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Characterisation of articular and growth plate cartilage collagens in porcine osteochondrosis

R. John Wardale* and Victor C. Duance

Muscle and Collagen Research Group, Department of Clinical Veterinary Science, University of Bristol, Langford, Bristol BS18 7DY, UK

*Author for correspondence

SUMMARY

The articular and growth plate cartilages of osteochondrotic pigs were examined and compared with those from clinically normal animals. Both types of osteochondrotic cartilage showed considerable localised thickening apparently due to a lack of ossification. Histological examination of cartilage lesions demonstrated a breakdown in the normal pattern of chondrocyte maturation. Articular cartilage lesions lacked mature clones of chondrocytes in the calcifying region. Growth plate cartilage showed an accumulation of disorganised hypertrophic chondrocytes rather than the well-defined columns seen in normal tissue.

The overall percentages of collagen in osteochondrotic lesions from both articular and growth plate cartilage were significantly reduced compared with levels in unaffected cartilage. There were substantial increases in the proportion of type I collagen in lesions from both osteochondrotic articular and growth plate cartilages and a reduction in the proportion of type II collagen. Type X collagen was detected in osteochondrotic but not normal articular cartilage. The proportion of type X collagen was unchanged in osteochondrotic growth plate cartilage.

The levels of the collagen cross-links, hydroxylysylpyridinoline, hydroxylysyl-ketonorleucine and dehydrohydroxylysinonorleucine were radically reduced in samples from osteochondrotic growth-plate cartilage lesions when compared with normal tissue. Less dramatic changes were observed in articular cartilage although there was a significant decrease in the level of hydroxylysyl-ketonorleucine in osteochondrotic lesions.

Immunofluorescence examination of osteochondrotic lesions showed a considerable disruption of the organisation of the collagenous components within both articular and growth-plate cartilages. Normal patterns of staining of types I and VI collagen seen at the articular surface in unaffected tissue were replaced by a disorganised, uneven stain in osteochondrotic articular cartilage lesions. Incomplete removal of cartilage at the ossification front of osteochondrotic growth plate was demonstrated by immunofluorescence staining of type IX collagen. Type X collagen was produced in the matrix of the calcifying region of osteochondrotic articular cartilage by small groups of hypertrophic chondrocytes, but was not detected in normal articular cartilage. The distribution of type X collagen was unchanged in osteochondrotic growth plate cartilage.

Key words: collagen, cartilage, growth plate, osteochondrosis, collagen cross-link

INTRODUCTION

Osteochondrosis is a generalised skeletal disease of growing animals. It has been described in several species of mammal but is most prevalent in commercially farmed pigs (Reiland, 1978) where it is responsible for considerable economic losses (Hill, 1990). The disease results from a disturbance in the articular and growth-plate cartilages and as the bone is only affected secondarily, dyschondroplasia is technically a more correct term (Olsson, 1978). A further condition, osteochondritis dissecans, which results in chipping of the articular surface is thought to be caused by an underlying weakness in the cartilage caused by an osteochondrotic lesion. Lesions are usually bilateral and in pigs the medial femoral condyle is one of the commonest sites although any of the long bones can be affected. The lesions are characterised by focal impaired endochondral ossification, resulting in areas of retained cartilage extending into the subchondral bone (Reiland, 1978). The perturbation of normal growth causes malformed bones with uneven articular surfaces resulting in lameness and leg weakness.

Overall there is still little evidence to indicate the cause of osteochondrosis. However, severe clinical osteochondrosis appears to be restricted to fast-growing animals with rapid weight gain. Improvements in the gait of pigs by breeders have reduced the amount of osteochondrosis in commercial herds. This suggests either a mechanical cause, which is supported by the fact that heavier, faster growing lines are more susceptible

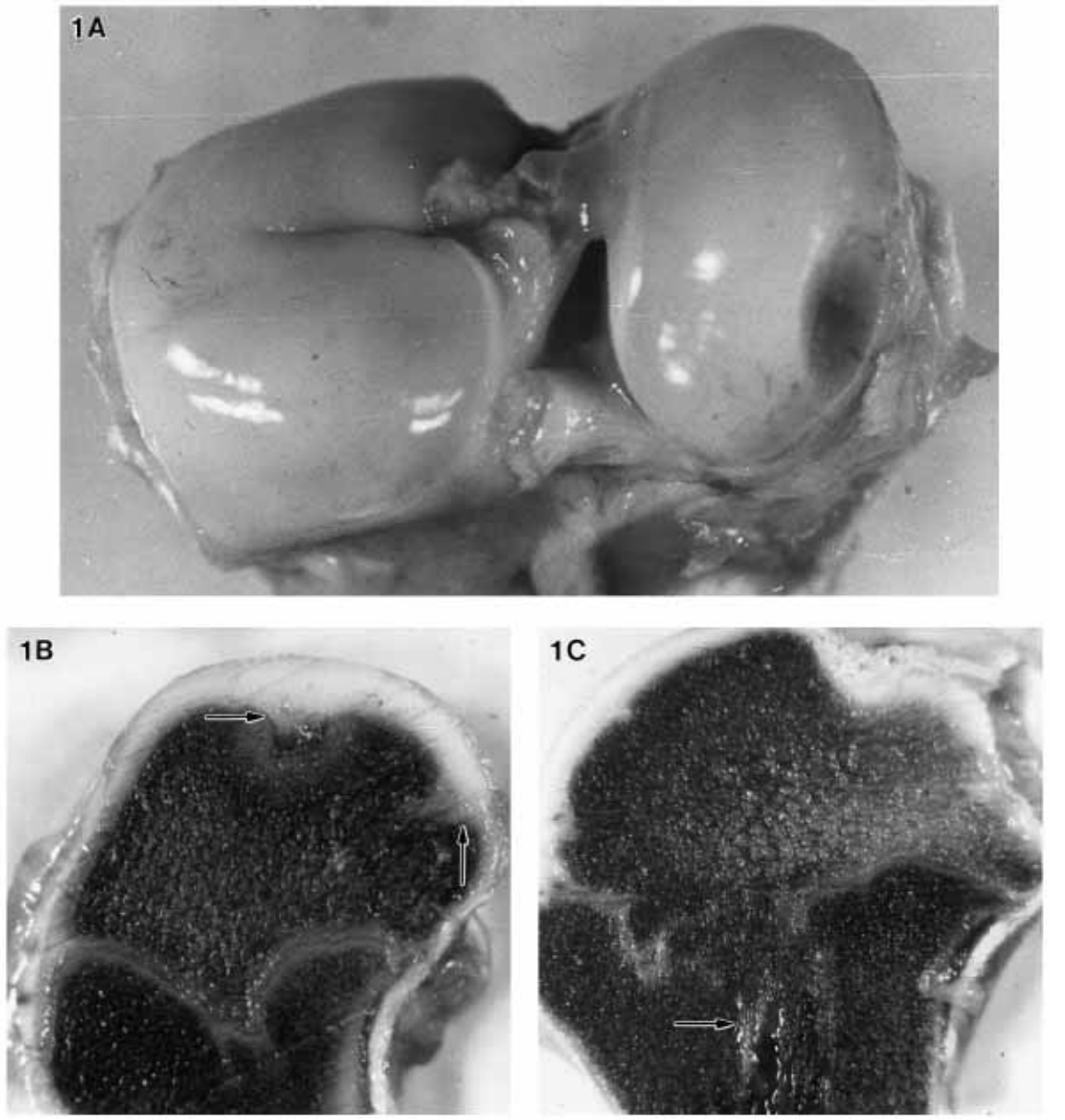
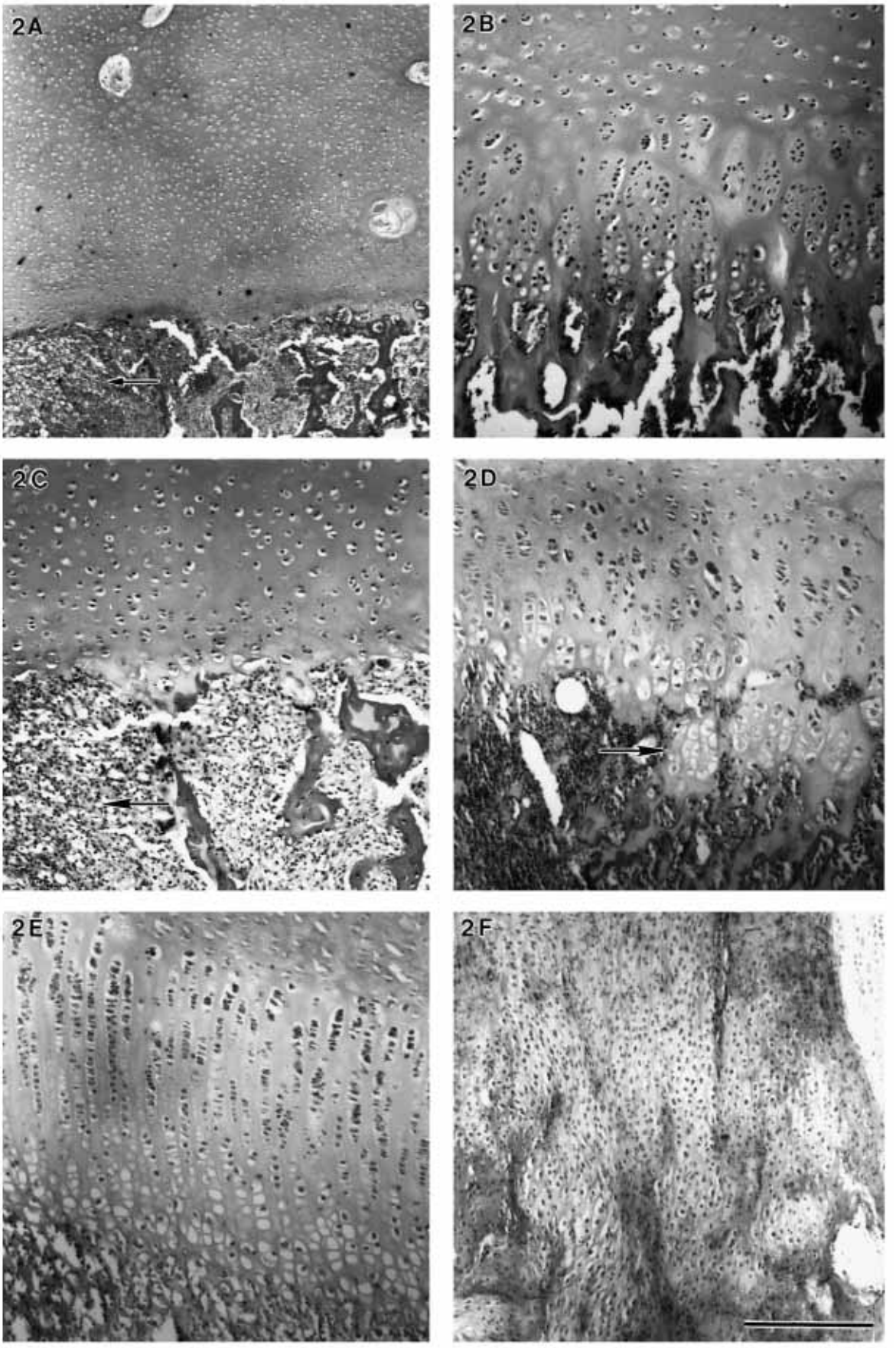


