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Original article

Differences in metabolic parameters and gene expression related to Osteochondrosis/Osteoarthrosis in pigs fed 25-hydroxyvitamin D_3

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Abstract – Osteochondrosis/osteoarthrosis (OC/OA) are common terms for various joint pathologies that occur in pigs. Pathologies that may contribute to these disorders have been described, but the primary cause(s) remain unknown. We hypothesised that as OC has some similarities to dyschondroplasia, which involves a failure of growth plate chondrocytes to fully differentiate and hypertrophy, treatment with 25-hydroxyvitamin D_3 (25-D) might reduce the incidence and/or severity of lesions in pigs, as it does in chickens with dyschondroplasia. Control pigs were fed a commercial diet ad libitum. In the treated group this diet was supplemented with 25-D at 0.1mg/kg. Ten pigs from each of the control and treated groups were sampled at 7, 12, 16 and 21 weeks. Treatment with 25-D had no effect on the incidence or severity of OC/OA lesions. Cartilage dry weight, total collagen content and proteoglycan content, and plasma levels of total calcium, inorganic phosphorous, vitamin C, insuline-like growth factor-I, parathyroid hormone and tumour necrosis factor alpha were unaffected by treatment. In addition, none of these parameters were correlated with the incidence or severity of OC/OA lesions. The mRNA expression levels of 21 out of 23 genes assayed by RT-PCR were unaltered in articular cartilage from OA lesion samples as compared to normal articular cartilage. However, collagen type II was reduced and collagen type X increased in OA lesion and near lesion samples. These results suggest that OA in pigs may share some features of osteoarthritis in other mammalian species.

pig / osteochondrosis / osteoarthrosis / articular cartilage / 25-hydroxy vitamin D_3

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Résumé – Différences observées dans les paramètres métaboliques et l'expression des gènes liés à l'ostéochondrite et à l'arthrose chez le porc ayant ingéré de la 25-hydroxyvitamine D_3. Ostéochondrite et arthrose (OC/AR) sont des termes courants désignant les diverses pathologies des articulations survenant chez le porc. Les maladies pouvant contribuer à ces troubles ont été décrites, mais les causes premières demeurent inconnues. Nous avons fait l'hypothèse que, puisque l'ostéochondrite a quelques similarités avec la dyschondroplasie, qui entraîne une incapacité de la plaque endochondriale à se différencier et à s'hypertrophier, le traitement par la 25-hydroxyvitamine D_3 (25-D) pourrait réduire l'incidence et/ou la sévérité des lésions chez le porc, comme elle le fait chez les poulets atteints de dyschondroplasie. Des porcs témoins ont été nourris à volonté par des rations commerciales. Dans le groupe des porcs traités, cette ration a été complétée par de la 25-D à 0,1 mg/kg. Dix porcs provenant de chaque groupe (témoin et traité) ont été examinés aux semaines 7, 12, 16 et 21. Le traitement par la 25-D n'a eu aucun effet sur l'incidence ou la sévérité des lésions OC/AR. Le poids sec des cartilages, le contenu total en collagène et en protéoglycanes, et les taux plasmatiques en calcium total, en phosphore inorganique, en vitamine C, en facteur de croissance proche de l'insuline-I (IGF-I), en hormone parathyroïdienne, et en facteur de nécrose des tumeurs-α (TNFα), n'ont pas été affectés par le traitement. De plus, aucun de ces paramètres n'était corrélé à l'incidence ou à la sévérité des lésions OC/AR. Les niveaux d'expression de l'ARNm de 21 gènes sur 23 testés en RT-PCR n'étaient pas modifiés dans les cartilages articulaires provenant de prélèvements sur des lésions d'arthrose, comparé au cartilage articulaire normal. Cependant, le collagène de type II était réduit et le collagène de type X était augmenté dans les lésions d'arthrose et autour de ces lésions. Ces résultats suggèrent que l'arthrose chez le porc pourrait partager certaines caractéristiques de l'arthrose chez d'autres espèces de mammifères.

porc / ostéochondrite / arthrose / cartilage articulaire / 25-hydroxyvitamine D_3

1. INTRODUCTION

Osteochondrosis and osteoarthrosis (OC/OA) are collective terms for various joint disorders in pigs. These joint disorders are a widespread problem resulting in lameness and poor welfare. The primary cause(s) of OC/OA are unknown, although joint diseases have been linked with various dietary deficiencies such as vitamin D, calcium and other minerals [19]. It has also been recognised that there is a genetic component to OC/OA [1, 14, 15, 19, 20, 43, 44]. Unfortunately, the criteria used for selection in commercial pig breeding programs, such as fast growth and reduced backfat thickness tend to be associated with OC/OA [25].

