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The spatial and temporal pattern of collagens I and II and keratan sulphate in the developing chick metatarsophalangeal joint

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Summary

Both intrinsic and extrinsic factors are known to be involved in the morphogenesis of diarthrodial joints. The use of specific antibodies to collagens I and II and keratan-sulphate-containing proteoglycans (KSPG) has enabled the distributions of these macromolecules to be followed during the development of the third metatarsophalangeal joint in the chicken embryo.

Our study shows that cartilage differentiation occurs as a continuous rod, which is then subsequently divided into separate elements. Further development also reveals that, unlike the matrix of the cartilaginous elements, there is a differential distribution of collagen (type II) and KSPG in the presumptive joint region. It is proposed that a decrease in KSPG in the presumptive joint region at stages 28/30 may be involved in the mechanism for the flattening of cells in formation of the interzone. Whereas, a decrease in collagen across the joint interzone region may provide an area of weakness, which might allow forces produced by the developing musculature to cause cavitation.

Key words: joint development, collagen I, collagen II, keratan sulphate, chick embryo, pattern.

Introduction

A characteristic feature of the cartilage skeleton of embryonic vertebrates is the formation of diarthrodial joints in a precise manner. Bernays (1878) divided joint morphogenesis into two separate phases, the first being formation of the anlage and the second being the completion of the joint. In this respect, it is of interest that studies on digit regeneration in the newt have shown that the cartilage is laid down as a solid rod and thereafter divided into separate digits (Smith, 1978).

Changes in histological staining properties have been described during these two phases by a number of authors (Andersen & Bro-Rasmussen, 1961; Andersen, 1962*b*; O'Rahilly & Gardner, 1956; Mitrovic, 1978). Prior to cavitation, Andersen (1962*a*), in the hip, and Andersen & Bro-Rasmussen (1961) in the joints of the hand and foot, describe large amounts of chondroitin sulphates A and C (shown by alcian blue staining) accumulated intercellularly in the three-layered interzone. When present at the site of the joint, the interzone consists of two chondrogenous layers and an intermediate layer. Just before cavitation the intercellular metachromasia of the intermediate interzonal layer decreases. Regional variations in collagen fibre density and arrangement have been described in joint condensations of the developing human elbow joint (Gray & Gardner, 1951).

Murray (1926), Murray & Selby (1930), Fell & Canti (1934), Hamburger & Waugh (1940) & Mitrovic (1982) demonstrated with organ culture experiments that intrinsic factors are largely responsible for the determination of the general form and architecture of the joint. Environmental factors also are postulated to play an important role in the formation of joints; some workers emphasize the role of movement in both formation (Lelkes, 1958; Drachman, 1964; Drachman & Sokoloff, 1966; Persson, 1983) and maintenance of the formed joint cavity (Drachman & Coulombre, 1962; Mitrovic, 1972, & see Thorogood, 1983 for review). The relationship of intrinsic and extrinsic factors, and their influence on joint morphogenesis is unclear.

The purpose of the present study was to visualize changes in the distribution of collagens I and II and keratan sulphate, (as a representative proteoglycan component) using polyclonal and monoclonal antibodies respectively, during the development of the

third metatarsophalangeal joint in the chick embryo in order to see if cartilage is initially a continuous rod which then subsequently segments, as in the newt. In view of their different mechanical properties, we suggest that changes in the distribution of these extracellular matrix components play a role in the initiation and completion of joint formation.

Materials and methods

Preparation of specimens

Fertilized White Leghorn embryos were incubated, humidified at 38°C (±1°C). Stage-27 to -41 embryos (Hamburger & Hamilton, 1951) were selected and the hind limbs dissected in phosphate-buffered saline. Prior to stage 32, whole leg primordia were fixed in 95% alcohol at 4°C for 2 h. From stage 32, a cut was made through the distal end of the tibia and the feet fixed as described above. The tissue was dehydrated at 4°C in three changes of absolute ethanol followed by three changes of xylene (30 min each) and allowed to reach room temperature before embedding in paraffin wax.

The embedded tissue was stored at 4°C until sectioned. Serial 5 μm sections were cut and four consecutive sections mounted on each glass slide. These were stored at 4°C until required for immunohistochemistry.

Additional leg primordia at the same stages were fixed in Bouins and processed for conventional histology. Serial 5 μm sections were cut and stained with 0·2% aqueous toluidine blue (Lillie, 1929) or picro–ponceau and haematoxylin (Gurr, 1956).