Fig. 1. Osteochondrotic femoral condyles of a 5-month-old Yorkshire Landrace hybrid pig. (A) Severe osteochondrosis of the medial femoral condyle showing flattening and creasing of the articular surface. (B) and (C) Longitudinal sections of the medial femoral condyle showing osteochondrotic lesions (arrowed) in the articular cartilage (B) and severe disruption of the growth plate with cartilage retained in the metaphyseal bone (arrow; C).

to the disease (Grondalen, 1974), or that there is a genetic influence. It is possible that the cartilages of rapidly growing animals are prone to minor defects, which are either repaired or are so small as to not cause any problems. Microscopic lesions have been reported in cartilage from the majority of pigs down to the age of 1 day and this indicates that defects are a normal occurrence (Hill, 1985). Trauma and infection of the joints are common among commercial pigs and it is likely that minor resultant damage, although transitory and largely untraceable, could be enough to tip the balance towards clinical osteochondrosis.

Several degenerative changes have been reported in osteochondrotic cartilage, including perivascular chondrolysis (Kincaid et al., 1985), degeneration of vascular channels

Fig. 2. Wax sections of articular and growth-plate cartilage from normal and osteochondrotic pigs. (A) Articular cartilage in an osteochondrotic lesion showing immature chondrocytes and extensive vascularisation. An area of non-ossified tissue is indicated (arrow). (B) Normal porcine articular cartilage showing mature chondrocyte clusters and an even ossification front. (C) Osteochondrotic lesion in articular cartilage showing lack of mature chondrocytes and an area with no bone formation (arrow). (D) Osteochondrotic lesion in articular cartilage showing swollen or hypertrophic chondrocyte columns and an area of cartilage being overtaken by the ossification front (arrow). (E) Normal porcine growth plate. (F) Part of an osteochondrotic lesion in growth plate cartilage showing massive disorganisation of the hypertrophic region. All sections are stained with haemotoxylin/Van Gieson. Bar: 600 μm (A); 250 μm (B-E).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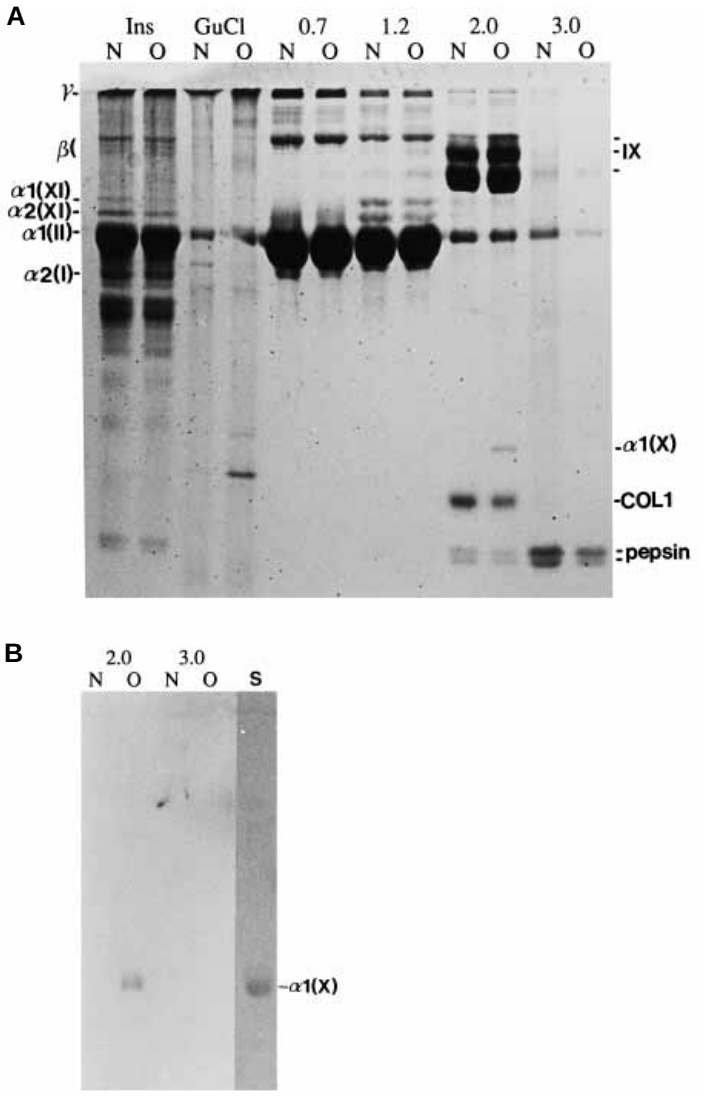


Fig. 3. Identification of collagens in normal and osteochondrotic articular cartilage. (A) SDS-PAGE of collagens purified from normal (N) and osteochondrotic (O) articular cartilage. Ins, pepsin-insoluble fraction; GuCl, material extracted by treatment with guanidine hydrochloride; 0.7, 1.2, 2.0, 3.0 refer to molarities of sodium chloride used to precipitate samples solubilised by pepsin in 0.5 M acetic acid; 7.5% acrylamide gel run under non-reducing conditions and stained with Coomassie Brilliant Blue R. A 20 μg sample of protein loaded per track. Main collagen types are indicated on both sides of the gel. (B) Western blot analysis of fractions shown in (A). Conditions and samples as described in (A), loaded with 5 μg protein per track and probed with a mouse anti-porcine type X collagen polyclonal antibody. S, standard track. The position of type X collagen is shown on the right of the blot.

