) where cartilage was sampled from one tibial plateau of each specimen within 60 min of operation. Similar age-matched, macroscopically normal cartilage samples were obtained from six early autopsy cases. Cartilage tissues were sampled down to the subchondral bone, fixed in Carnoy's fixative for 2 h at 20°C, dehydrated in alcohol and processed to paraffin wax. 5 μm cartilage tissue sections from the different specimens were stained with acidified toluidine blue to determine histological grading. Each specimen was examined by two independent assessors and graded as 0, 1, 2 or 3, defined in Table I and adapted from previous studies of clinical grading[4,18]. Sixteen specimens were selected for an immunological study of VDR and MMP distributions; these comprised 11 osteoarthritic cartilage samples representing grades 1, 2, 3, and five non-arthritic normal cartilage samples, grade 0. Two cartilage zones were identified, viz. a 'superficial zone' beneath the articular surface and a 'deep zone' as previously defined and illustrated[2,4]. The minimum number of cells examined in any zone was 40 (range 40–225, mean value 95). All cartilage specimens were derived from individuals in the age range 47–76 years (mean 68). 5 μm sections were cut and consecutive sections were immunostained for VDR, MMP-1, MMP-3 and MMP-9, respectively.

IMMUNOLOCALIZATION OF VDR

Tissue sections were pre-treated with 2M HCl at 37°C for 30 min, an antigen retrieval procedure recommended by the supplier of the primary antibody. Non-immune rabbit serum at 10% (v/v) in Tris-buffered saline (TBS) was applied to the tissue sections for 20 min at 20°C. Rat monoclonal antibody to chick VDR (known to cross-react with human VDR; Cambridge BioScience, U.K.) was applied for 2 h at 20°C after dilution to 5 μg/ml in TBS. After 3×10 min washes in TBS, biotinylated rabbit antirat IgG (DAKO Ltd, Cambridge, U.K.) diluted 1:200 in TBS was applied to the sections for 45 min at 20°C. After further washing in TBS, alkaline phosphatase (AP) conjugated ABC (Avidin-Biotin Complex system, DAKO) was applied to the sections for 45 min at 20°C. After further washing in TBS the AP was developed using new fuchsin substrate to give a red color. Sections were lightly counterstained with toluidine blue before dehydrating and mounting in Histomount permanent mountant (National Diagnostics, Hull, U.K.).

IMMUNOLOCALIZATION OF MATRIX METALLOPROTEINASES -1, -3, AND -9

Tissue sections were first incubated for 30 min with 10% (v/v) donkey serum. Sheep polyclonal antibodies to MMPs-1, -3 and -9 (Biogenesis, Poole, U.K.) were diluted 1:300, 1:500 and 1:250, respectively, in TBS and applied to the sections for 2 h at 20°C followed by 3×10 min washes

in TBS. Biotinylated donkey anti-sheep IgG (Sigma, Poole, U.K.) was diluted 1:300 in TBS and applied to the sections for 45 min at 20°C. After further washing AP-conjugated ABC was applied and developed as described above. Sections were counterstained in toluidine blue or Harris's haematoxylin and mounted.

IMMUNOLOCALIZATION OF S-100 PROTEIN IN NORMAL CARTILAGE SPECIMENS

The S-100 protein is a recognized marker for human articular chondrocytes[19] and its antibody was shown to effectively access the chondrocytes of normal cartilage. Tissue sections were first incubated for 30 min with 10% (v/v) swine serum. Rabbit polyclonal antibody to S-100 protein (Dako, U.K.) was diluted 1:100 in TBS and applied to the sections for 2 h at 20°C followed by 3×10 min washes in TBS. Biotinylated swine antirabbit IgG (Dako, U.K.) was diluted 1:300 in TBS and applied to the sections for 45 min at 20°C. After further washing AP-conjugated ABC was applied and developed as described above. Sections were lightly counterstained in toluidine blue.

DUAL IMMUNOLOCALIZATION OF VDR AND MMP-3

Tissue sections were pretreated with rabbit serum 10% (v/v) for 30 min after which primary antibodies to VDR and MMP-3, diluted in TBS as described above were applied together at 4°C overnight. The sections were washed 3×10 min in TBS followed by incubation with a combination of biotinylated rabbit antirat IgG and horseradish peroxidase (HRP)-conjugated rabbit anti-sheep IgG for 1 h at 20°C. Following a further 3×10 min washes inTBS the sections were incubated with AP-conjugated ABC complex as described above for 45 min at 20°C. After further washing the HRP was developed first using 3-amino-9-ethylcarbazole (AEC; Sigma, U.K.) to give a red color, followed by development of the AP using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT; Sigma, U.K.) to give a blue-black color. Sections were mounted in Immunomount aqueous mountant (Shandon, U.K.).

IMMUNOHISTOCHEMISTRY CONTROLS

For controls the primary antibody was replaced by the appropriate non-immune rat or sheep IgGs at concentrations corresponding to those of the primary antibodies. Immunoadsorption of each antibody with its relevant purified antigen subsequently produced negative observations, as described previously[20,21].

CELL CULTURES

Human articular cartilage from OA specimens was enzymically digested to provide chondrocyte cultures as previously described[22]. Primary chondrocyte cultures grown to confluence in Dulbecco's Modified Eagle's Medium (DMEM)+10% (v/v) fetal calf serum (FCS), were harvested and seeded into 12-well culture dishes (Nunc, Gibco, U.K.). Triplicate wells of sub-confluent HAC in DMEM+2% FCS were treated with $1\alpha25(OH)_2D_3$ (10^{-8} M); TNFα (20 ng/ml); or TNFα+$1\alpha25(OH)_2D_3$ (20 ng/ml and 10^{-8} M, respectively) and incubated for 48 h at 37°C. Similarly, triplicate wells of sub-confluent HAC in DMEM+2%FCS were treated with either PMA (25 ng/ml) or PMA+$1\alpha,25(OH)_2D_3$ (25 ng/ml and 10^{-8} M, respectively). The conditioned medium was collected after 48 h and assayed for MMPs -1, -3, and -9, and prostaglandin E_2 (PGE_2) using enzyme-linked immunosorbent assay(ELISA) methodology. Cell numbers/well were counted at the end of each experiment following 70% ethanol fixation and toluidine blue staining (six fields of 1 mm^2, total area=6 mm^2).