Immunofluorescence

Cartilage characteristic type II and type I collagens were localized in tissue sections by indirect immunofluorescence (von der Mark, von der Mark & Gay, 1976*a*). Affinity-purified rabbit antisera to chick type I and II collagens were kindly donated by C. Cottrill (both these antisera were prepared at the Max-Planck Institut für Biochemie, under the supervision of Dr K. von der Mark). MZ15, a

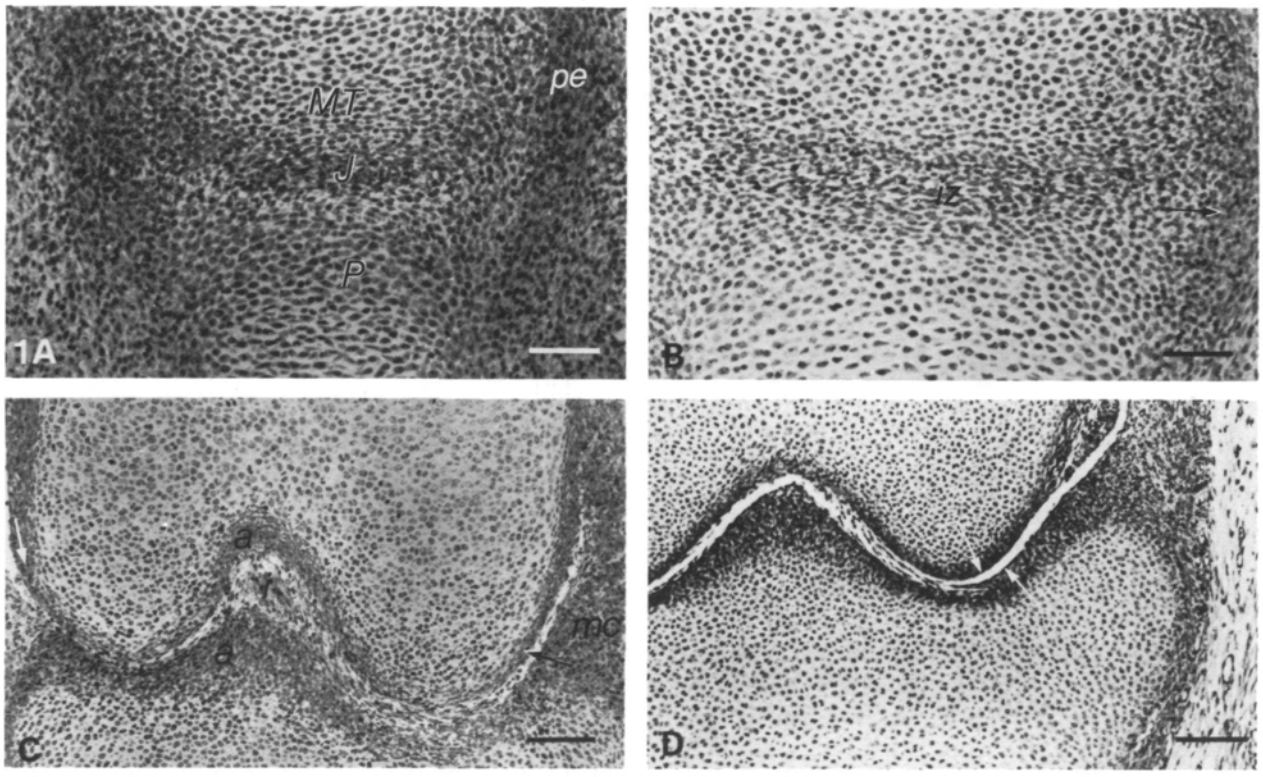

Fig. 1. Longitudinal section of chick metatarsophalangeal joint (MTPJ). All micrographs are presented with the metatarsal (*MT*) lying above the phalynx (*P*). (A) Stage 28, stained with picro–ponceau/haematoxylin. Bar, 43 μm. The presumptive joint region (*J*) is seen as a cellular condensation lying between the metatarsal (*MT*) and phalangeal (*P*) cartilage elements. The developing perichondrium can be seen as a more darkly staining layer surrounding the cartilage anlage (*pl*). (B) Stage-30 MTPJ, stained with picro–Ponceau/haematoxylin. The region of relative high cell density between the two opposed skeletal elements represents the developing interzone (*iz*). Increased intercellular matrix secretion is seen in the interzone in comparison with stage 28, especially peripherally (see arrow). Bar, 33 μm. (C) Stage-37 MTPJ, picro–Ponceau/haematoxylin. Bar, 33 μm. The interzone is divided into three layers, the intermediate layer (*i*) lying between the two darkly staining, developing endochondral layers (articular surfaces, *a*). The developing meniscus/capsule (*mc*) can be seen on the right-hand side of the micrograph. Cavitation has begun at the periphery of the joint (see arrow). Bar, 100 μm. (D) Stage-41 MTPJ, toluidine blue staining. Cavitation in the joint is virtually complete; the articular surfaces being well defined (arrows). Bar, 33 μm.

monoclonal antibody that has been shown to be specific for keratan sulphate (Zanetti, Ratcliffe & Watt, 1985; Mehmet, Saudder, Tang, Hounsell, Caterson & Feizi, 1986), was used as a marker of cartilage proteoglycan synthesis, and was a gift kindly donated by Fiona Watt (Kennedy Institute, London).