A number of studies have described various pathologies that may ultimately contribute to OC/OA. OC in pigs involves a failure of growth plate chondrocyte hypertrophy, leading to a perturbation of endochondral ossification in the growth plate. This is often assumed to be the cause of, or lead to, various pathologies associated with OC, including osteoarthritis and osteoarthrosis involving the articular-epiphyseal joint cartilage of the fore– and hind limbs [6, 19, 32, 35]. Meat-type (broiler) poultry exhibit a similar inhibition of endochondral ossification in the growth plate termed dyschondroplasia [8], the incidence of which can be reduced by including 25-hydroxyvitamin D_3 (25-D) in the feed [40].

We postulated that feeding 25-D to pigs might alleviate OC and associated joint pathologies. To investigate this possibility, we studied the effect of feeding 25-D to pigs on the prevalence of OC/OA from weaning until slaughter. In addition, we determined the correlation of a number of biochemical and molecular parameters with the occurrence of OC/OA. This was achieved by a combination of several different approaches: (1) gross and histopathological examination of joint articular-epiphyseal cartilage at different time points from weaning until slaughter; (2) measurement of the levels of various biochemical markers of bone metabolism/pathology in samples of blood or

cartilage; (3) comparison of the levels of expression of putative candidate genes for OC/OA in lesion and normal articular cartilage from control pigs.

2. MATERIALS AND METHODS

2.1. Animals

Two hundred 3-week old weaned male pigs reared on a commercial farm, with a history of a high incidence of OC/OA in growing and finishing pigs, were divided at random into two groups, control and treated. The pigs were the offspring of White German Duroc sires and Pig Improvement Company Camborough dams. Control animals were fed a standard commercial grower diet ad libitum during the growing period before being transferred to a commercial finishing diet. Treated animals were fed the same diets supplemented with 0.1 mg/kg 25-D. The pigs were housed in pens (50 pigs per pen) with part solid concrete floors and part concrete slats with straw. Ten pigs chosen at random from each group were weighed and slaughtered at 7, 12, 16 and 21 weeks. The leg joints (elbow and stifle) from all 4 legs were scored for OA (gross pathology) and sections from these joints processed for histopathology (OC/OA). In addition, samples of blood and articular cartilage were taken for biochemical analysis, and separate articular cartilage samples processed for the extraction of total RNA. Blood samples were collected in EDTA coated tubes with thorough mixing. Plasma was isolated from aliquots of blood by centrifugation at 3000 $\times g$ for 10 min and stored at –70 °C until analysis. In addition, 12 control pigs were sampled at 24 weeks of age solely to obtain RNA from normal and OA lesion articular cartilage.

2.2. Gross pathology

Elbow and stifle joints were opened and the articular surfaces examined for evidence of surface lesions. Joints were scored for severity of OA using an arbitrary scoring system from 0 to 5 as follows: 0 – no lesion, 1 – very mild or early lesion, 2 – mild lesions, 3 – moderate lesions, 4 – marked lesions, 5 – severe lesions [38].

2.3. Histopathology

Bones from the joints were sawn into 5 mm slices to encompass both the articular cartilage and growth plate. The slices were fixed in 10% neutral buffered formalin and then subjected to decalcification in EDTA. Decalcified bones were trimmed and processed through to paraffin wax for histopathological examination using standard techniques. Sections (4 μm) were mounted on glass slides, stained with haematoxylin and eosin and examined using light microscopy. Representative sections were scored as above for both articular (OA) and growth plate (OC) lesions.

2.4. Biochemical analysis

Plasma: (1) Total calcium and inorganic Phosphorous. Both were determined by colorimetric methods using commercially available kits (Wako Chemicals GmbH, Neuss, Germany). *(2) Vitamin C*. Ascorbic acid concentrations were measured by colorimetric assay using folin phenol reagent [22].