Chondrocytes were also grown on glass coverslips in DMEM+2% FCS and at various stages of confluence the cells were briefly fixed in Carnoy's fixative and immunostained for VDR and S-100 as described for the cartilage tissue sections. Cells were also immunostained for type II cartilage collagen using rabbit polyclonal antibody (Chemicon, Harrow, U.K.), diluted 1:20 and visualized with the ABC detection system as described.

ENZYME LINKED IMMUNOSORBENT ASSAYS (ELISA)

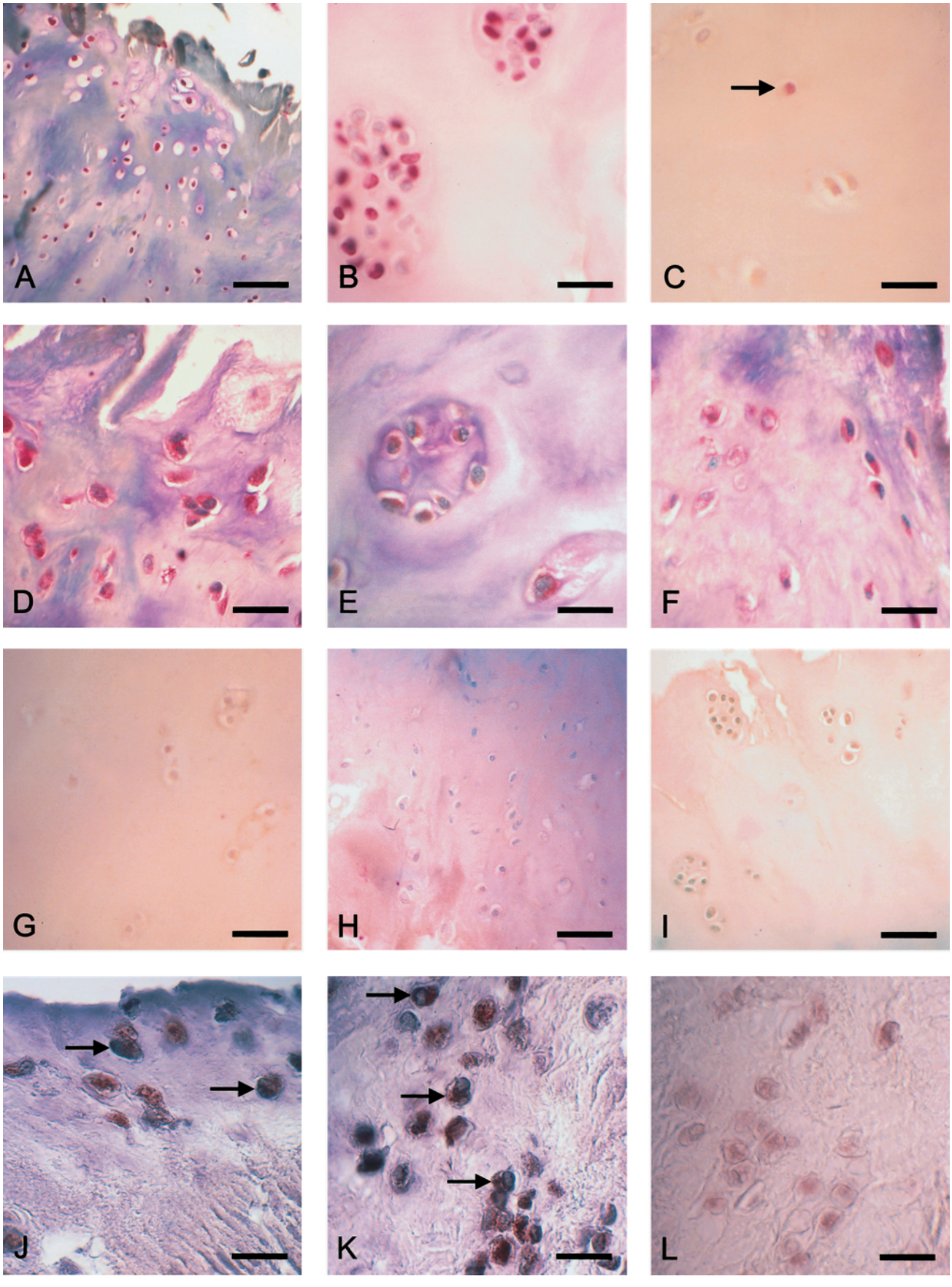
ELISA methodology was used to determine protein levels of MMP-1 (collagenase 1, sensitivity: 0.8–50 ng/ml), MMP-3 (stromelysin, sensitivity: 0.4–25 ng/ml) and MMP-9 (gelatinase B, sensitivity: 1.5–100 ng/ml) as previously described[23]. PGE_2 was measured using a competitive binding assay kit purchased from R & D Systems Europe Ltd, Abingdon, U.K., with a sensitivity range of 39–5000 pg/ml. All ELISA results were calculated as ng or pg antigen/ml culture medium/10^6 cells/48 h.