Sections were dewaxed and then rehydrated through a series of alcohols and rinsed in two changes of Tris-buffered saline (TBS, 15 min each). The sections were pretreated with chondroitinase ABC (Sigma, 0·25 i.u. ml^{-1}) and testicular hyaluronidase at 37°C (Sigma, 1·45 i.u. ml^{-1}), washed (TBS, three times 10 min), and then incubated with the primary antibody (collagen I, 1:20; collagen II, 1:20; MZ15, 1:1000 diluted in TBS) for 45 min at room temperature. The removal of chondroitin sulphate by chondroitinase treatment enhances staining by making keratan sulphate more accessible to the MZ15 antibody (Oike, Kimata, Shinomura, Nakazawa & Suzuki, 1980; Smith & Watt, 1985). After three washes in TBS (10 min each) the secondary antibodies were applied for 45 min at room temperature; fluorescein isothiocyanate (FITC) conjugated swine anti-rabbit immunoglobulins (IgG) (Dako Ltd, 1:50 dilution) were used to visualize the collagens and tetramethylrhodamine isothiocyanate (TRITC) conjugated rabbit anti-mouse IgG (Dako Ltd, 1:30 dilution) to visualize the keratan sulphate. The sections were then washed in TBS (three times 10 min) mounted in glycerol/TBS (1:9) containing 1,4-diazobicyclo [2.2.2.] octane (DABCO, 25 $g l^{-1}$; supplied by Sigma). The latter retards the fading of fluorescence during microscopy (Johnson, Davidson, McNamee, Russell, Goodwin & Halbarow, 1982). The sections were then viewed with a Zeiss photomicroscope III with an epifluorescence attachment.

Controls

Control sections for all three antibodies were processed without the primary antibody. Further controls were performed for MZ15 specificity; these included treatment of sections with 2 i.u. ml^{-1} keratanase (Miles Laboratories Ltd) for 60 min at 37°C. In order to assess the extent of masking of the MZ15 epitope by other matrix components, the above control was also performed with pre- or post-treatment with chondroitinase/hyaluronidase as described above. Finally, an additional control involved preabsorption of MZ15 with corneal keratan sulphate I (Miles, 100 $\mu g\, ml^{-1}$ TBS) for 30 min at room temperature. In photographing these controls, the automatic exposure time for sections prepared as in the immunofluorescence section was ascertained. Each control was then photographed for an equivalent exposure time. Thus the levels of fluorescence shown in Fig. 6 are relative.

Results

The onset of overt cartilage differentiation occurs at stage 25 in the chick leg when viewed in terms of immunolocalized collagen type II synthesis (von der