Cartilage extracellular matrix: (1) Dry weight. Weighed samples of articular cartilage were heated at 107 °C for 48 hours and re-weighed. This was repeated after a further 24 hours to check that there was no further change in weight. *(2) Proteoglycans*. Articular cartilage samples were weighed and analysed for proteoglycan content using 1,9-dimethylmethylene blue [9]. *(3) Collagen*. The collagen content of articular cartilage was assayed by measuring the amount of hydroxyproline in weighed samples [4].

Immunoassays: 1,25-dihydroxyvitamin D (1,25-D) and 25-D were measured in plasma samples using commercial radioimmunoassay kits (IDS, Boldon, UK). Plasma samples were further analysed by ELISA for tumour necrosis factor alpha (TNFα) (Endogen Inc, Massachusetts, USA) and insulin-like growth factor-I (IGF-I) (IDS). Parathyroid hormone (PTH) was assayed in plasma by a specific two-site radioimmunoassay (Immutopics, California, USA).

2.5. Molecular analysis

RNA extraction: Chondrocytes were isolated from articular cartilage samples using a method previously developed for growth plate chondrocytes [7]. None of the pigs fed 25-D were sampled so as to avoid possible treatment effects. Cartilage was taken from the sites of OA lesions and also from areas of normal cartilage adjacent to OA lesions, where possible. These samples were referred to as lesion and near lesion, respectively. Control samples from pigs without clinical signs of lesions from matched areas of the joint surface were also taken. Sterile scalpel blades were used at all times. The articular cartilage was chopped into small pieces and the tissue incubated in a shaking water bath for 4 hours at 37 °C in Dulbecco's modified Eagle's medium (DMEM), containing 0.4% collagenase and 0.2% hyaluronidase. After passage through a 100 μm filter to remove undigested material, the cells were washed 3 times with DMEM and pelleted by centrifugation. Total RNA was extracted from isolated chondrocytes using Ultraspec II according to the manufacturers instructions (Biotecx, USA). Briefly, cells were resuspended in 1 mL of Ultraspec II and the suspension passed repeatedly through an 18-gauge needle. After incubation on ice the RNA was extracted with chloroform and the aqueous phase recovered. The RNA was then bound to RNATack resin (Biotecx) in the presence of isopropanol and recovered by brief centrifugation. After washing twice with 75% ethanol the RNA was eluted in a small volume of 10 mM Tris (pH 7.0). The RNA was then treated with DNAse I (DNA-Free; Ambion, USA) to remove any contaminating genomic DNA before RT-PCR.

RT-PCR: Total RNA was reverse transcribed with random hexamers in aliquots of 1 μg using the Superscript preamplification system (Invitrogen, Paisley, UK) Control reactions were processed identically except for the omission of Superscript (reverse transcriptase) enzyme. Polymerase chain reaction (PCR) was carried out in a volume of 10 μL in 200 μL thin-walled tubes using a Robocycler PCR machine (Stratagene, UK). The reaction contained TLA buffer [23, 24], 0.05% Tween-20 (Sigma, UK), one unit of Taq polymerase (ABgene, UK), in the presence of Taqstart antibody (Clontech, UK) and the equivalent of 20 ng of target RNA. The final concentration of the primers was 1 μM. The temperature profile of the PCR was 95 °C for 30 s, 60 °C for 60 s and 72 °C for 60 s. The number of PCR cycles was carefully titrated to ensure that the reactions were in the exponential phase. PCR products were run out on 3% NuSieve agarose gels (Flowgen, UK), stained with ethidium bromide, and a record of the gel made using the BioRad gel documentation system (BioRad, UK). For comparison of OA lesion and control samples, relative levels of expression were calculated by densitometric analysis of gel images using the Scion Image software package (Scion Corporation, USA). The expression levels were estimated in arbitrary units, with the control band assigned a value of one. The housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or actin were used as invariant internal control genes. Each gene was assayed at least three times and the results collated and analysed statistically using a one-sample t-test within the Statview

statistics package (SAS Institute Inc.). The two genes that showed differences in expression levels between samples, collagen types II and X, were assayed at least a further 3 times, comparing mRNA abundance between control, lesion and near lesion samples.