Results

IMMUNOHISTOCHEMISTRY

All eleven specimens of osteoarthritic cartilage showed chondrocytes positive for the vitamin D receptor [Fig. 1(a),(b)], in contrast to the infrequent expression shown by the control non-arthritic specimens [Fig. 1(c)]. The proportion of VDR^+ cells in osteoarthritic cartilage was variable, not only between specimens but also within the different zones of a single specimen (Table I). The two zones described as the 'superficial' or 'deep' zone have been reported by other studies[2,4,24]. We recognize that the original 'superficial' zone of grade 1 lesions has mainly been lost on progression to grade 3 and will not represent the same tissue location as described for the non-arthritic control cartilage specimens. We therefore use the term 'superficial' to describe the location at and just beneath the articular surface of each specimen irrespective of the grading, thereby allowing a comparative assessment for VDR and the three MMPs (Table I). The most prominent VDR^+ staining of chondrocytes occurred within the superficial zone of Grades 1 and 3 (Table I). Although some chondrocytes were observed to be VDR^+ within the deep zones their numbers were consistently less than in the superficial zone. However, the deep zone of grade 3 osteoarthritic cartilage usually contained markedly more VDR+ cells than those for Grades 1 and 2. VDR expression was observed for both single chondrocytes [Fig. 1(a)] and those found in typical OA chondrocyte clusters [Fig. 1(b)], but seldom in the specimens of normal articular cartilage.

The extent of immunostaining for MMPs -1, -3 and -9 was variable in the osteoarthritic cartilage specimens [Fig. 1(d–f)]. MMP-1 was most prominent in Grade 3 superficial zone cartilage, with the deep zone of Grades 1 and 2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showing little staining [Fig. 1(d)]. MMP-3 was most prominent in the superficial zone of Grades 2 and 3 with markedly less staining in the deep zone [Fig. 1(e)], whereas MMP-9 immunostaining was less prevalent than that for MMPs -1 and -3 [Fig. 1(f) and Table I]. By contrast normal, non-arthritic cartilage revealed negligible immunostaining for MMP-1 [Fig. 1(g)]. Control tissue sections for the immunostaining of VDR and MMPs consistently gave negative results [Fig. 1(h) and (i), respectively]. The S-100 antibody effectively stained most chondrocytes in normal cartilage thereby demonstrating that the weak immunostaining of these specimens was not due to lack of penetration of antibodies (Fig. 2).

Table I summarizes the immunohistochemical results obtained for VDR and each of the three MMPs; all four antigens were significantly elevated in the osteoarthritic compared with non-arthritic specimens, and especially so for VDR. Whereas significant differences were observed between the two cartilage zones for the extent of VDR and MMP immunostaining, in general Grade 3 was greater than 2 and 1, and the superficial zone was greater than the deep zone (see legend to Table I). Since these observations suggested some association between VDR^+ cells and MMP expression *in vivo*, dual immunolocalization studies have subsequently demonstrated that a proportion of VDR^+ cells were also producing MMP-3 [Fig. 1(j) and (k)]; these representing approximately 20% of chondrocytes in the superficial zone of specimens 697/3 and 805/2. Recognizing the unlikelihood that all VDR^+ cells would be responding to the locally available ligand $1\alpha,25(OH)_2D_3$ at specimen sampling time, the potential relationship of VDR and MMP expression was further examined by assessing the effects of $1\alpha,25(OH)_2D_3$ on VDR and MMP expression by HAC in culture.

CHONDROCYTE CULTURES

Chondrocytes prepared from osteoarthritic cartilage specimens and grown on coverslips were immunostained for VDR. Whereas most chondrocytes of sub-confluent HAC were VDR^+ (Fig. 2), this decreased as the cells approached confluence (Fig. 2). Thus to determine the effects of $1\alpha,25(OH)_2D_3$ on MMP and PGE_2 production the chondrocytes were used at sub-confluence to ensure VDR expression by a major proportion of the cells. No noticeable differences in VDR^+ immunostaining was observed following TNFα- and PMA-stimulation of the HAC cultures compared to controls, and all three preparations of OA derived HAC were shown to be VDR^+ at sub-confluence. In addition, to confirm the chondrocytic phenotype of these cultures, the cells were stained for both S-100 and type II cartilage collagen, demonstrating the continued expression of both antigens (Fig. 2).

The data in Fig. 3 represents the mean values from experiments made with sub-confluent HAC from three different specimens in which the responses to the treatments were qualitatively similar. $1\alpha,25(OH)_2D_3$ alone had little effect on MMP-1, -9 or PGE_2 production, but when added to cells stimulated with TNFα or PMA more marked responses were observed (Fig. 3). $1\alpha,25(OH)_2D_3$ had no significant effect on TNFα-stimulated MMP-1 production by HAC, but when stimulated with PMA the resultant increase in MMP-1 was significantly increased by the addition of $1\alpha,25(OH)_2D_3$ (Fig. 3(a)). By contrast, MMP-3 production by HAC was increased by $1\alpha,25(OH)_2D_3$ treatment alone, and its addition to TNFα or PMA-stimulated cells significantly enhanced MMP-3 production [Fig. 3(b)]. MMP-9 was not produced by the HAC with or without $1\alpha,25(OH)_2D_3$ or TNFα exposure. However, PMA stimulation significantly increased MMP-9 production, this being reduced by approximately 75% by the simultaneous addition of $1\alpha,25(OH)_2D_3$ [Fig. 3(c)].

$1\alpha,25(OH)_2D_3$ had no effect on control values of PGE_2 production by HAC *in vitro*, and TNFα also showed only a marginal increase. However, PMA stimulation of chondrocytes resulted in more than a five-fold increase in PGE_2 production, this being reduced by more than 60% by the concomitant addition of $1\alpha,25(OH)_2D_3$ (Fig. 4).