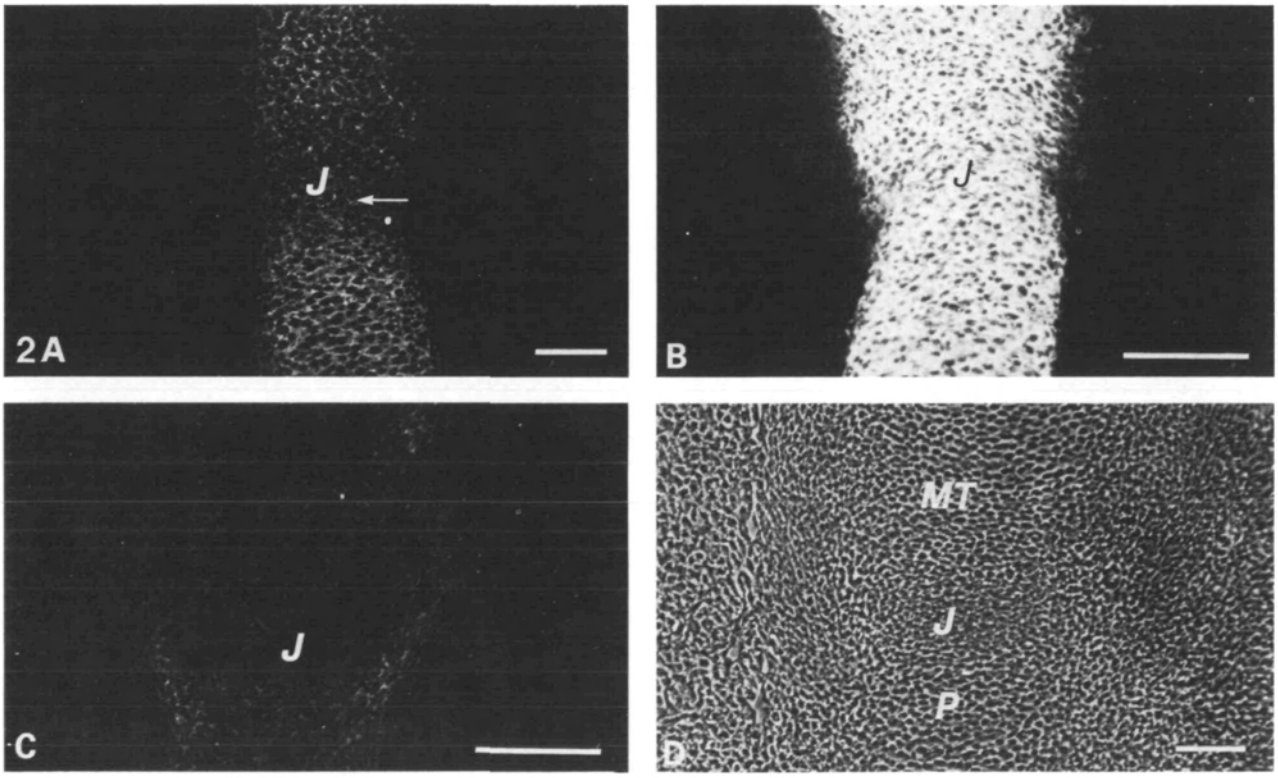

Fig. 2. (A–C) Fluorescent micrographs of stage-28 MTPJ. Bar, 100 μm. Staining for collagen II (A) and KSPG (B) can be seen throughout the cartilage anlage and is continuous across the presumptive joint region (*J*), although staining here is less for collagen (see arrow, A). (C) Staining for collagen I is found throughout the cartilage matrix but is absent in the central region of the presumptive joint region (*J*). (D) Phase-contrast micrograph of 2A. Bar, 61 μm.

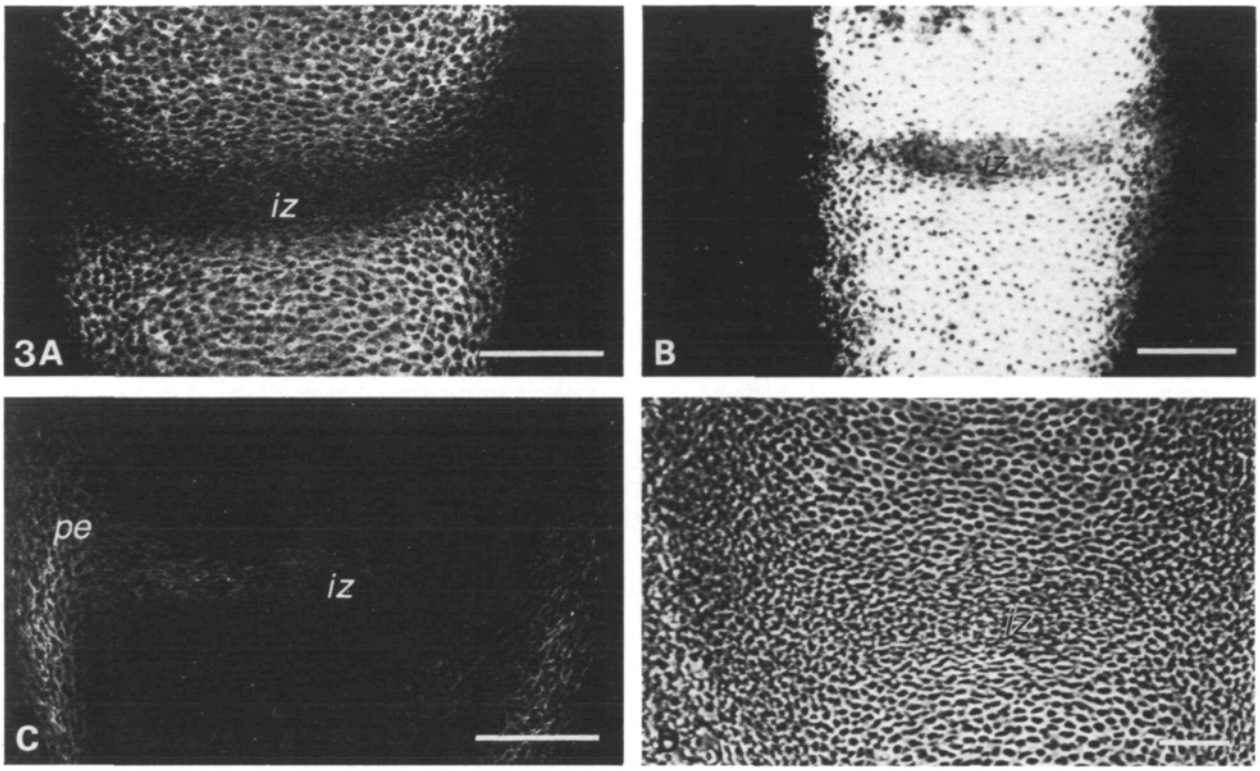

Fig. 3. (A–C) Fluorescent micrographs of longitudinal sections of stage-30 MTPJ. Collagen II and KSPG staining is found throughout the cartilage matrix, although collagen II (A) is not found at the interzone (*iz*), and that for KSPG is greatly reduced (B). Collagen I staining (C) is mainly restricted to the interzone and developing perichondrium (*Pe*), with a sparse distribution in the cartilage anlage matrix. Bar, 100 μm. (D) Phase-contrast micrograph of 3B at the same stage. Bar, 74 μm.