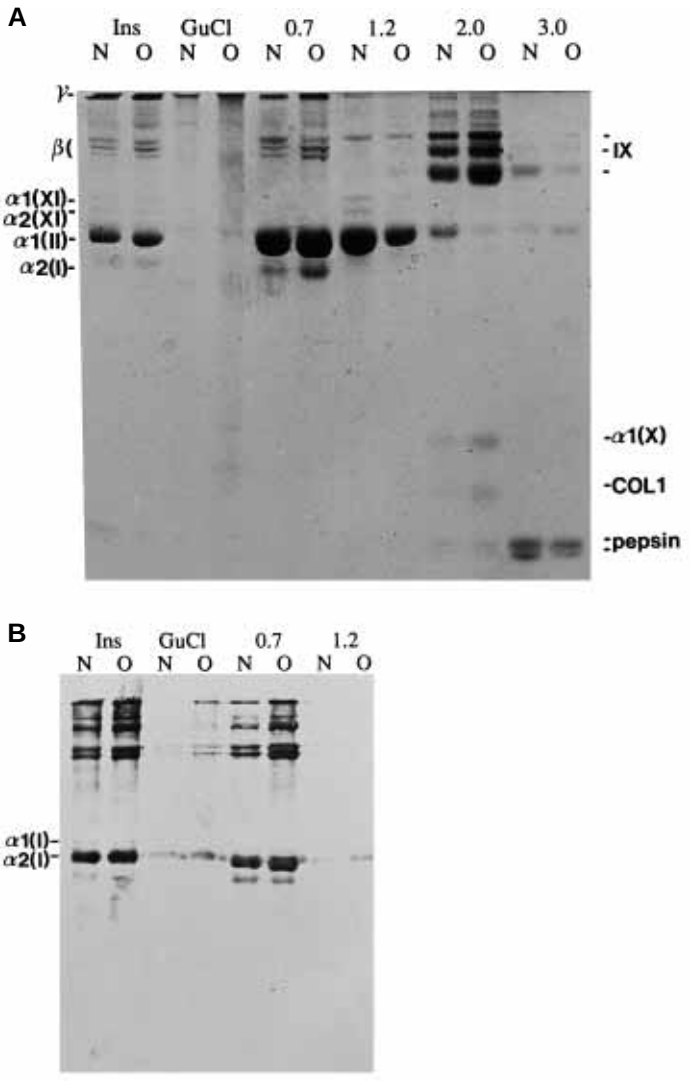


Fig. 4. Identification of collagens in normal and osteochondrotic growth plate cartilage. (A) SDS-PAGE of collagens purified from normal (N) and osteochondrotic (O) growth plate cartilage. Ins, pepsin-insoluble fraction; GuCl, material extracted by treatment with guanidine hydrochloride; 0.7, 1.2, 2.0, 3.0 refer to molarities of sodium chloride used to precipitate samples as in Fig. 3; 7.5% acrylamide gel run under non-reducing conditions and stained with Coomassie Brilliant Blue R; 10 μg protein loaded per track. Main collagen types are shown on both sides of the gel. (B) Western blot analysis of fractions shown in (A). Conditions and samples as described in (A), loaded with 5 μg protein per track and probed with a goat anti-human type I collagen polyclonal antibody that reacts predominantly with the alpha 2(I) chain. Alpha chains of type I collagen are shown on the left of the blot.

(Woodward et al., 1987), intracellular lipid accumulation and chondronecrosis (Carlson et al., 1986). Ultrastructural studies have shown that chondrocytes in osteochondrotic lesions are disordered, produce an abnormal pericellular matrix and fail to undergo normal hypertrophic maturation (Farnum and Wilsman, 1986). Recently, a fault in chondrocyte maturation has been implicated in the progression of the disease (Ekman and Heinegard, 1992).

Articular and growth plate cartilages consist principally of water, collagen and proteoglycan (Muir et al., 1977). The genetically distinct collagen types, being major components, clearly play an important role in the structure and function of these tissues. However, although some have been implicated in the processes of calcification and ossification, an investigation into their nature and distribution in osteochondrotic cartilage has not been previously carried out. We have shown recently (Wardale and Duance, 1993) that types I, II, VI, IX and XI exist in porcine articular and growth-plate cartilages, with type X collagen only appearing in the growth plate.

Studies on articular cartilage have revealed the possible functions of some of the minor collagens. Type IX is found attached to the surface of type II fibrils (Eyre et al., 1987; Vaughan et al., 1988) and strong evidence exists that it has a role in the regulation of fibril diameter (Wotton et al., 1988). Type VI collagen may be involved in maintaining the structural integrity of the tissue (Bonaldo et al., 1990) and is seen to be elevated in diseases such as osteoarthritis (Ronziere et al., 1990). Type X collagen is found exclusively in the hypertrophic region of growth plate cartilage (Gibson and Flint, 1985) and is only rarely detected in the calcifying region of articular cartilage (Gannon et al., 1988). It is found associated with type II collagen (Chen et al., 1990) and its location suggests an important role in the process of calcification or chondrocyte maturation.

Collagen undergoes numerous post-translational modifications, one of which is the formation of intermolecular cross-links, which confers strength and stability to connective tissues (Eyre et al., 1984). A disturbance in the normal processes of cross-linking could result in a structurally weakened tissue as can be seen in osteochondritis dissecans. Conversely, an excessively cross-linked cartilagenous matrix could be resistant to the processes of resorption that occur at the ossification front giving rise to a lack of endochondral ossification.

Osteochondrosis is not only a disease of considerable economic importance but it also may be useful as a model with which to investigate endochondral ossification. The purpose of this study is to establish whether changes in the nature, ratio or distribution of the collagenous components of the articular and growth plate cartilages occur during osteochondrosis.

MATERIALS AND METHODS

Cartilage preparation

Long bones were removed from clinically lame Yorkshire/Landrace hybrid pigs of average weight 60 kg within 24 hours of slaughter and dissected clean of adherent muscle and tendon. The articular surfaces were examined for defects indicative of osteochondrosis and then the bones were cut longitudinally into approximately 0.5 cm slices using a band saw. The articular and growth plate cartilages from both proximal and distal epiphyses of the femur and humerus, and the proximal epiphyses of the tibia and ulna, were examined for osteochondrotic lesions. Lesions visible to the naked eye were carefully dissected from the surrounding tissue and pooled for biochemical analysis or processed individually for microscopy. Lesions of varying severity from up to ten animals were used for each cartilage preparation. Apparently unaffected cartilage was removed as control material or samples were taken from age-matched clinically normal animals.

Collagen preparation

Articular and growth plate collagens were prepared by treatment of cartilage samples with 4 M guanidine hydrochloride followed by pepsin extraction at acid pH as described previously (Wardale and Duance, 1993). Collagens were partially purified by sequential salt precipitation at acid pH and all samples were dialysed extensively against 0.05 M acetic acid before lyophilisation.

Collagen types were separated by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and either stained with Coomassie Brilliant Blue R or subjected to western blotting (Towbin et al., 1979). Blots were probed using collagen type-specific antibodies as described previously (Wardale and Duance, 1993).