Discussion

This study has demonstrated VDR expression by chondrocytes within osteoarthritic cartilage, particularly near to the articular surface and fibrillations, an observation in contrast with normal cartilage specimens where VDR^+ cells were rarely observed. This up-regulation of VDR^+ in osteoarthritic cartilage, an observation which was mirrored in the cartilage derived from rheumatoid joints[11], supports the idea that $1\alpha,25(OH)_2D_3$ may play a regulatory role in the modulation of chondrocyte behavior in these pathologies[23,25]. Recent studies have reported $1\alpha,25(OH)_2D_3$ synthesis by synovial fluid macrophages from patients with inflammatory arthritis[15], and that the receptor:metabolite complex interacts with nuclear DNA to evoke a cellular response[26,27]. Thus the marked nuclear staining observed for some osteoarthritic cartilage specimens might well suggest local activation of VDR^+ chondrocytes. The extent of VDR expression was variable between specimens, but as yet the factors responsible for its regulation remain unclear. Although a noticeable down-regulation of VDR in chondrocytes approaching confluence in culture was noted, little difference was observed for VDR expression

Fig. 1. Immunolocalization of the vitamin D receptor (VDR) and matrix metalloproteinases (MMPs) -1, -3 and -9 in osteoarthritic and normal, age-matched cartilage. (A) Immunolocalization of VDR in osteoarthritic cartilage (grade 3). Positive cells stained red. Bar=65 μm. (B) High power micrograph of chondrocyte clusters within osteoarthritic cartilage (grade 3) immunostained for VDR. Note both positive (red stain) and negative cells within the clusters. Bar=25 μm. (C) Immunolocalization of VDR in normal cartilage (grade 0), Note only one cell in the field is positively stained (arrow). Bar= 25 μm. (D) Chondrocytes in osteoarthritic cartilage (grade 3) immunostained for MMP-1. Note cytoplasmic positive staining (red). Bar=25 μm. (E) Chondrocytes in osteoarthritic cartilage (grade 2) immunostained for MMP-3. Note cytoplasmic positive staining (red). Bar=25 μm. (F) Chondrocytes in osteoarthritic cartilage (grade 2) immunostained for MMP-9. Note both positively and negatively stained cells. Bar=25 μm. (G) Normal cartilage (grade 0) immunostained for MMP-1. Note absence of positively stained cells. Bar=40 μm. (H) Control tissue section for VDR staining in A, B and C (grade 2) treated with non-immune rat IgG. Bar=65 μm. (I) Control tissue section for MMP staining in D, E, F and G (grade 2) treated with non-immune sheep IgG. Bar=65 μm. (J) and (K) Dual immunolocalization of VDR (blue-black staining) and MMP-3 (reddish-brown) in osteoarthritic cartilage grade 2. Cells observed to be positive for either one or both (arrrows) of the antigens. Bar=25 μm. (L) Control tissue section for (J) and (K) treated with non-immune rat and sheep IgG. Bar=25 μm.