Gene specific primers: Candidate genes were selected from searches of the EMBL nucleotide sequence database (http://www.ebi.ac.uk). Gene specific primers were then designed using the GCG program *Prime* (Wisconsin Package version 10.1, Genetics Computer Group (GCG), Madison, Wisconsin). Genes that were considered possible candidate genes for OA included those thought to be involved in cartilage differentiation and degradation, such as growth factors and matrix metalloproteinases, as well as components of the extracellular matrix that may be altered during cartilage degeneration.

Heterologous cloning of pig collagen type-II: Using primers specific for the chicken collagen type II gene and total RNA from pig articular cartilage, RT-PCR was carried out as described above, but using a lower stringency annealing temperature of 55 °C, to take account of the possible differences in the sequences of the pig and chicken genes.

2.6. Statistical analysis

Pig OC/OA scores: Mean scores were calculated and the scores of control and treated pigs were compared statistically using the Mann-Whitney U test for non-parametric data.

Pig weights: Pig weights were analysed using analysis of variance according to treatment and age within the Statview statistics software package (SAS Institute Inc., USA).

Biochemical parameters: The effects of group (control and treated), age and their interaction, plus covariates for the effects of elbow and stifle joint lesion score on the parameters measured in cartilage and blood were assessed using analysis of covariance (Genstat statistics package, Lawes agricultural trust, Rothamsted experimental station, Harpenden, UK).

3. RESULTS

3.1. Gross and histopathology

Scores for each joint were assigned on the basis of gross and histopathological examination. Histopathology scores for the control and 25-D treated groups at each time point are shown in Table I, as well as the number of pigs with lesions and the range of lesion scores. Only two animals (25-D treated group at 12 weeks of age) had a lesion that involved the growth plate (OC), where there was a small focus of disruption to growth plate chondrocyte maturation with fibrosis. All other lesions involved the articular cartilage and included thinning of the articular cartilage, pitting of the articular surface, necrosis and cartilage degeneration at both the gross and cellular levels. Overall scores for the prevalence of OC/OA in each age group revealed that there were few lesions apparent in pigs from the early age groups (Tab. I). Lesions became prevalent at 16 weeks with the incidence and severity increasing at 21 weeks. There were no statistically significant differences in incidence or severity of OC/OA between the control and treated groups at any age point. However, the mean weight of the control and treated groups, which did not differ during the early part of the experiment, was significantly higher in the treated group at 21 weeks of age ($P < 0.01$) (Tab. II).

3.2. Biochemical parameters (Tabs. III, IV)

None of the parameters measured showed a significant correlation with OC/OA score

Table I. Frequency and severity of DJD lesions in leg joints of control and 25-D treated pigs at different ages.

Age (weeks)	Elbow						Stifle					
	Control			Treated			Control			Treated		
	ms ± sd	nl	r	ms ± sd	nl	r	ms ± sd	nl	r	ms ± sd	nl	r
7	0.0 ± 0.0	0	–	0.2 ± 0.6	1	0 – 2	0.1 ± 0.3	1	0 – 1	0.0 ± 0.0	0	–
12	0.0 ± 0.0	0	–	0.2 ± 0.6	3	0 – 2	0.6 ± 1.1	3	0 – 3	0.5 ± 0.8	4	0 – 2
16	1.3 ± 1.5	6	0 – 3	1.8 ± 1.6	7	0 – 4	0.3 ± 0.7	2	0 – 3	0.4 ± 1.0	2	0 – 3
21	2.6 ± 2.3	10	0 – 5	3.2 ± 1.5	10	0 – 5	1.5 ± 1.7	8	0 – 5	1.1 ± 1.0	7	0 – 2

ms ± sd: mean score ± standard deviation (n =10). nl: number of pigs in group with lesions. r: range of lesion scores.

Table II. Pig weights in the 25-D treated and control groups.

Age (weeks)	Weight (kg)	
	Control	Treated
7	15.30 ± 2.25	13.88 ± 3.71
12	41.20 ± 3.62	40.75 ± 4.98
16	72.10 ± 5.82	69.80 ± 7.84
21	83.50 ± 4.60	92.50 ± 6.06

Weights are given as the mean ± standard deviation, n = 10.

Table III. Summary of results for biochemical parameters measured in articular cartilage with respect to treatment.