Quantification

Lyophilised whole cartilage or salt-fractionated samples were hydrolysed in 6 M HCl for 20 hours at 115°C and the total collagen content estimated by automated hydroxyproline analysis (Woessner, 1976). Ratios of whole alpha chains were estimated from SDS-polyacrylamide gels stained with Coomassie Brilliant Blue R and analysed by an LKB Ultroscan XL scanning laser densitometer as described previously (Wardale and Duance, 1993).

Collagen cross-link analysis

For analysis of collagen cross-links, lyophilised whole cartilage was reduced using potassium borohydride prior to hydrolysis as above. Hydrolysed samples were subjected to column chromatography on a cellulose CF1 column. Analyses of the reduced forms of the intermediary collagen cross-links hydroxylysyl-ketonorleucine (HLKNL) and dehydro-hydroxylysinonorleucine (deH-HLNL), and the mature non-reducible collagen cross-links hydroxylysyl-pyridinoline (HYL-PYR) and lysyl-pyridinoline (LYS-PYR) were carried out on a LKB 4400 amino acid analyser as described previously (Sims and Bailey, 1992).

Histology

Blocks of tissue were dissected from fresh samples and fixed in 0.25% glutaraldehyde, 0.4% paraformaldehyde, 0.1 M sodium cacodylate, pH 7.0, for 3-4 hours at 4°C before rinsing in 0.1 M sodium cacodylate, pH 7.0, and wax embedding using a Histokinet automated tissue processor. Where necessary, tissue was decalcified in 10% formic acid for several days. Sections, 5-10 μm thick, were cut on a Leitz sledge microtome and stained using a haematoxylin/Van Gieson stain.

Immunolocalisation

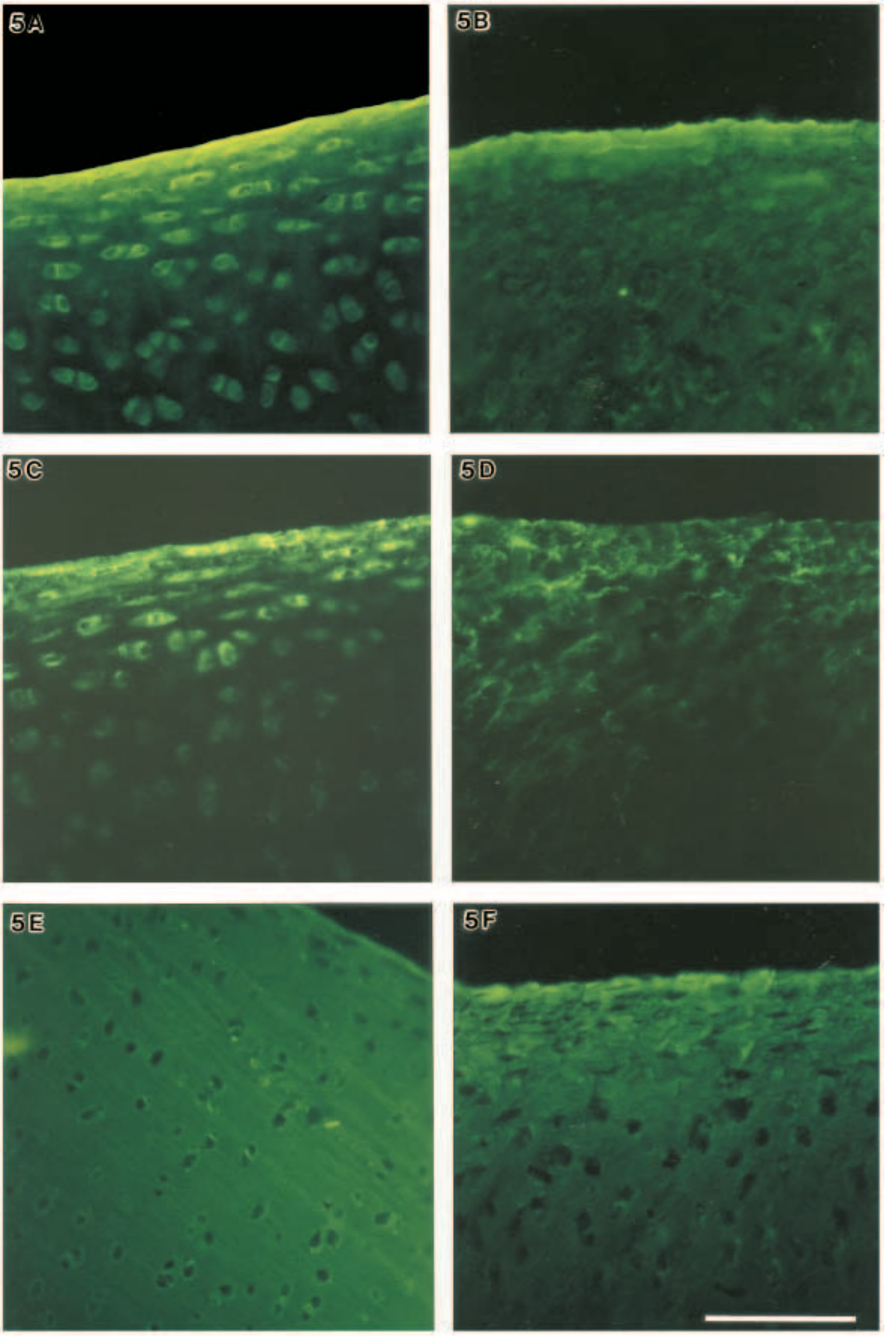
Unfixed, full-thickness cartilage blocks were snap-frozen in isopentane cooled in liquid nitrogen and 5-10 μm cryostat sections were prepared. Sections were treated with testicular hyaluronidase (1 mg/ml) in phosphate buffered saline (PBS), pH 7.4, for 16 hours at 20°C, washed extensively with PBS, pH 7.4, and a primary, collagen type-specific antibody applied for 4 hours. Non-immune serum of the appropriate species or unrelated monoclonal supernatants were used as controls. After further washes a secondary, species-specific antibody conjugated with fluoroscein isothiocyanate (FITC) was applied for 1 hour. Sections were washed exhaustively with PBS, pH 7.4, before mounting in Citifluor AF1, and viewing by epifluorescence on a Leitz Dialux microscope.

RESULTS

Gross morphology and histology

A severe case of osteochondrosis affecting the femoral condyles of a 5-month-old Yorkshire Landrace hybrid pig is shown in Fig. 1A. The cartilage shows characteristic deformation with a flattening of the medial femoral condyle and creasing of the articular surface. A section through the femoral condyle reveals localised thickening of the articular cartilage with a change in the appearance of the epiphyseal bone directly underlying the lesion (Fig. 1B). Growth plate cartilage is also affected by osteochondrosis, defects appearing as disruptions to the growth plate with a breakdown of cartilage structure and inclusion of wedges of cartilage that persist in the metaphyseal bone (arrowed) (Fig. 1C). Osteochondrotic bones usually contained lesions of both articular and growth plate but occasionally only one type of cartilage was affected.