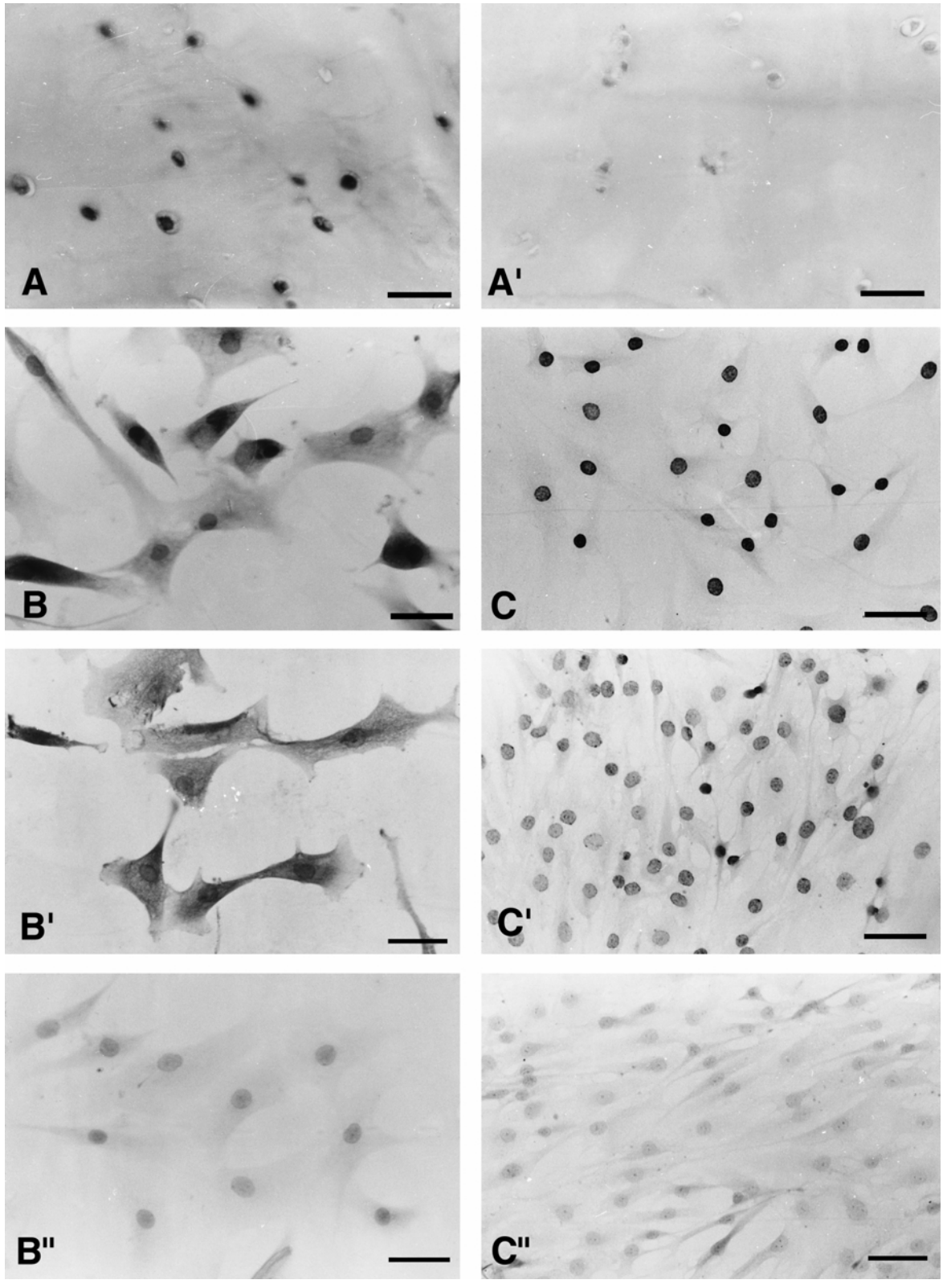

Fig. 2. Immunolocalization of the chondrocyte markers S-100 and cartilage type II collagen, and VDR expression by human chondrocytes *in vitro*. (A) S-100 immunostaining of normal cartilage chondrocytes (specimen NC/98/1). Bar=40 μm. (A′) Control tissue section for S-100 staining in (A) treated with non-immune rabbit IgG. Bar=40 μm. (B) S-100 immunostaining of sub-confluent HAC used in the *in vitro* experiments. Bar=30 μm. (B′) Cartilage type II collagen immunostaining of sub-confluent HAC as described for (B). Bar=30 μm. (B″) Negatively stained control sub-confluent culture of HAC treated with non-immune rabbit IgG for comparison with (B) and (B′). Bar=30 μm. (C) VDR^+ immunostaining of sub-confluent culture of HAC used for *in vitro* experiments. Bar=30 μm. (C′) VDR immunostaining of confluent HAC culture. Note fewer cells positive for VDR than observed in (C). Bar=30 μm. (C″) Negatively stained control HAC culture treated with non-immune rat IgG for comparison with (C) and (C′). Bar=30 μm.

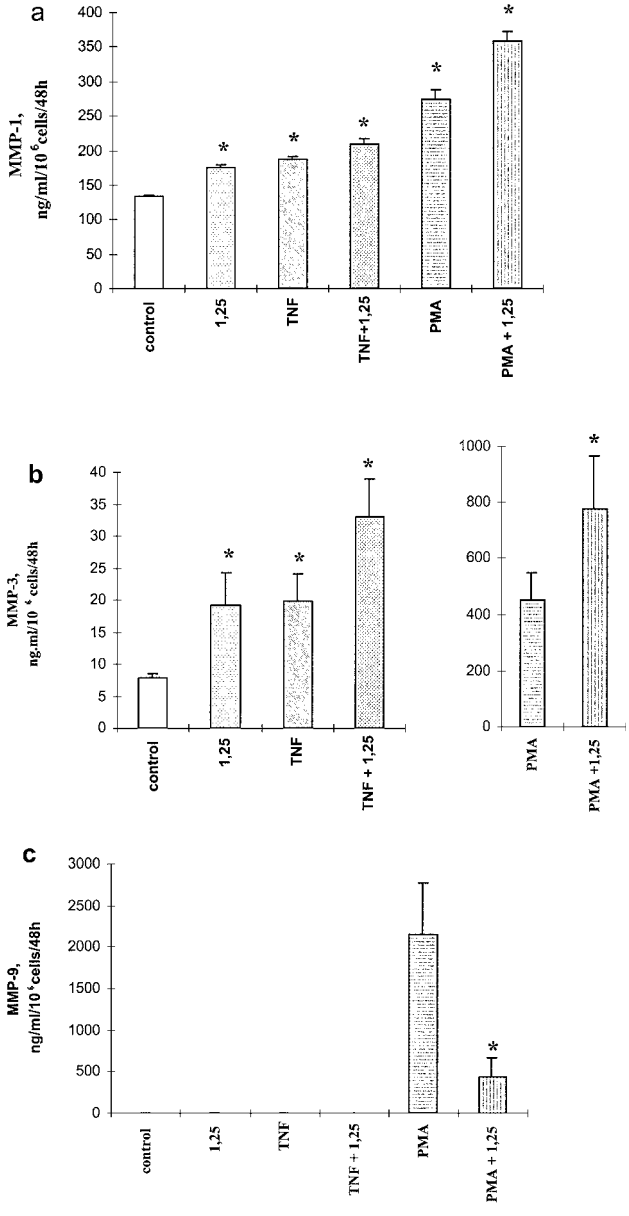

Fig. 3. Effects of 1α,25dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) on matrix metalloproteinase (MMP) -1, -3 and -9 production by human articular chondrocytes (HAC) with and without stimulation by TNFα or PMA. (a) MMP-1 production by HAC (N=3) expressed as ng/ml/10^6 cells/48 h showing values for control; +1α,25$(OH)_2D_3$ (10^{-8} M); +TNFα (20 ng/ml); +TNFα and 1α,25$(OH)_2D_3$ (20 ng/ml and 10^{-8} M, respectively); +PMA (25 ng/ml); and +PMA and 1α,25$(OH)_2D_3$ (25 ng/ml and 10^{-8} M, respectively). Student t-test showed significant differences (*****) in MMP-1 production between control and treatments, viz. +1α,25$(OH)_2D_3$, P=0.01; +TNFα, P=0.003; +PMA, P=0.007. For TNFα *vs*. TNFα+1α,25$(OH)_2D_3$, P=0.05; and for PMA *vs*. PMA +1α,25$(OH)_2D_3$, P=0.005. (b) MMP-3 production by HAC (N=3) expressed as ng/ml/10^6 cells/48 h, showing values for control and treatments as described in (a). Student t-test showed significant differences (*) between control and treatments, viz. +1α,25$(OH)_2D_3$, P=0.01; +TNFα, P=0.03; +PMA, P=0.0002. For TNFα *vs*. TNFα+1α,25$(OH)_2D_3$, P=0.009; and for PMA *vs*. PMA+1α,25$(OH)_2D_3$, P=0.03. (c) MMP-9 production by HAC (N=3) expressed as ng/ml/10^6 cells/48 h, showing values for control and treatments as described in (a). Student t-test showed a significant difference (*)between the PMA and PMA+1α,25$(OH)_2D_3$ treated values, P=0.0054. All values shown are mean±S.E.M.