Parameter	Age (weeks)	Control[a]	Treatment[a]	S.e.d.[b]
%PG	7	7.76	9.60	0.855
	12	9.65	9.62	
	16	6.69	7.27	
	21	ND	ND	
%Collagen	7	10.11	9.37	1.127
	12	11.55	11.53	
	16	12.27	11.94	
	21	ND	ND	
%DW	7	20.54	21.41	2.106
	12	21.14	22.77	
	16	21.26	21.11	
	21	ND	ND	

[a] Values given are the means (n = 10). [b] Standard error of the difference of the means. PG: proteoglycan. DW: dry weight. ND: not determined.

Table IV. Summary of results for biochemical parameters measured in blood with respect to treatment.

Parameter	Age (weeks)	Control[a]	Treated[a]	S.e.d.[b]
Total Ca (mM)	7	2.35	2.38	0.124
	12	2.14	2.12	
	16	1.93	2.17	
	21	2.00	1.85	
P_i (mg/dL)	7	11.08	11.26	0.947
	12	11.09	11.80	
	16	9.53	9.07	
	21	12.02	10.56	
Vit C (mg/dL)	7	1.291	1.328	0.096
	12	1.318	1.357	
	16	0.976	0.859	
	21	0.739	0.700	
1,25 D (pmol/mL)	7	592.4	673.9	41.84
	12	446.4	550.7	
	16	377.6	444.6	
	21	400.7	446.5	
25-D (pmol/mL)	7	50.2	328.2	18.74
	12	108.3	343.8	
	16	178.5	390.2	
	21	117.9	363.7	
IGF-I (ng/mL)	7	30.2	24.0	1.39
	12	63.0	66.7	
	16	114.8	127.9	
	21	99.3	87.5	
PTH (pg/mL)	7	0.37	0.64	2.47
	12	3.10	2.42	
	16	1.15	1.84	
	21	7.72	2.68	
TNFα (pg/mL)	7	8.0	6.2	1.63
	12	26.7	24.0	
	16	6.7	6.4	
	21	8.0	7.7	

[a] Values given are the means ($n = 10$). [b] Standard error of the difference of the means. Ca: total calcium. P_i: inorganic phosphorous. Vit C: vitamin C.

of the elbow or stifle joints at any age. However, a number of the parameters measured showed statistical significance with age. For example, PTH ($P < 0.05$) and IGF-I ($P < 0.001$) increased, and calcium ($P < 0.001$), 1, 25-D ($P < 0.001$) and vitamin C ($P < 0.001$) decreased significantly with age. However, several other correlations with age were not due to trends, but to increased or decreased measurements at an isolated time point (inorganic phosphorous, $P < 0.01$; 25-D, $P < 0.001$; TNFα, $P < 0.001$). Only two parameters showed statistical significance with treatment. Levels of the vitamin D

metabolites, 25-D ($P < 0.001$) and 1,25-D ($P < 0.001$) were both significantly higher in the treated group (Tab. IV).

3.3. Cloning of the pig homologue of collagen type II

After PCR at the reduced annealing temperature of 55 °C agarose gel electrophoresis revealed a complex pattern of minor bands with a major band of approximately the correct size at 300 base pairs. This band was cloned and sequenced and the resulting 330 base pair sequence compared with sequences in the EMBL database. This revealed that the cloned band had high homology with a number of vertebrate collagen type II genes, including human, horse, rabbit, rat, mouse and chicken at the nucleotide and translated amino acid sequence levels (86% to 96% identity over 248 to 330 base pairs/110 amino acids) and it was therefore concluded that this was a fragment of the pig collagen type II gene. Oligonucleotide primers specific for this sequence were synthesised and included in the candidate gene screen described in Section 3.4.

3.4. Gene expression

Gene expression levels were assayed by RT-PCR for 25 different putative candidate genes, including collagen type II. The sequence of the primers and the size of the PCR product generated are given in Table V. In an initial screen 23 of the 25 pig genes assayed were amplified from articular cartilage, with only two genes, mmp and msp1, giving no detectable signal under the assay conditions used. Of these 23 genes assayed further using semi-quantitative RT-PCR, 21 showed no difference between control and OA lesion samples of articular cartilage (e.g. Fig. 1). The remaining two genes, collagen types II and X, showed consistently altered levels of abundance in OA lesion and near lesion samples, as compared to controls. Collagen type II was downregulated ($P < 0.01$) and collagen type X upregulated ($P < 0.01$) in lesion and near lesion samples of articular cartilage (Fig. 2).