A section taken through an osteochondrotic lesion in articular cartilage is shown in Fig. 2A. Histological examina-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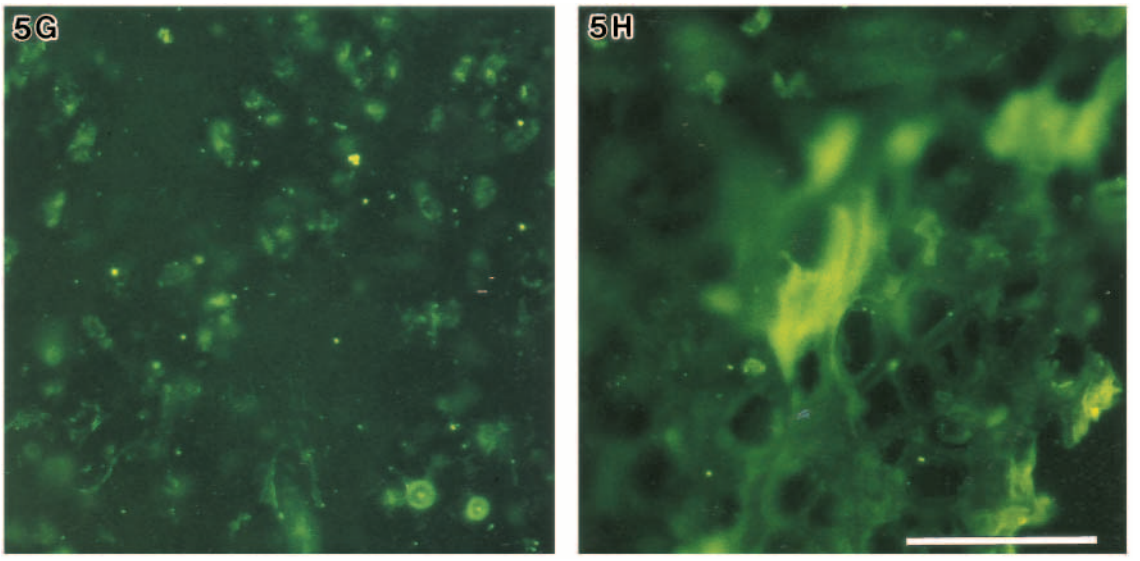

Fig. 5. Immunofluorescence localisation of collagens in porcine articular cartilage. (A-F) articular surface; (G,H) calcifying region of articular cartilage. (A,C,E and G) Normal cartilage. (B,D,F and H) Osteochondrotic cartilage. (A,B) Staining for type I collagen. (C,D) Staining for type VI collagen. (E,F) Staining for type IX collagen. (G,H) Staining for type X collagen. Bar, 100 μm.

tion shows that the majority of the lesion is composed of apparently healthy chondrocytes resembling those of the middle zone of normal articular cartilage. Vascularisation is usually absent from normal articular cartilage but all samples of articular cartilage taken from osteochondrotic lesions show the presence of numerous vascular channels. Areas of subchondral bone underlying the lesions are frequently replaced with large areas of mononuclear cells interspersed with fibrous tissue. The chondrocytes of the articular surface are normal in appearance and distribution (not shown). The most significant alteration is in the calcifying region of the lesion cartilage where the large, oval, mature chondrocyte clusters that are normally present in unaffected articular cartilage (Fig. 2B) are absent. Articular chondrocytes of the calcifying region in osteochondrotic lesions either resemble immature, mid-zone cells (Fig. 2C), or take on an appearance similar to hypertrophic growth plate chondrocytes, aligned in short columns (Fig. 2D).

Normal growth plate cartilage has a well-ordered structure with the chondrocytes of the proliferative and hypertrophic regions arranged into columns (Fig. 2E). Osteochondrotic growth plate cartilage lesions reveal that the main changes occur in the hypertrophic region, which appears disorganised and greatly extended compared to normal tissue (Fig. 2F). Some vascularisation is usually evident in lesions and there are areas of fibrosis within the cartilage and adjacent bone. Areas of uncalcified cartilage are retained deep within the metaphyseal bone.

Collagen preparation and quantification

Samples of the pepsin-insoluble residue, guanidine hydrochloride extracts and salt-precipitated fractions appeared to be completely soluble in SDS-sample buffer, allowing all the collagenous components of each cartilage to be identified by SDS-PAGE (Figs 3A and 4A). Few differences are evident between normal and osteochondrotic articular cartilage by staining with Coomassie Blue (Fig. 3A). However, a weak band corresponding to pepsinised type X collagen is evident in the osteochondrotic sample precipitated with 2.0 M sodium chloride (Fig. 3A, track 10). This was confirmed by western blotting with a specific mouse anti-porcine collagen type X antibody (Fig. 3B).

In growth plate cartilage, type I collagen appears to be more abundant in the osteochondrotic samples (Fig. 4A, track 6), a fact reinforced by the appearance of the western blot (Fig. 4B). A band corresponding to pepsinised type X collagen is present in both normal and osteochondrotic samples.

Collagen type VI was also identified by western blotting in both cartilages but no differences were observed between normal and osteochondrotic samples (data not shown).

The amounts of total collagen in cartilage samples were calculated by hydroxyproline assays and the results expressed as a percentage of dry weight of tissue. There is a substantial decrease in total collagen in both articular and growth plate cartilages from osteochondrotic lesions when compared with normal cartilage. In articular cartilage, the percentage collagen decreases from 52.4%±2.7% to 38.2%±0.5% and in growth plate there is a decrease from 32.4%±3.1% to 18.4%±3.4%

The major collagen types (I, II, IX, X and XI) were quantified as described previously (Wardale and Duance, 1993) and the results shown in Table 1.

The quantification results show changes in the ratio of the major collagens from osteochondrotic articular or growth plate cartilage lesions when compared with normal cartilage. There are substantial increases in the level of type I collagen in both osteochondrotic cartilages compared to normal. Type I collagen is particularly increased in osteochondrotic growth plate to almost double the level found in normal growth plate cartilage. Both osteochondrotic cartilages show a concomitant decrease in the percentage of type II collagen. There is also a decrease in type IX content in lesion material from both cartilages. Type X collagen is present in osteochondrotic articular cartilage lesions but only in very low amounts. Type XI collagen levels appear slightly reduced in both osteochondrotic cartilages.

Table 1. Relative percentages of major collagen types in porcine articular and growth plate cartilage

Collagen type	Articular cartilage (normal)	Articular cartilage (OCD)	Growth plate cartilage (normal)	Growth plate cartilage (OCD)
I	11.6±3.8	21.6±6.0	18.3±3.7	43.3±5.5
II	75.5±5.0	71.7±4.7	63.3±5.0	44.8±6.8
IX	3.9±1.8	0.8±0.1	1.3±0.2	0.6±0.1
X	0	0.05±0.03	0.3±0.2	0.2±0.03
XI	9.0±2.9	5.9±1.5	16.8±1.6	11.2±2.1

We have previously reported differences in the extraction of collagen types following pepsin treatment and this was examined for osteochondrotic samples (Table 2).

The data show that for both articular and growth plate cartilage, types I and II collagen from osteochondrotic cartilage are less readily extracted by pepsin than from normal cartilage. Type II collagen from osteochondrotic growth plate is particularly resistant to extraction by pepsin treatment, indicating an overall change in the structure of the cartilage during osteochondrosis.

Collagen cross-link analysis

Articular and growth plate cartilage from normal and osteochondrotic animals were examined to determine whether the changes in the amounts of collagen extracted by pepsin were related to changes in the amount or nature of collagen cross-links present in the diseased tissue (Table 3).

There is a significant fall in the levels of both mature and intermediary cross-links in osteochondrotic growth plate cartilage. deH-HLNL is most affected, being reduced to only 13% of the original. HLKNL is reduced to 32% of normal growth-plate levels and the mature cross-link HYL-PYR is at 46% of the original. Articular cartilage shows less significant changes with only HLKNL being reduced to 58% of the normal level whereas deH-HLNL and HYL-PYR are slightly increased in osteochondrotic tissue.

Immunolocalisation

Age-matched clinically normal controls and apparently unaffected areas of articular cartilage from the osteochondrotic joints reveal a normal distribution of type I collagen (Fig. 5A). However, the articular surface of osteochondrotic lesions show considerable disruption with the normally well-defined area of surface staining replaced by a disorganised matrix stain (Fig. 5B). Type I collagen is also present in the walls of blood vessels, which are apparent only in the osteochondrotic lesions (not shown). The distribution of type II collagen appears unaffected in the areas of osteochondrotic lesions although the matrix stain is slightly granular in appearance (not shown). Type III collagen colocalises with type I collagen in the walls of the invading blood vessels in osteochondrotic lesions and also produces a weak signal at the articular surface (not shown). No signal is seen with an antibody to type V collagen in either normal or osteochondrotic tissue. The normal pattern of staining for type VI collagen at the articular surface (Fig. 5C) appears to be disrupted in osteochondrotic tissue in a similar manner to that of type I collagen (Fig. 5D). The staining pattern also progresses deeper into the cartilage, suggesting a greater number of cells secreting this collagen type. The matrix stain for type IX collagen in osteochondrotic lesions is more granular and intense than that seen in the upper regions of normal articular cartilage (Fig. 5E and F). Staining in the calcified region is little changed although a slightly stronger matrix stain is seen in the osteochondrotic lesions (not shown). Type X collagen is absent from normal articular cartilage (Fig. 5G) but is evident in the calcifying region of osteochondrotic lesions surrounding areas of chondrocytes that are hypertrophic in appearance (Fig. 5H). The staining pattern for type XI collagen appears unchanged in the diseased state (not shown).