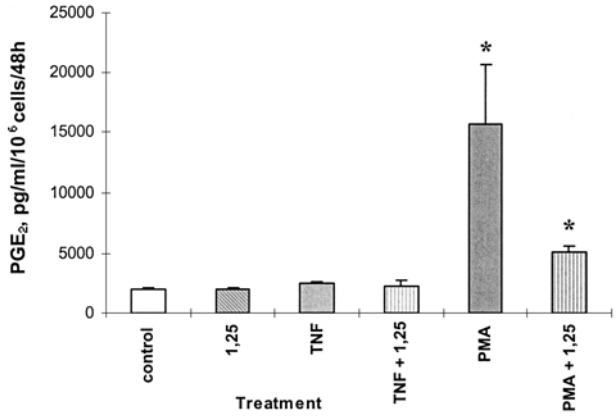

Fig. 4. Effects of 1α,25$(OH)_2D_3$ on PGE_2 production by human articular chondrocytes with and without stimulation by TNFα or PMA. Mean values±S.E.M. are expressed as pg PGE_2/ml/10^6 cells/48 h and are shown for control; +1α,25$(OH)_2D_3$ (10^{-8} M); +TNFα (20 ng/ml); +TNFα and 1α,25$(OH)_2D_3$ (20 ng/ml and 10^{-8} M, respectively); +PMA (25 ng/ml); and +PMA and 1α,25$(OH)_2D_3$ (25 ng/ml and 10^{-8} M, respectively). No statistically significant differences were seen except between the PMA and PMA +1α,25$(OH)_2D_3$ treated cultures where P=0.05* (Student t-test).

in situ between single chondrocytes and those in clusters; most VDR^+ differences being noted between the deep and superficial zones of the same cartilage specimen.

The importance of MMPs in the pathophysiology of OA is well established, both at protein and mRNA level[4,28,29]. Collagenase 1 (MMP-1),collagenase 3 (MMP-13) and stromelysin 1 (MMP-3) have all been demonstrated both intra-and extracellularly in localized sites within osteoarthritic cartilage[28,29]. In this study MMPs -1, -3 and -9 were demonstrated in cartilage sites where a proportion of the chondrocytes were VDR^+; an association which suggested the possibility that 1α,25$(OH)_2D_3$ could be involved in the regulation of MMP and cytokine production at sites of cartilage degradation in OA. Such a proposal could be considered fortuitous and unrelated. However, the dual localization study showing that some VDR^+ cells were also expressing MMP-3 suggests otherwise, this being supported to some extent by the *in vitro* studies where 1α,25$(OH)_2D_3$ was shown to directly modulate MMP-3 production by HAC. For the *in vitro* chondrocyte studies it was important to use VDR^+ chondrocytes; thus the demonstration that sub-confluent chondrocytes appeared to be more positive than confluent cells was the prime reason for using the former in the MMP and PGE_2 experiments.

Previous studies regarding a relationship between 1α,25$(OH)_2D_3$ and MMP regulation are mainly derived from animal studies or experiments with immortalized cell lines. For example, the interstitial collagenase gene expression in rat chondrocytes was increased by the administration of 1α,25$(OH)_2D_3$ to the animals[7]; 1α,25$(OH)_2D_3$ significantly up-regulated MMP-3 and MMP-2 in growth zone rat chondrocytes[12], and Lacraz *et al*.[30] demonstrated the discoordinate regulation of MMP-1 and -9 production by human mononuclear phagocytes. A plenitude of agents are known to have regulatory effects on MMP expression. The cytokines IL-1β and TNFα, and the prostanoids, such as PGE_2, have all been implicated in the enhanced production of specific MMPs. The increased levels of MMP-1, -3 and -9 produced by IL-1β-stimulated chondrocytes *in vitro* was further enhanced upon addition of 1α,25$(OH)_2D_3$[23], an observation in accord with the TNFα and PMA-induced

increase for MMP-1 and MMP-3 reported here. By contrast, the PMA-induced increase in MMP-9 and PGE_2 production by the same chondrocytes was suppressed by exposure to $1\alpha,25(OH)_2D_3$, thereby indicating both enhancing and suppressive roles for the vitamin D metabolite in the regulation of these four chondrocyte products. Such disparate modulatory effects of $1\alpha,25(OH)_2D_3$ on 'activated' chondrocytes may be of relevance to the chondrolytic processes which potentiate intrinsic cartilage damage.

$1\alpha,25(OH)_2D_3$ appears to have multifunctional properties which relate to specific cell types. $1\alpha,25(OH)_2D_3$ has different effects on cytokine production and some inflammatory responses. For example, $1\alpha,25(OH)_2D_3$ acts synergistically with bone matrix components to stimulate IL-1β production by mononuclear cells[31], inhibits IL-1β production by myeloid cells[32], and can regulate IL-1β production by rat chondrocytes[8]. It seems plausible, therefore, that IL-1β and TNFα production by OA chondrocytes might be affected by $1\alpha,25(OH)_2D_3$, but this has yet to be examined.