Mark *et al.* 1976*a*) and histological metachromasia. However, it is not until stage 27 that collagen type II is localized in the distal limb regions.

At stages 26–27 the metatarsophalynx is seen as a densely packed homogenous avascular tissue. At stage 28, the presumptive joint region first becomes histologically identifiable. It is seen as a cellular condensation lying between the opposing metatarsal and phalangeal cartilage elements (Fig. 1A). These observations confirm those made by Mitrovic (1978). At this stage staining for collagen II and keratan-sulphate-containing proteoglycan (KSPG) is seen throughout the cartilage anlage matrix and is continuous across the presumptive joint region (Fig. 2A,B,D). It is noticeable, however, that collagen type II stains less intensely in the presumptive joint region when compared with the cartilage anlage (Fig. 2A). Collagen I positive staining is also found throughout the matrix, but is absent in the central region of the presumptive joint region (Fig. 2C).

In comparison with stage 28, at stage 30 matrix secretion is more widespread, especially in peripheral areas of the developing joint (Fig. 1B). In addition, an area of relative high cell density is seen in the region between the two opposed skeletal elements, which represents the interzone (Figs 1B, 3D). In contrast to stage 28, collagen II staining is not found at the interzone and that for KSPG is greatly reduced, although both are seen throughout the cartilage matrix (Fig. 3A,B). The staining for collagen I, however, is mainly restricted to the interzone and developing perichondrium, with a sparse distribution in the cartilage anlage matrix (Fig. 3C).

Between stages 35 and 37, clefting at the periphery of the joint occurs, and by stage 37, the morphology of the future joint is apparent. The three layers of the interzone are well defined, the intermediate layer being sandwiched between the two endochondral layers (Fig. 1C). Collagen II staining is only seen in the matrix and not at the articular surface region (Fig. 4A). Unlike stage 30, when a decrease in KSPG staining is seen at the interzone (Fig. 3B), at stage 37, anti-KSPG stains both the cartilage matrix and interzone to a similar degree except in areas of cavitation (Fig. 4B,D). Collagen I is found in the perichondrium, presumptive joint capsule and meniscal structures and developing articular surfaces (endochondral layers). However, there is a marked decrease in the intermediate layer of the interzone (Fig. 4C,D).