4. DISCUSSION

This study tested the hypothesis that 25-D, which is known to decrease the occurrence of

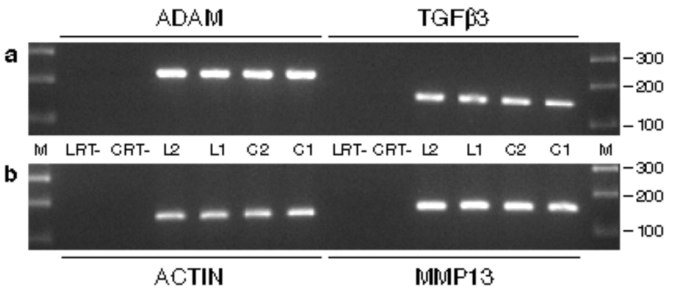

Figure 1. Relative levels of gene expression in total RNA from normal control (lanes C1 and C2) and DJD lesion (lanes L1 and L2) pig articular cartilage samples. Lanes CRT– and LRT– are negative control reactions. 100, 200 and 300 base pair markers are indicated on right-hand side of gel. Genes assayed are: (a) ADAM and TGFβ3. (b) MMP13 and the control gene actin.

Table V. Genes assayed in cartilage samples from normal and DJD lesion joints.

Gene	Primer	Primer sequence (5' – 3')[a]	Fragment size (bp)[b]
tenascin	PTEN	ACAGAGGTGACGGAAGAAAC TTCAGGTGTAGGCAGGTAAGG	255
collagen VII	PCOL7	ACTGAGTACCAAGTGACAGTG TGTGGTATTCTGGATGGTCAG	210
link protein	PLINK	CCAAGCTAACTTCAGATTACC TCACCTCACACTTATATCTCC	180
aggrecan	PAGG	CGAGGAGCAGGAGTTTGTCAAC ATCATCACCACGCAGTCCTCTC	177
osteopontin	POST	GAGCAGTCCAAAGAATACACG TCCAGAGCCACACACATATAC	332
TGF-α	PTGFα	AAGAAGCAGGCCATTACCGC AGTGTTTTCGGACCTGGCAG	103
TGF-β1	PTGF1	AAGGAAAAGGAAGCCCATAG CACCCCAAATTCATCTTCAC	276
TGF-β2	PTGF2	GTTGTTGCCCTCCTACAGAC CTGAGAACCCTGCTATGCTG	243
TGF-β3	PTGF3	AAAGCAACAGACCTCACCTCG CCTTCCCCTAATCAAACCCAC	153
TGF-β type III-R	PTGFR	GTCGTGTTCCTGCTCAACTC CCTTTCCTCTGTTTCTGCTG	156
GHR	PGHR	ACCACAGAAAGCCTTACCAC TCACATAGCCACACGATGAG	184
PTHR	PPTHR	CCTCCATTGTGCTCAACTTC AAAGTCCAGTGCCAATGTCC	344
IGF-I	PIGFI	ACAAGCCCACAGGGTACGGCT CCTGAACTCCCTCTACTTGTG	220
GEMBP	PGEM	GCTCAAGTCCAAATCCTGCC TGAACACCAACCACGCTCTC	299
c-myc	PMYC	GAGACACTTTTCCCTGTCGC CTTCCAGATATCCTCGCTGG	261
MMP	PMMP	TACACACCTTCCATGACTCCT ACCATTCATTCCCATAGTCCT	267
MMP-1	PMMP1	TCTCACCCTTGACCTTCACC GAGCAGCCACACGATACAAG	222
MMP-3	PMMP3	ACCCAAGAAGTATCCACACCCT TGCTTCAAAGACAGCATCCACT	215
MMP-13	PMMP13	GATGATGAAACCTGGACAAG ATCAGGAAGCATAAAGTGGC	162

Table V. (*Continued*)

MSP	PMSP	CGCACACTGCTTCCAAAATTC AGCGACACCGATTCTTTCAAC	177
ADAM	PADAM	CACATGATTCTGGAACAGAGTG CATTCCATTTTCCACAGATAGGC	218
PIAP	PPIAP	CCAAAGGATGATGCTATGAC ACTCACACCTTGGAAACCAC	313
endoglin	PEND	CGTCCACATCCTCTTCTTGG AGCAGCAGAGATGATGGAGC	295
collagen type II	PCOLII	GCTATGGAGATGACAACCTGGCTC CACTTACCGGTGTGTTTCGTGCAG	256
collagen type X	PCOLX	GCTGCCACATTCTGACACAATC TGCGCTGAGCATCATTTGAGAC	225
GAPDH	PGAP	ACCCCTTCATTGACCTCCAC ATACTCAGCACCAGCATCGC	179
ACTIN	PACT	TGGTGGGTATGGGTCAGAAAG TCGTTGTAGAAGGTGTGGTGC	148

[a]Primers are listed as sense followed by antisense. [b]Base pairs.