Prostaglandins are primary mediators in inflammation, are recognized as having prominent roles in the immune response, and PGE_2 has been implicated in the potentiation of MMP production in some cell cultures[17]. $1\alpha,25(OH)_2D_3$ was reported to stimulate PGE_2 production by rat growth zone chondrocytes[33], but in the present study $1\alpha,25(OH)_2D_3$ had little effect on basal production of PGE_2 by human chondrocytes. However, the enhanced PGE_2 production observed following chondrocyte stimulation with PMA was markedly suppressed by the simultaneous addition of $1\alpha,25(OH)_2D_3$.

In summary, our *in vitro* data suggest that $1\alpha,25(OH)_2D_3$ has more pronounced effects on chondrocytes in states of activation or proliferation, where it appears to modulate MMP and PGE_2 production. Whether 'activated' chondrocytes manifest increased VDR expression remains uncertain at present, as does the nature of its regulation. Although $1\alpha,25(OH)_2D_3$ has the potential to modify MMP-1, MMP-3, MMP-9 and PGE_2 production by activated chondrocytes *in vitro*, these represent only a minority of the proteinases, prostanoids and cytokines elaborated by such cells. Further studies are needed to examine the immunomodulatory effects of this vitamin D metabolite on the various chondrolytic or reparative processes of relevance to the degenerative changes within osteoarthritic cartilage.

Acknowledgments

We thank consultant orthopaedic surgeons T. Dunningham (Tameside Hospital, Manchester) and M. Morris (Devonshire Royal Hospital, Buxton) for the supply of joint tissues; Professor A. J. Freemont for help with the supply of normal cartilage; Professor E. B. Mawer for her support and advice; and Roddy McColl and Mark Jotham (The Medical Illustration Unit, Manchester Royal Infirmary) for preparation of the color figures. This work was supported by grant No. WO541 from the Arthritis Research Campaign.

Abbreviations

ABC, Avidin-Biotin Complex; AEC, 3-amino-9-ethylcarbazole; AP, Alkaline phosphatase; BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium; DMEM, Dulbecco's Modified Eagle's Medium; ELISA, Enzyme-linked immunosorbent assay; FCS, Fetal calf serum; HAC, Human articular chondrocytes; HBSS, Hank's balanced salt solution; HRP, Horseradish peroxidase; IgG, Immunoglobulin G; IL-1β, Interleukin-1 beta; MMP, Matrix metalloproteinase; OA, Osteoarthritis; PMA, Phorbol myristate acetate; PGE_2, Prostaglandin E_2; TBS, Tris-buffered saline; TNFα, Tumor necrosis factor-alpha; VDR, Vitamin D receptor; v/v, volume/volume; $1\alpha25(OH)D_3$, 1-alpha,25,dihydroxyvitamin D_3.

References

1. Howell DS, Pelletier J-P. Etiopathogenesis of osteoarthritis. In: McCarty DJ, Koopman WJ, Eds. Arthritis and Allied Conditions. A Textbook of Rheumatology. 12th ed. London: Lea & Febiger 1993:1723–34.
2. Poole AR. Cartilage in Health and Disease. In: McCarty DJ, Koopman WJ, Eds. Arthritis and Allied conditions. A Textbook of Rheumatology, 12th ed. London: Lea & Febiger 1993:279–333.
3. Martel-Pelletier J, McCollum R, Fujimoto N, Obata K, Cloutier JM, Pelletier J-P. Excess of metalloproteinase over tissue inhibitor of metalloproteinases may contribute to cartilage degradation in osteoarthritis and rheumatoid arthritis. Lab Invest 1994;70:807–15.
4. Freemont AJ, Hampson V, Tilman R, Goupille P, Taiwo Y, Hoyland JA. Gene expression of matrix metalloproteinases 1, 3 and 9 by chondrocytes in osteoarthritic human knee articular cartilage is zone and grade specific. Ann Rheum Dis 1997;56:542–9.
5. Arend WP, Dayer J-M. Inhibition of and the effects of interleukin-1 and tumour necrosis factor alpha in rheumatoid arthritis. Arthritis Rheum 1995;38:151–60.
6. Dean DD, Schwartz Z, Schmitz J, Muniz OE, Lu YH, Calderon F, *et al*. Vitamin D regulation of metalloproteinase activity in matrix vesicles. Conn Tiss Res 1996;35:385–90.
7. Grumbles RM, Shao L, Jeffrey JJ, Howell DS. Regulation of rat interstitial collagenase gene expression in growth cartilage and chondrocytes by vitamin D3, interleukin 1β and okadaic acid. J Cell Biochem 1996;63:395–409.
8. Dean DD, Schwartz Z, Muniz OE, Arsenis CH, Boyan BD, Howell DS. Interleukin-1 alpha and beta in growth plate cartilage are regulated by vitamin D metabolites *in vivo*. J Bone Min Res 1997;12:1560–9.
9. Lemire JM. Immunomodulatory role of 1,25dihydroxyvitamin D_3. J Cell Biochem 1992;49:26–31.
10. Norman AW, Roth J, Orci L. The vitamin D endocrine system: steroid metabolism, hormone receptors and biological response (calcium binding proteins). Endocrine Reviews 1982;3:31–6.
11. Tetlow LC, Smith SJ, Mawer EB, Woolley DE. Vitamin D receptors in the rheumatoid lesion: expression by chondrocytes, macrophages and synoviocytes. Ann Rheum Dis 1999;58:118–21.
12. Dean DD, Boyan BD, Muniz OE, Howell DS, Schwartz Z. Vitamin D metabolites regulate matrix vesicle metalloproteinase content in a cell maturation-dependent manner. Calcif Tissue Int 1996;59:109–16.
13. Goldring MB. Degradation of articular cartilage in culture: regulatory factors. In: Woessner JF Jr, Howell D, Eds. Joint Cartilage Degradation: Basic and Clinical Aspects. New York: Marcel Decker 1993: 281–345.