In the growth plate, a similar disruption to the type I staining pattern is evident in osteochondrotic areas (Fig. 6A and B). Type II collagen is unaffected and there is no evidence of any type III collagen except in blood vessels as seen in the articular cartilage lesions. No staining for types V or VI was detected. The staining pattern of type IX collagen is similar to that in the articular lesions in that it is more intense and granular in the matrix of the diseased tissue than in the normal tissue. The hypertrophic region of normal growth plate stained with anti-type IX collagen (Fig. 6C) is compared with the same region of an osteochondrotic lesion (Fig. 6D). There appears to be little difference between the distribution of type X collagen in normal (Fig. 6E) and osteochondrotic growth plate (Fig. 6F). There is no evidence of any intracellular accumulation of type X as reported for vitamin D-deficient chickens (Bashey et al., 1989). As with articular cartilage, there is little change in the staining pattern for type XI collagen (not shown).

It was apparent from the immunofluorescence studies that the removal of cartilage from the osteochondrotic growth plate cartilage/bone interface is not as complete as in the normal interface. This was particularly evident from the staining pattern revealed using the antibody against type IX collagen. By combining immunofluorescence with a low light level phase-contrast image it is possible to see the ossification front with respect to the immunofluorescence for type IX collagen

Table 2. Percentage of major collagen types extracted from articular and growth plate cartilage by pepsin treatment

Collagen type	Articular cartilage (normal)	Articular cartilage (OCD)	Growth plate cartilage (normal)	Growth plate cartilage (OCD)
I	41.4±9.8	9.7±2.3	26.3±7.0	16.5±3.8
II	88.0±3.4	71.3±5.4	55.5±1.7	16.2±8.4
IX	~100	~100	~100	~100
X	–	~100	~100	~100
XI	28.2±11.4	27.3±9.1	0	0.9±0.5

Table 3. Quantification of cross-links in porcine cartilage (moles per mole of collagen)

Cross-link	Articular cartilage (normal)	Articular cartilage (OCD)	Growth plate cartilage (normal)	Growth plate cartilage (OCD)
HYL-PYR*	0.45±0.04	0.73±0.15	0.35±0.04	0.16±0.05
LYS-PYR	0.10±0.02	0.07±0.01	0.01±0	0.03±0.01
HLKNL	1.15±0.16	0.67±0.04	1.95±0.28	0.63±0.09
deH-HLNL	0.43±0.28	0.63±0.07	0.68±0.04	0.09±0.01

*Abbreviations: HYL-PYR, hydroxylysyl-pyridinoline; LYS-PYR, lysyl-pyridinoline; HLKNL, hydroxylysyl-ketonorleucine; deH-HLNL, dehydro-hydroxylysinonorleucine.

(Fig. 7A-E). It is clear that the well-defined demarcation of type IX staining at the normal growth plate resting zone/epiphyseal bone junction (Fig. 7A) is lost in osteochondrotic lesions, with areas of immunoreactive type IX collagen extending into the bone (Fig. 7B). This is also true for the ossification front where the chondrocytes of the hypertrophic region are normally rapidly eroded (Fig. 7C). In osteochondrotic lesions a proportion of the chondrocytes appears to remain intact within the advancing bone and are not removed until later (Fig. 7D).

DISCUSSION

The complex sequence of events that lead to the calcification and subsequent ossification of hyaline cartilage are still not fully understood. However, it is likely that the composition of the cartilage is critical in that it needs to be resorbed and remodelled to provide a scaffolding on which to build trabecular bone. In the growth plate, the rate of expansion of the cartilage is matched by the rate of resorption at the ossification front, giving rise to a cartilage that remains at a near constant thickness while the animal is growing. A system such as this is obviously critically balanced in both types of biochemical processes and also the rates with which these processes take place. Collagen makes up approximately 50% and 30% of the dry weight of articular and growth-plate cartilages, respectively, and it is likely that any perturbation in the collagenous matrix of the cartilage will upset this delicate balance.

Our results show changes in the overall ratios of collagen types in osteochondrotic articular and growth plate cartilage when compared with normal levels. If the whole cartilage from joints in which osteochondrotic lesions are present is examined, the changes in collagen ratios are largely masked (Wardale, unpublished data). This indicates the localised nature of the disease and the possible reason why biochemical differences have been rarely reported in the past. The large increase in the levels of type I at the expense of type II collagen in osteochondrotic growth plate is particularly striking. It is possible that there is some contamination with type I collagen from bone during sampling as the two tissues are difficult to separate in large lesions. However, no type V collagen could be detected in our samples even using enhanced western blotting techniques and therefore it is probable that there is little or no influence from bone collagens. Type I collagen production is quite common in diseases of cartilage and is generally viewed as an attempt at repair by the chondrocytes as in osteoarthritis (Adam and Deyl, 1983; Aigner et al., 1993a). In pig cartilage, where type I collagen already exists (Wardale and Duance, 1993), a similar mechanism may be taking place and increasing the already high levels to those seen in osteochondrotic samples. However, the dramatic drop in the percentage of total collagen seen in both osteochondrotic articular and growth plate cartilages may be the major cause of the change in collagen ratios. A loss of type II collagen from the cartilage would result in a higher ratio of type I to type II collagen without a large increase in type I synthesis. Type II collagen is thought to be an inhibitor of angiogenesis and the increase in vascularisation of osteochondrotic cartilage may be due in part to this reduction in type II collagen content. Whether vascularisation precedes the changes in collagen ratios or whether there is a change in the collagenous composition of the cartilage allowing vascularisation to occur will be a subject of future studies.

There are quite dramatic changes in the overall arrangement of chondrocytes within the diseased tissue, which suggests an alteration in phenotype or in the normal pathways of maturation. Normal porcine articular cartilage of this age is characterised by having ovoid clumps of chondrocytes in the area of calcification adjacent to the epiphyseal bone. In osteochondrotic articular cartilage these clumps are either altered in appearance, greatly reduced in number or completely absent. The thickened articular cartilage is mainly made up of chondrocytes of the type found in the middle regions of a normal cartilage, i.e. single or paired cells. This suggests that the chondrocytes have been halted in an earlier state of maturation than normal. The cells usually appeared healthy and there were rarely any signs of the chondronecrosis that has been reported in the past (Carlson et al., 1986; Ekman et al., 1990). Numerous samples revealed chondrocytes in the calcifying region of osteochondrotic articular cartilage with an appearance similar to hypertrophic growth plate chondrocytes. Immunofluorescence indicated that a number of articular hypertrophic cells express type X collagen and are presumably the source of type X collagen detected by SDS-PAGE and western blots of osteochondrotic articular cartilage samples. This is a clear indication of an altered chondrocyte lineage and suggests that the factors that normally act on growth plate chondrocytes to produce their characteristic hypertrophic maturation may also be acting on osteochondrotic articular cartilage.

In growth plate, the thickening occurs mainly with a build up of chondrocytes in the hypertrophic region. These cells fail to remain in the well-ordered columns characteristic of normal tissue and become a disorganised mass. The unaffected parts of the ossification front extend past these areas, resulting in the characteristic osteochondrotic lesions.

The extraction data presented here suggest changes in the overall interactions between the main collagen types and other components of the extracellular matrix. We have previously shown differences in the susceptibility to pepsin extraction between articular and growth plate cartilage and it seems clear that there are considerable differences between the normal and diseased states. Types I and II collagen were less readily extracted by pepsin from osteochondrotic cartilage than from normal cartilage although all the collagen types were soluble in SDS. Type II collagen was substantially less extractable from osteochondrotic growth plate and this may have implications for resorption of the cartilage at the ossification front.