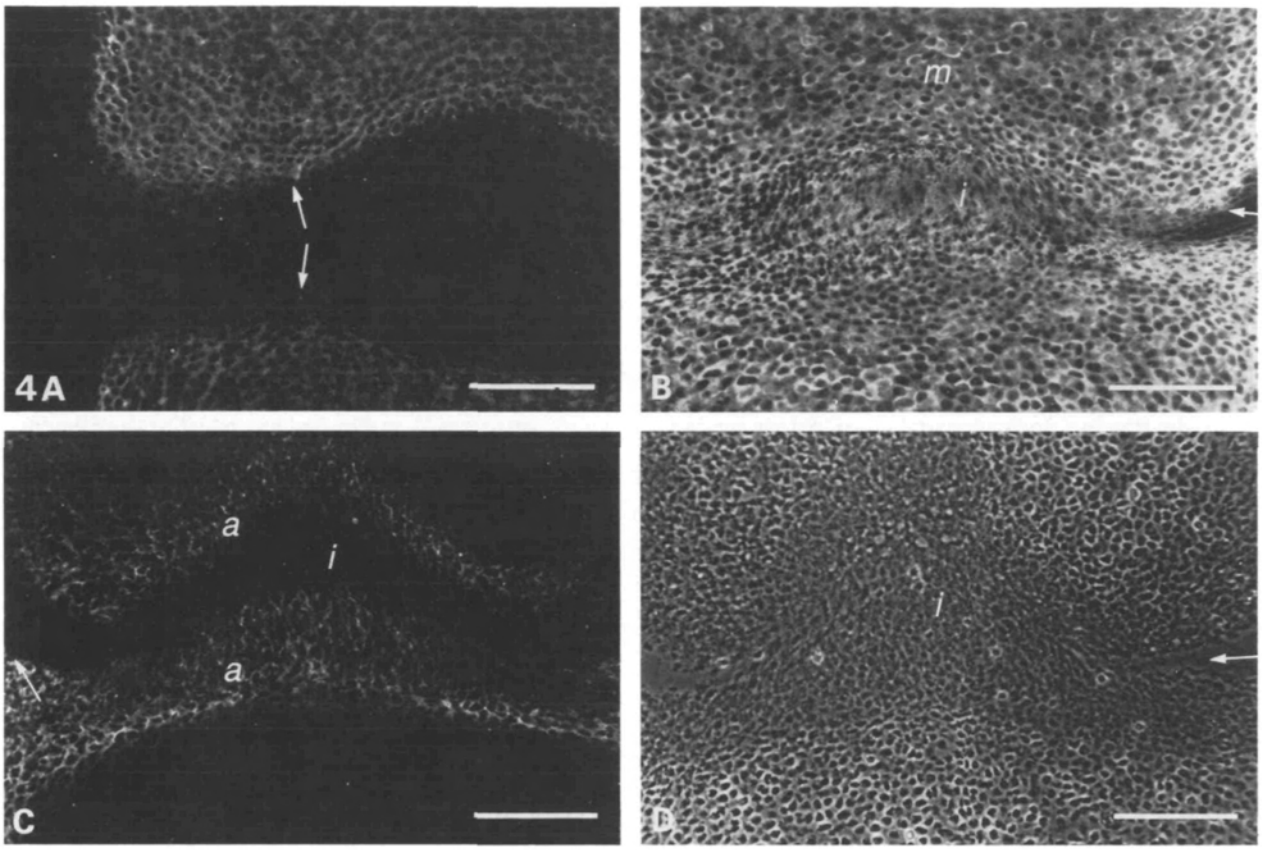

Fig. 4. (A–C) Fluorescent micrographs of stage-37 MTPJ. (A) Collagen II staining is only seen in the cartilage matrix, and is not found at the articular surfaces (see arrow). Bar, 100 μm. (B) Shows KSPG staining throughout the cartilage matrix (*m*) and interzone (*i* is intermediate layer) to a similar degree, except where cavitation is occurring (see arrow). (C) Collagen I staining is restricted to the developing articular surfaces (*a*) and meniscal structures (arrow). There is a marked decrease in collagen I staining in the intermediate layer of the interzone (*i*). (D) Phase-contrast micrograph of Fig. 4B. Cavitation has started at the periphery of the joint (arrow). The intermediate layer of the interzone is still intact (*i*). Bar, 100 μm.

By stage 41, clefting of the third metatarsophalangeal joint (MTPJ) is virtually complete (Fig. 1D, 5D). KSPG staining is found in the cartilage, articular surfaces, tendons and developing meniscus and capsule (Fig. 5A), but not in the perichondrium (not shown). Collagen I colocalizes with KSPG in the articular surfaces (approx. eight cell layers deep), tendons and meniscus and capsule but is also found in the perichondrium (Fig. 5B). There is also some colocalization of the two collagen types at the articular surfaces (approximately two cell layers deep, Fig. 5B,C). Similar results were obtained in unfixed frozen sections.

All control sections treated without primary antibody showed no staining (for KSPG, Fig. 6A). Preincubation of MZ15 antibody with excess keratan sulphate abolished staining (Fig. 6B). Pretreatment with either, or both, chondroitinase ABC and hyaluronidase enhanced KSPG staining. Pretreatment with keratanase abolished MZ15 staining in the cartilage but not where KSPG codistributed with collagen I (Fig. 6C), thus staining was still seen six to eight cells deep at the articular surfaces (Figs 5B, 6C). If keratanase treatment preceded incubation with hyaluronidase/chondroitinase, staining at the articular surfaces was enhanced (not shown), however, if hyaluronidase/chondroitinase treatment was performed before that with keratanase, the staining at the articular surfaces was reduced almost to background levels (not shown), presumably because the hyaluronidase/chondroitinase treatment increases the amount of keratan sulphate accessible for digestion.

Discussion

We have described the changing distributions of three components in the developing chick metatarsophalangeal joint; collagens I and II and keratan-sulphate-containing proteoglycan (KSPG). At the region of the presumptive joint all three matrix components are present to a lesser or greater degree. However, concomitant with the morphogenesis of the joint