14. Mawer EB, Hayes ME, Still PE Davies M, Lumb GA, Palit J, *et al*. Evidence for non-renal synthesis of 1,25-dihydroxyvitamin D in patients with inflammatory arthritis. J Bone Min Res 1991;6:733–9.
15. Hayes ME, Denton J, Freemont AJ, Mawer EB. Synthesis of the active metabolite of vitamin D, $1,25(OH)_2D_3$, by synovial fluid macrophages in arthritic diseases. Ann Rheum Dis 1989;48:723–9.
16. Dickson IR. Bone. In: Royce PM, Steinmann B, Eds. Connective Tissue and its Heritable Disorders: Molecular, Genetic and Medical Aspects. New York: Wiley-Liss 1993:249–85.
17. Shapiro SD, Kobayashi DK, Pentland AP, Welgus HG. Induction of macrophage metalloproteinases by extracellular-matrix. Evidence for enzyme-specific and substrate-specific responses involving prostaglandin dependent mechanisms. J Biol Chem 1993;268(11):8170–5.
18. Kammermann JR, Kincaid SA, Rumph PF, Baird DK, Visco DM. Tumor necrosis factor-alpha (TNF-alpha) in canine osteoarthritis: immunolocalization of TNF-alpha, stromelysin and TNF receptors in canine osteoarthritic cartilage. Osteoarthritis Cartilage 1996;4:23–34.
19. Kahn HJ, Marks A, Thom H, Baumal R. Role of antibody to S-100 protein in diagnostic pathology. Am J Clin Pathol 1983;79:341–7.
20. Tetlow LC, Woolley DE. Mast cells, cytokines and metalloproteinases at the rheumatoid lesion: dual immunolocalisation studies. Ann Rheum Dis 1995;54:896–903.
21. Tetlow LC, Woolley DE. Comparative immunolocalisation studies of collagenase 1 and collagenase 3 production in the rheumatoid lesion, and by human chondrocytes and synoviocytes in vitro. Br J Rheumatol 1998;37:64–70.
22. Meats JE, McGuire MK, Russel RGG. Human synovium releases a factor which stimulates chondrocyte production of PGE and plasminogen activator. Nature 1980;286:891–92.
23. Tetlow LC, Woolley DE. The effects of 1α,25dihydroxyvitmain D_3 on matrix metalloproteinase and prostaglandin E2 production by cells of the rheumatoid lesion. Arthritis Res 1999;1:63–70.
24. Poole AR, Alini M, Hollander AH. Cellular biology of cartilage degradation. In: Henderson B, Edwards JCW, Pettipher ER, Eds. Mechanisms and Models in Rheumatoid Arthritis. London: Academic Press 1995:163–203.
25. Schmitz JP, Schwartz Z, Sylvia VL, Dean DD, Calderon F, Boyan BD. Vitamin D3 regulation of stromelysin (MMP-3) in chondrocyte cultures is mediated by protein kinase C. J Cell Physiol 1996;168:570–9.
26. Minghetti PP, Norman AW. $1,25(OH)_2D_3$-Vitamin D_3 receptors: gene regulation and genetic circuitry. FASEB J 1988;2:3043–53.
27. Haussler M, Jurutka P, Hsieh JC, Thompson PD, Selznick SH, Haussler CA, *et al*. Receptor mediated genomic actions of $1,25(OH)_2D_3$: Modulation by phosphorylation. In: Norman AW, Boiullon R, Thomasset M, Eds. Vitamin D—A Pluripotent Steroid Hormone: Structural studies, molecular endocrinology and clinical applications. New York: de Gruyter 1994:209–16.
28. Okada Y, Shinmei M, Tanaka O, Naka K, Kimura A, Nakanishi I, *et al*. Localisation of matrix metalloproteinase (stromelysin) in osteoarthritic cartilage and synovium. Lab Invest 1992;66:680–90.
29. Reboul P, Pelletier J-P, Tardif G, Cloutier JM, Martel-Pelletier J. The new collagenase, collagenase 3 is expressed and synthesised by human chondrocytes but not by synoviocytes: a role in osteoarthritis. J Clin Invest 1996;97:2011–19.
30. Lacraz S, Dayer J-M, Nocod I, Welgus HG. 1,25dihydroxyvitamin D_3 dissociates production of interstitial collagenase and 92kDa gelatinase in human mononuclear phagocytes. J Biol Chem 1994;269:6485–90.
31. Hogasen AM, Nordsletten L, Aasen AO, Falch JA. 17-beta oestradiol and 1α,25dihydroxycholecalciferol modulate constitutive and bone-matrix-induced interleukin-1 (IL-1beta) production by peripheral blood mononuclear cells isolated from postmenopausal women. Scand J Clin Lab Invest 1998;58:97–102.
32. Peleg S, Qui HY, Reddy S Harris D, Van Q, Estey EH, *et al*. 1,25 dihydroxyvitamin D_3 and its analogues inhibit acute myelogenous leukaemia progenitor proliferation by suppressing interleukin-1 beta production. J Clin Invest 1997;100:1716–24.
33. Schwartz Z, Swain LD, Kelly DW, Brooks B, Boyan BD. Regulation of prostaglandin E_2 production by vitamin D metabolites in growth zone and resting zone chondrocyte cultures is dependent on cell maturation. Bone 1992;13:395–401.