Analysis of collagen cross-link data also suggested considerable changes in the nature of the collagenous matrix of osteochondrotic cartilage. The changes in collagen cross-links in osteochondrotic articular cartilage were variable compared to normal tissue, with slight increases in both the mature cross-link HYL-PYR and the intermediary cross-link deH-HLNL. However, there was a significant drop in the amount of the intermediary cross-link HLKNL. Osteochondrotic growth-plate cartilage showed considerable decreases in all of the major cross-links, particularly deH-HLNL. A reduction of this size in the collagen cross-linking would have the effect of producing a considerably weakened tissue, which could give rise to the fissuring and fibrous repair tissue that is frequently reported in cases of osteochondrosis in the growth plate. The

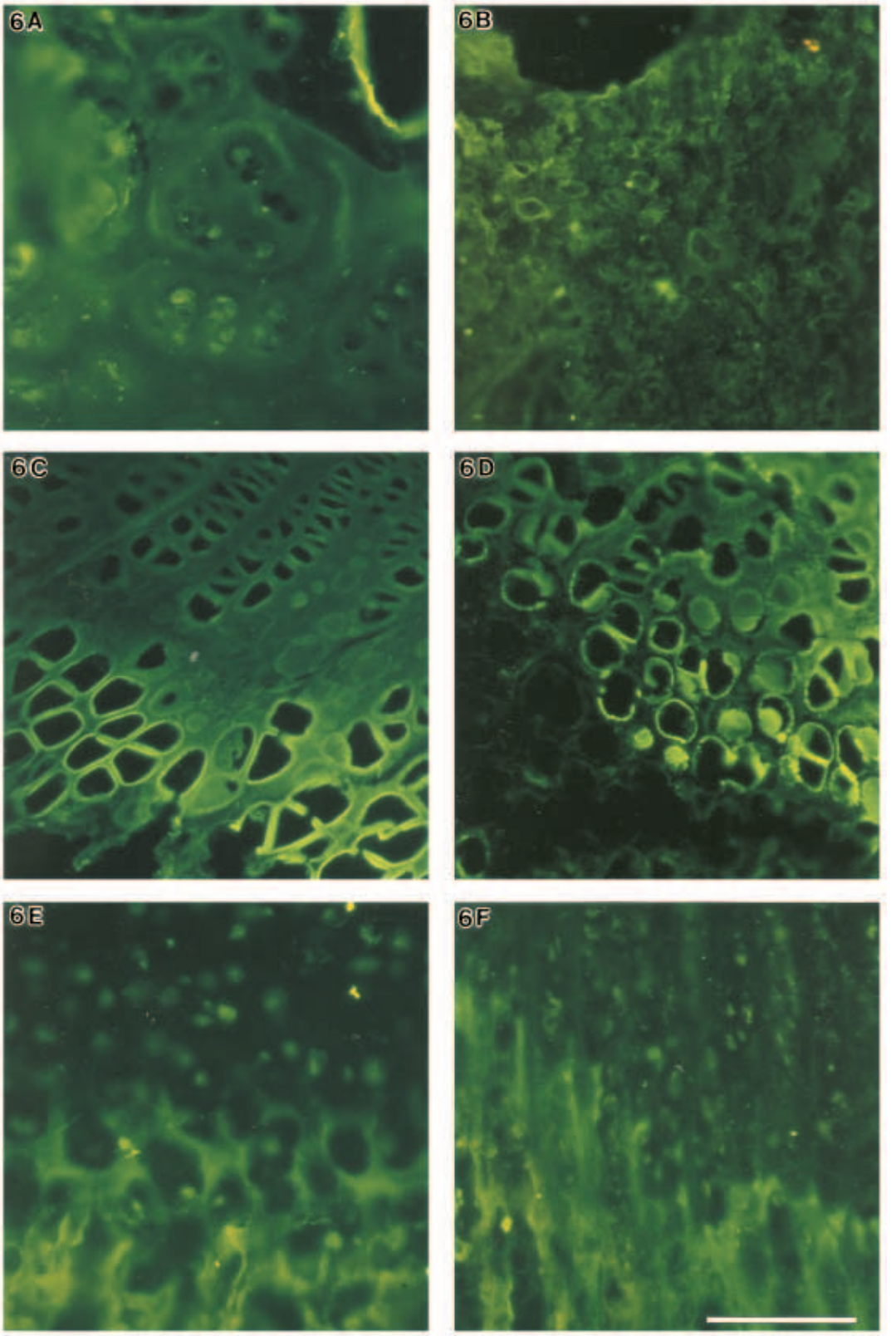

Fig. 6. Immunofluorescence localisation of collagens in porcine growth plate cartilage. (A,B) Growth plate resting zone. (C-F) Growth plate hypertrophic region. (A,C and E) Normal cartilage. (B,D and F) Osteochondrotic cartilage. (A,B) Staining for type I collagen. (C,D) Staining for type IX collagen. (E,F) Staining for type X collagen. Bar, 100 μm.

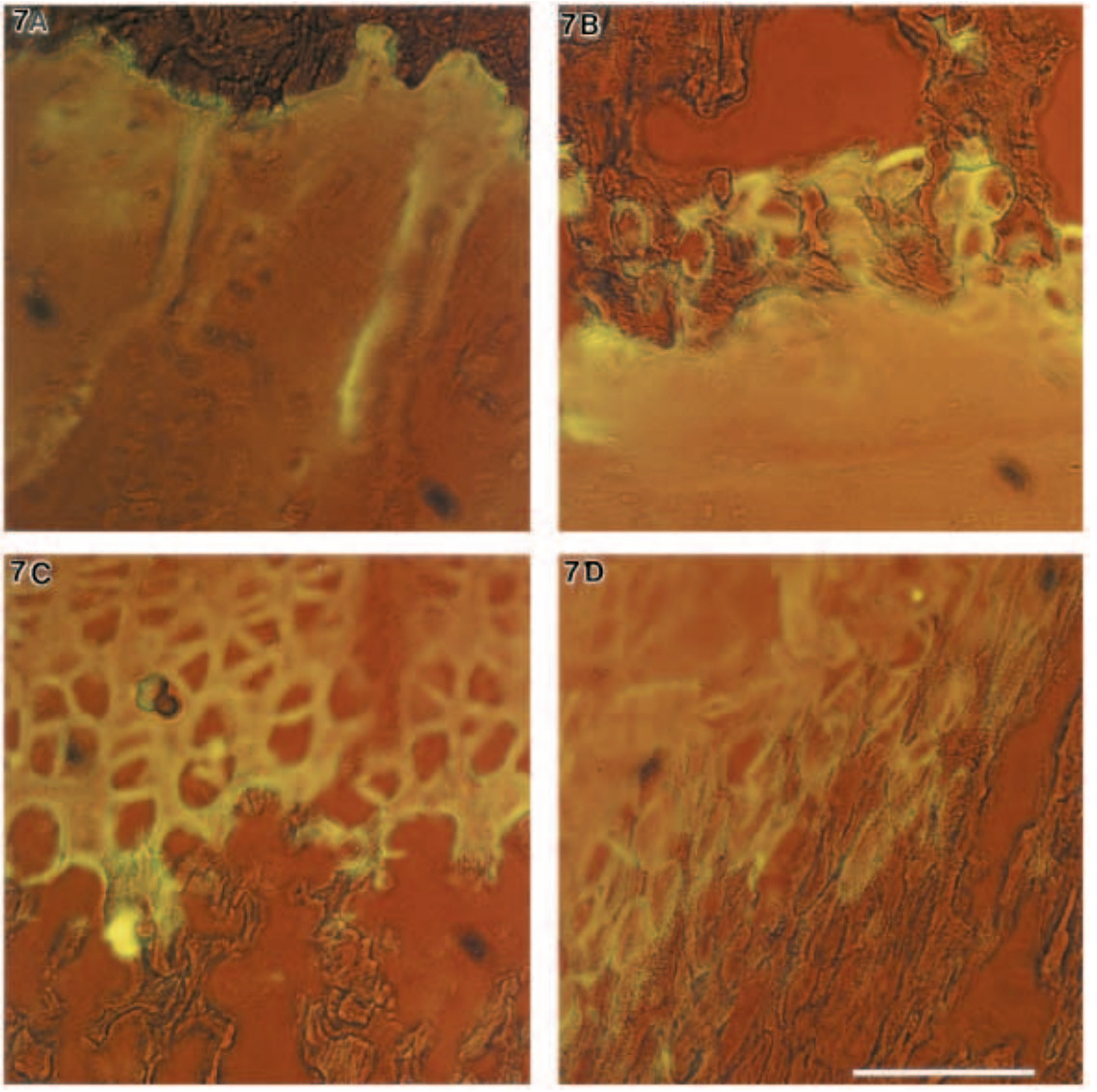

Fig. 7. Immunofluorescence/phase-contrast microscopy of the type IX collagen staining pattern in normal and osteochondrotic growth plate. (A,C) Normal cartilage. (B,D) Osteochondrotic cartilage. (A,B) Growth plate resting zone. (C,D) Growth plate hypertrophic region. Bar, 100 μm.