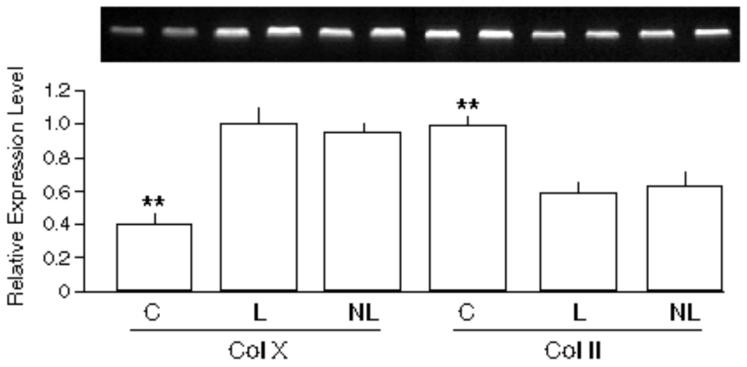

Figure 2. Relative levels of gene expression in total RNA from normal control (C), DJD lesion (L) and near lesion (NL) pig articular cartilage samples. Col X = type X collagen. Col II = type II collagen.

dyschondroplasia in chickens [40], could reduce the incidence or severity of OC/OA in pigs. No evidence was found that 25-D had an influence on the type or severity of OC/OA lesions that developed in the pigs examined in the present study. However, in chickens the success of 25-D in preventing dyschondroplasia is due to its positive effect on the differentiation of growth plate chondrocytes, which fail to fully differentiate in this disorder. As few lesions typical of OC (dyschondroplasia) in the growth plate of the pigs were encountered during this study, the hypothesis that 25-D can reduce

the incidence of growth plate lesions of OC was not properly tested. In addition, 25-D supplementation of the diet did not significantly alter any of the parameters measured in blood and articular cartilage, with the exception of 25-D and 1,25-D, which as expected, were significantly increased in the treated group. It should be noted that an early study on the effect of vitamin D on the severity of OC in pigs also found no evidence of a reduction in the pathological changes associated with OC, although only 12 pigs were examined in this experiment [39].

The virtual absence of detectable growth plate lesions was unexpected given the generally accepted view that early onset lesions of the epiphyseal growth cartilage of the A-E complex give rise to later degenerative changes in articular cartilage [19, 21]. However, a number of studies have reported the occurrence of articular lesions, more typical of osteoarthrosis [13, 19, 26, 37]. The changes in gene expression that were observed in the present study seem to indicate that there may be similarities between the molecular changes that occur in OC/OA in pigs and degenerative changes in the articular cartilage of other mammals in osteoarthrosis or osteoarthritis. The expression of both collagen types II and X is altered in osteoarthritic/osteoarthrotic articular cartilage, with type II being down regulated or degraded [27, 42] and type X upregulated [11, 30], respectively. There is also evidence that the changes in mRNA levels observed in the present study may lead to alterations in the abundance of collagen proteins within the extracellular matrix. Immunolocalisation of collagens within the cartilage of pigs with articular as well as growth plate lesions of osteochondrosis showed that there was a reduction in the proportion of type II collagen, and that type X collagen was detected in osteochondrotic but not normal articular cartilage [45]. Other studies have found no evidence of altered expression of type II or type X collagen in OC [5, 43]. However, this may reflect differences in sampling techniques, the individual joints sampled and the depth to which cartilage is sampled, or the heterogeneity of the lesions classed as osteochondritic within and between species.