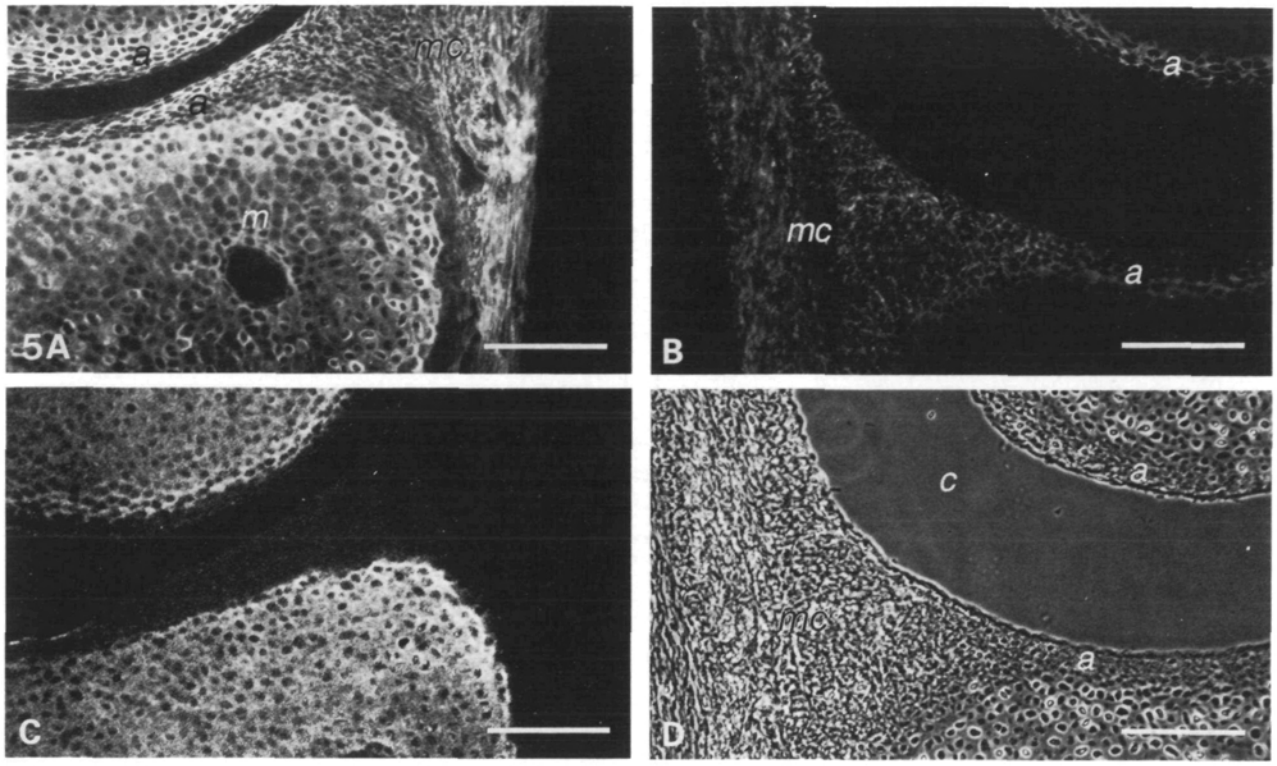

Fig. 5. (A–C) Fluorescence micrograph of lateral, stage-41 MTPJ. Bar, 100 μm. (A) KSPG staining is found in the cartilage matrix (*m*), articular surfaces (*a*) and developing meniscus/capsule (*mc*). (B) Collagen I is localized in the meniscus/capsule (*mc*), tendons, perichondrium and at the articular surfaces (*a*) to a depth of approximately eight cell layers. (C) Collagen II staining is only found in the cartilage matrix. (D) Phase-contrast micrograph of 5B showing a lateral aspect of a well-defined joint cavity (*c*). The tissue of the meniscus/capsule (*mc*) is continuous with the articular surfaces (*a*). Bar, 100 μm.

there is a restriction of the matrix components to specific structures. Our results have confirmed the initial observations of von der Mark, von der Mark & Gay (1976*b*) that both type I and II collagens are synthesized in developing articular cartilage, and that there is a region where codistribution of the two types occurs. Interestingly, using the antibody MZ15 as an indicator of proteoglycan synthesis, we have shown that these two populations of chondrocytes are synthesizing at least one common glycosaminoglycan species.

After stage 30, collagen II is never found in the presumptive joint region, whereas KSPG remains in the interzone until cavitation is complete, at stage 42. Other workers using histochemical techniques, have shown the presence of other glycosaminoglycan species in the interzones of developing human joints (Andersen, 1961; Andersen & Bro-Rasmussen, 1961; Hinchliffe & Griffiths, 1983). It is an intriguing possibility, therefore, that the cells in the joint interzone are synthesizing cartilage-specific proteoglycan capable of aggregation in the presence of hyaluronic acid, but not type II collagen. We have also shown that other type I synthesizing structures such as the developing capsule and meniscus, and the tendons also synthesize keratan sulphate; the presence of KSPG in the latter has not previously been described and is under further investigation. However, keratan sulphate synthesis is markedly absent from the metaphyseal perichondrium and diaphyseal fibrous periosteum, which also synthesize type I collagen.

In the human, prior to cavitation, Andersen & Bro-Rasmussen (1961) describe a decrease of chondroitin sulphates A and C in the intermediate region of the interzone. Using MZ15, we see no such decrease in KSPG staining prior to cavitation in the chick (Fig. 4B), but have no data for the other major cartilage glycosaminoglycans, chondroitin sulphates A and C.

Our series of controls for MZ15 specificity may indicate differences in the associations of keratan sulphate with type I and II collagens. In association with type II collagen, pretreatment with keratanase abolishes MZ15 binding. However, this is not the case when keratan sulphate is in association with type I collagen.

