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-artritic, normal cartilage; and to assess the in vitro effects of 1α,25 dihydroxyvitamin D3 (1α,25(OH)2D3) on MMPs-1, -3 and -9 and prostaglandin E2 (PGE2) 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)2D3 on MMP and PGE2 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)2D3 alone had no effect on MMP-1, -9 and PGE2 production by HAC, but MMP-3 production was up-regulated by 1α25(OH)2D3 either with or without stimulation with TNFα or PMA. By contrast the increased production of MMP-9 and PGE2 induced by PMA was significantly suppressed by concomitant treatment with 1α25(OH)2D3.

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 1α,25(OH)2D3 contributes to the regulation of MMP and PGE2 production by HAC in osteoarthritic cartilage.

Key words: Osteoarthritis, Vitamin D receptors, 1α,25-dihydroxyvitamin D3, Matrix metalloproteinases, Chondrocytes, Prostaglandin E2.
**Table I**

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**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, obtained from six early autopsy cases. Cartilage tissues were removed at joint replacement surgery and transferred to the laboratory in Hanks balanced salt solution (HBSS). Tissue 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 Histo-mount 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 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 x 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 x 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 x 10 min washes in TBS 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.

CELL CULTURES

Human articular cartilage from OA specimens was enzymically digested to provide chondrocyte cultures as previously described. 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 either PMA (25 ng/ml) or PMA+1α,25(OH)2D3 (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 E2 (PGE2) 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 mm², total area=6 mm²).

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 collagen cartilage 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. PGE2 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/10⁶ 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. 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–l)]. MMP-1 was most prominent in Grade 3 superficial zone cartilage, with the deep zone of Grades 1 and 2...
showing little staining [Fig. 1(d)]. MMP-3 was most prominent in the superficial zone of Grades 2 and 3 with marked 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 1α,25(OH)2D3 at specimen sampling time, the potential relationship of VDR and MMP expression was further examined by assessing the effects of 1α,25(OH)2D3 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 1α,25(OH)2D3 on MMP and PGE2 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. 2 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. 1α,25(OH)2D3 alone had little effect on MMP-1, -9 or PGE2 production, but when added to cells stimulated with TNFα or PMA more marked responses were observed (Fig. 3). 1α,25(OH)2D3 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 1α,25(OH)2D3 (Fig. 3(a)). By contrast, MMP-3 production by HAC was increased by 1α,25(OH)2D3 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 1α,25(OH)2D3 or TNFα exposure. However, PMA stimulation significantly increased MMP-9 production, this being reduced by approximately 75% by the simultaneous addition of 1α,25(OH)2D3 [Fig. 3(c)].

1α,25(OH)2D3 had no effect on control values of PGE2 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 PGE2 production, this being reduced by more than 60% by the concomitant addition of 1α,25(OH)2D3 (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 joints11, supports the idea that 1α,25(OH)2D3 may play a regulatory role in the modulation of chondrocyte behavior in these pathologies23,29. Recent studies have reported 1α,25(OH)2D3 synthesis by synovial fluid macrophages from patients with inflammatory arthritis15, and that the receptor:metabolite complex interacts with nuclear DNA to evoke a cellular response26,27. 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. 2. Immunolocalization of the chondrocyte markers S-100 and cartilage type II collagen, and VDR expression by human chondrocytes \textit{in vitro}. (A) S-100 immunostaining of normal cartilage chondrocytes (specimen NC/98/1). Bar=40 \mu m. (A') Control tissue section for S-100 staining in (A) treated with non-immune rabbit IgG. Bar=40 \mu m. (B) S-100 immunostaining of sub-confluent HAC used in the \textit{in vitro} experiments. Bar=30 \mu m. (B') Cartilage type II collagen immunostaining of sub-confluent HAC as described for (B). Bar=30 \mu m. (B'') Negatively stained control sub-confluent culture of HAC treated with non-immune rabbit IgG for comparison with (B) and (B'). Bar=30 \mu m. (C) VDR$^+$ immunostaining of sub-confluent culture of HAC used for \textit{in vitro} experiments. Bar=30 \mu m. (C') VDR immunostaining of confluent HAC culture. Note fewer cells positive for VDR than observed in (C). Bar=30 \mu m. (C'') Negatively stained control HAC culture treated with non-immune rat IgG for comparison with (C) and (C'). Bar=30 \mu m.
MMP-9 production by HAC \((=3)\) expressed as ng/ml/10\(^6\) cells/48 h and are shown for control; +1\(\alpha_25\)(OH)\(_2\)D\(_3\) (10\(^{-8}\) M); +TNF\(_a\) (20 ng/ml); +TNF\(_a\) and 1\(\alpha_25\)(OH)\(_2\)D\(_3\) (20 ng/ml and 10\(^{-8}\) M, respectively); +PMA (25 ng/ml); and +PMA and 1\(\alpha_25\)(OH)\(_2\)D\(_3\) (25 ng/ml and 10\(^{-8}\) M, respectively). No statistically significant differences were seen except between the PMA and PMA +1\(\alpha_25\)(OH)\(_2\)D\(_3\) treated cultures where \(P=0.05\) (Student t-test).