Despite the apparent reduction in type II collagen detected at the mRNA level in the present study, there appeared to be no overall reduction in the collagen content of the articular cartilage samples. There are two possible explanations for this discrepancy. Firstly, the cartilage samples were analysed for total collagen content and reduction in type II collagen may have been compensated for by the increase in type X. Secondly, the samples analysed were from the earlier time points of the trial. It was not possible to process samples for total collagen content from the pigs at 21 weeks and most RNA samples were from pigs taken at the end of the trial when the most severe lesions were seen. Therefore, as the pathology associated with earlier lesions was less severe, it is possible that the changes in collagen abundance did not occur until the later stages of disease progression. One mechanism by which type II collagen gene expression may be reduced is through vitamin C deficiency [12]. In one study dietary supplementation with vitamin C appeared to lead to a reduction in the severity of OC/OA lesions, although the reduction in overall incidence of lesions was not statistically significant [16]. However, in the present study circulating levels of ascorbic acid appeared normal, although they did decline with age, but there was no correlation with increasing OC/OA lesion score. IGF-I depletion has also been shown to lead to a reduction in collagen gene expression [12], but systemic levels of IGF-I actually increased with age in this study and this appears to be the norm [29, 36]. Local levels of IGF-I production were also unchanged, as measured by mRNA abundance in articular cartilage. It is possible that different levels of IGF binding proteins might alter

the levels of active IGF-I, but unfortunately the reagents to test this were not available.

No changes in the total proteoglycan (PG) content of the articular cartilage of OA lesion pigs as compared to normal pigs were detected in the present study. This is in agreement with a study of the effect of recombinant pig growth hormone, which induces high levels of OC, on the glycosaminoglycan content of joint cartilage in pigs. Total PG content of articular cartilage was unchanged, but was reduced in the deeper layers of the epiphyseal cartilage [18]. There are a number of conflicting reports documenting either increased or decreased levels of general PGs, or individual PG components of the extracellular matrix in OC. However, changes were restricted to the growth cartilage and were either unchanged due to the absence of lesions or not determined in the articular cartilage of the joints examined [5, 17, 18, 33]. Nakano et al. [31] concluded that biochemical changes in PGs only occur in severely affected joint cartilage, and not in cartilage with early OC/OA.

TNFα is known to promote the degradation of cartilage and bone [2]. It has been implicated in the inhibition of 1,25-D stimulated extracellular matrix protein synthesis [34] and the activation of catabolic MAP kinase pathways in articular chondrocytes [10], which can lead to focal loss of cartilage glycosaminoglycans [46]. Despite this we found no evidence that TNFα is involved in cartilage destruction in OC/OA. Systemic levels of TNFα were unaltered in OA lesion affected pigs compared to unaffected controls. However, local production of TNFα is known to be important in the destruction of cartilage in osteoarthritis [46] and future studies should consider measuring concentrations of TNFα in the synovial fluid of affected joints.

Due to its central role in calcium homeostasis PTH is of fundamental importance in the regulation of skeletal integrity [41]. However, although articular chondrocytes express PTH receptor mRNA they appear to be unresponsive to treatment with PTH [3]. This would explain the results of the present study, in which no correlation between systemic levels of PTH and OC/OA lesion status was apparent and no difference in PTH receptor mRNA levels was detected between lesion and non-lesion samples.

The increase in the weight of the pigs was similar in both groups until 16 weeks, but at 21 weeks was significantly higher in the treated group, as compared to the controls (Tab. II). This was a rather surprising result considering that the weight gain of the treated animals over this 5 week period was almost double that of the control group. Clearly this result must be treated with caution, particularly as the number of pigs in the groups was so small. It would need to be repeated on a much larger scale to be considered significant.

The changes in gene expression observed in the present study in the articular cartilage of pigs with OA lesions indicate that the condition may have similarities with osteoarthritis/osteoarthrosis in mammals. Although it is clear from numerous studies that OA is not an inflammatory condition. Mice with a targeted inactivation of one allele of the collagen type II alpha I gene have an increased prevalence of osteoarthritis [28]. This is of interest in the light of the results of the present study that indicate that the reduction of type II collagen mRNA expression occurred not only in OA lesion cartilage, but also in adjacent phenotypically normal articular cartilage. Although this assessment was based on a macroscopic examination of the joint cartilage, if true, this would suggest that the cartilage of the affected joints is predisposed to develop OA. Further studies will be necessary to test this hypothesis and to document more fully the molecular changes that characterise the various pathologies associated with OC/OA in pigs.

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