decrease in mature collagen cross-links may be an indication that there is increased turnover of collagen in the diseased tissue but this is not reflected by an increase in the levels of intermediary cross-links. The reduction in both mature and intermediary cross-links in osteochondrotic growth plate could be caused by a failure of the overall cross-linking mechanism. This is supported by a piece of work in which lesions identical to those found in osteochondrosis were induced by feeding a copper-deficient diet to foals (Bridges and Harris, 1988). The authors suggested that the reduction in serum copper levels affected the copper-catalysed lysyl oxidase reaction that is necessary for collagen cross-link formation.

Despite the low levels of collagen cross-links, osteochondrotic cartilage is more resistant to pepsin extracton than normal cartilage. The increase in collagen insolubility that is observed with increasing age of cartilage is often ascribed to an increase in the levels of mature collagen cross-links. Our data suggest that other interactions within the cartilage may be responsible for alterations in collagen solubility. An as yet unidentified cross-link may be responsible but the total solubility of our samples in detergents such as SDS suggest a membrane-collagen interaction.

It should be noted that while the upper regions of both cartilages appear histologically normal, the overall appearance of osteochondrotic cartilage as shown by immunofluorescence localisation is one of a generalised disruption to the organisation of the extracellular matrix. Staining for type I collagen in articular cartilage shows clearly that the very well-defined patterns of a continuous layer at the articular surface and pericellular staining of the surface chondrocytes seen in normal tissue become broken and patchy in the diseased state. In both growth plate and articular osteochondrotic cartilage there is no longer any pericellular staining and the type I collagen is dispersed into the matrix. It was noted that the staining pattern for type VI collagen is deeper into the osteochondrotic articular cartilage than normal cartilage and this suggests an increase in

the number of cells expressing this collagen type. This should lead to an overall increase in the amount of type VI collagen in osteochondrotic articular cartilage, but this could not be confirmed biochemically as the overall levels of this collagen type were too low to measure. The staining pattern of type VI collagen showed a similar disruption to that of type I collagen, again suggesting that the originally ordered pattern of collagenous matrix had been altered. As in normal growth plate cartilage, no type VI collagen staining was seen in osteochondrotic growth plate. Type I appears to be dispersed more widely throughout the growth plate than in articular cartilage and it is likely, given the fact that types I and VI co-localise in most tissues, that the low levels of growth plate type VI detected by immunoblotting are too widely distributed to be visible by immunofluorescence. Disruption to the ultrastructure of the extracellular matrix in osteochondrosis has been previously reported (Farnum and Wilsman, 1986) and they put forward the hypothesis that a breakdown of pericellular matrix components preceded the failure of ossification. An alteration in extracellular matrix composition caused by a failure of chondrocyte maturation has also been suggested as a cause of osteochondrosis (Ekman and Heinegard, 1992). The widespread disruption of the collagenous components of osteochondrotic cartilage that we observed in all lesions, irrespective of size, supports this theory.

Type IX collagen appears to stain considerably more intensely in osteochondrotic cartilage than in normal cartilage despite the apparently reduced percentage in the tissue. There is a suggestion of some disruption to the overall matrix stain of type IX, but it is less marked than for types I or VI collagen. The change observed in the organisaton of the cartilage may make the type IX molecule more accessible to antibody labelling, which would account for the increased immunofluorescent stain. Of more significance is the apparent failure of type IX collagen to be completely resorbed at the ossification front. Normal cartilage has a remarkably well-defined demarcation between cartilage and bone, which can clearly be seen using a strongly fluorescent cartilage stain such as that for type IX collagen. Hypertrophic cells and their associated matrix are normally completely eroded with few traces of cartilage collagens remaining in the trabecular bone. However, in osteochondrosis, hypertrophic cells that still possess a pericellular type IX collagen survive into the newly formed bone, suggesting that there is impaired digestion of the cartilaginous matrix. It is probable that type II collagen, to which type IX is covalently bound, and the other cartilage collagens are also not fully resorbed, but the signals derived from the other collagen-specific antibodies were too weak to be visualised by this method. As this occurs at the border of the growth plate with the epiphysis as well as at the metaphysis, it is likely that there is an overall inhibition of growth plate resorption, possibly due to the observed disruption in the collagenous structure of the cartilage or to a decrease in the enzymes responsible for the removal of specific components of the cartilage. The lack of resorption of whole cartilage would not necessarily cause the alterations in collagen ratios that we have demonstrated and therefore it is likely that the observed ratios are due to changes within the cartilage rather than at the ossification fronts.

The existence of type X collagen in osteochondrotic porcine articular cartilage is interesting in that it coincides with areas of chondrocytes that are hypertrophic in appearance and do not resemble chondrocytes normally found in this species of articular cartilage. This fact combined with the overall histological appearance of osteochondrotic lesions in both cartilages suggests that the normal pattern and progression of chondrocyte maturation has been affected. Type X collagen has been shown to be expressed in osteoarthritic articular cartilage (Hoyland et al., 1991; Aigner et al., 1993b) and may be a product of attempted repair by the cell population. Expression of type X collagen by articular chondrocytes may be a valuable marker for investigating some cartilage disease processes.

A similar disease to osteochondrosis exists in commercially reared chickens and turkeys. Tibial dyschondroplasia is characterised by a lack of growth plate ossification and a build up of hypertrophic growth plate chondrocytes. Dyschondroplasic chicken hypertrophic chondrocytes show a build up of intracellular type X collagen similar to that shown in vitamin D-deficient birds (Bashey et al., 1989), but this is not the case for porcine osteochondrotic growth plate hypertrophic chondrocytes, which appear normal in their expression of type X collagen. A significant difference between the two diseases is that chondrocytes from avian dyschondroplasia appear to be halted at an early stage of hypertrophy, whereas in mammalian osteochondrotic growth plate the cells appear to hypertrophy normally but lack the characteristic columnar organisation of unaffected growth plate. In osteochondrosis, endochondral ossification is impaired despite the presence of a normal level and distribution of type X collagen in growth plate and the increase of type X in articular cartilage. This indicates that the existence of this collagen type alone is not enough to cause ossification of cartilage. It had originally been suggested that type X collagen had a role either in cartilage calcification or chondrocyte maturation, but it is now thought that type X collagen has a structural role in which the molecule provides a scaffolding of support to the cell while the extracellular matrix is eroded away at the ossification front (Kwan et al., 1991; Schmid and Linsenmayer, 1990).

It is evident from our work that there is a considerable change in both the nature of the chondrocytes and the characteristics of the collagenous matrix in osteochondrotic cartilage. The significant reduction in the proportion of collagen in the cartilage combined with the reduction in cross-linking seen in the growth plate would produce highly unstable areas of tissue. In an animal such as the commercially raised pig, which has an extremely rapid weight gain, the stresses on such a weakened cartilage undoubtedly lead to serious defects. Further studies are now necessary to identify those factors that are acting on the chondrocytes to produce these changes.

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