**Fig. 3.** Effects of 1\(\alpha_25\)dihydroxyvitamin D\(_3\) (1\(\alpha_25\)(OH)\(_2\)D\(_3\)) on matrix metalloproteinase (MMP) -1, -3 and -9 production by human articular chondrocytes (HAC) with and without stimulation by TNF\(_a\) or PMA. Mean values\(\pm\)S.E.M. are expressed as pg PGE\(_2\)/ml/10\(^6\) cells/48 h and are shown for control; +1\(\alpha_25\)(OH)\(_2\)D\(_3\) (10\(^{-8}\) M); +TNF\(_a\) (20 ng/ml); +TNF\(_a\) and 1\(\alpha_25\)(OH)\(_2\)D\(_3\) (20 ng/ml and 10\(^{-8}\) M, respectively); +PMA (25 ng/ml); and +PMA and 1\(\alpha_25\)(OH)\(_2\)D\(_3\) (25 ng/ml and 10\(^{-8}\) M, respectively). Student t-test showed significant differences (*) in MMP-1 production between PMA and PMA +1\(\alpha_25\)(OH)\(_2\)D\(_3\) treated cultures where \(P=0.05\) (Student t-test).

**Fig. 4.** Effects of 1\(\alpha_25\)(OH)\(_2\)D\(_3\) on PGE\(_2\) production by human articular chondrocytes with and without stimulation by TNF\(_a\) or PMA. Mean values\(\pm\)S.E.M. are expressed as pg PGE\(_2\)/ml/10\(^6\) cells/48 h.

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 1\(\alpha_25\)(OH)\(_2\)D\(_3\) 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 more positive than control or PMA+1\(\alpha_25\)(OH)\(_2\)D\(_3\) treated cultures where \(P=0.05\) (Student t-test).
increase for MMP-1 and MMP-3 reported here. By contrast, the PMA-induced increase in MMP-9 and PGE2 production by the same chondrocytes was suppressed by exposure to 1,25(OH)2D3, 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 1,25(OH)2D3 on ‘activated’ chondrocytes may be of relevance to the chondrolytic processes which potentiate intrinsic cartilage damage.

1,25(OH)2D3 appears to have multifunctional properties which relate to specific cell types. 1,25(OH)2D3 has different effects on cytokine production and some inflammatory responses. For example, 1,25(OH)2D3 acts synergistically with bone matrix components to stimulate IL-1β production by mononuclear cells31, inhibits IL-1β production by myeloid cells32, and can regulate IL-1β production by rat chondrocytes33. It seems plausible, therefore, that IL-1β and TNFα production by OA chondrocytes might be affected by 1,25(OH)2D3, but this has yet to be examined.

Prostaglandins are primary mediators in inflammation, are recognized as having prominent roles in the immune response, and PGE2 has been implicated in the potential for MMP production in some cell cultures34. 1,25(OH)2D3 was reported to stimulate PGE2 production by rat growth zone chondrocytes33, but in the present study 1,25(OH)2D3 had little effect on basal production of PGE2 by human chondrocytes. However, the enhanced PGE2 production observed following chondrocyte stimulation with PMA was markedly suppressed by the simultaneous addition of 1,25(OH)2D3.

In summary, our in vitro data suggest that 1,25(OH)2D3 has more pronounced effects on chondrocytes in states of activation or proliferation, where it appears to modulate MMP and PGE2 production. Whether ‘activated’ chondrocytes manifest increased VDR expression remains uncertain at present, as does the nature of its regulation. Although 1,25(OH)2D3 has the potential to modify MMP-1, MMP-3, MMP-9 and PGE2 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

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Abbreviations

ABC, Avidin-Biotin Complex; AEC, 3-amino-9-ethylcarbazole; AP, Alkaline phosphatase; BCIP/NBT, 5-bromo-4-chloro-3-indoly 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, Prostaglandin E2; TBS, Tris-buffered saline; TNFα, Tumor necrosis factor-alpha; VDR, Vitamin D receptor; v/v, volume/volume; 1α25(OH)2D3, 1-alpha,25-dihydroxyvitamin D3.

References