The precise mechanism whereby the cells at the interzone flatten is unknown. The higher cell density at the presumptive joint region, seen at stage 28, is

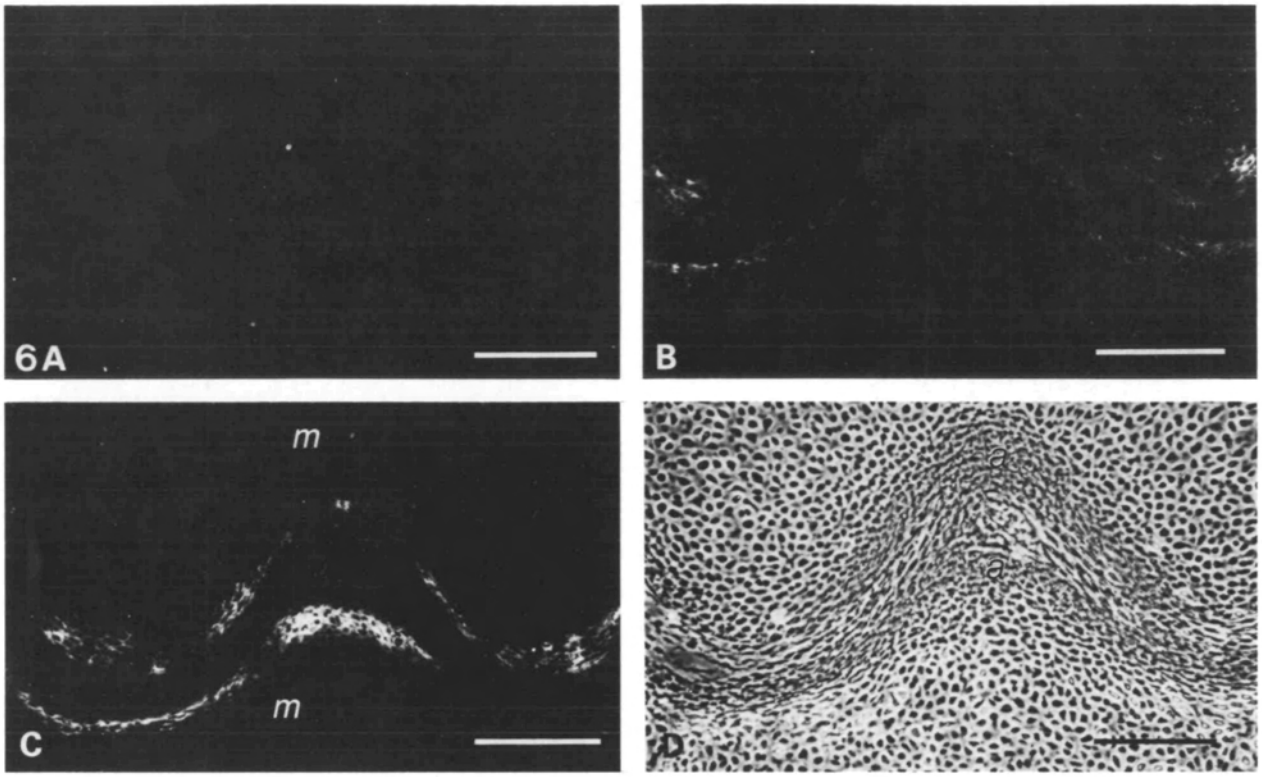

Fig. 6. Control sections, of stage-38 MTPJ, for MZ15 specificity. Bar, 100 μm. (A) Fluorescence micrograph of section prepared without the addition of the first antibody (MZ15). No staining can be seen. (B) Fluorescence micrograph of staining after preabsorption of MZ15 with corneal keratan sulphate. Staining is virtually all abolished. (C) Fluorescence micrograph of KSPG staining after pretreatment with keratanase. All KSPG staining has been abolished in the cartilage matrix (*m*). Staining only remains at the articular surfaces (approx. eight cell layers deep) and in the tendons (not shown). (D) Phase-contrast micrograph of 6C confirming that KSPG staining in the former is restricted to the articular surfaces (*a*).

indicative of less matrix secretion compared with that of the surrounding anlage cartilage. Immunohistochemically, collagen I and II staining is less intense at the presumptive joint. However, this region cannot be differentiated after staining for KSPG. At stage 30 the presumptive interzone cells are clearly flattened and secrete type I collagen. Concomitant with this, there is a total paucity in type II collagen staining and a considerable decrease in KSPG. We tentatively propose that the decrease in extracellular matrix, particularly of the proteoglycan moiety, provides a mechanism whereby the cells of the interzone flatten. In particular, the reduction in compressively stiff proteoglycan in this region may bring about passive flattening of the interzone cells by localized mechanical pressures, which lends support to the interpretation of Fell & Canti (1934) & Holder (1977) who suggest that these are generated by the matrix secretion of the opposing cartilage elements. The process of cell flattening in the interzone may also be important in the initiation of collagen type I synthesis by the cells. It has been shown that a rounded cell shape is conducive to chondrogenesis (Benya, Padilla & Nimni, 1978; Archer, Rooney & Wolpert, 1982; Solursh, Lisenmayer & Jensen, 1982). Whereas, cell flattening and elongation is often associated with a switch from type II to type I collagen synthesis (von der Mark, Gauss, von der Mark & Müller, 1977; Benya *et al.* 1978).

Whilst proteoglycans provide tissues with compressive stiffness (Kempson, Muir, Swanson & Freeman, 1970), collagen gives a tissue the capacity to withstand tensile and shear forces (Kempson, Muir, Pollard & Tuke, 1973; Kempson, 1979). Therefore, the paucity of collagen in the presumptive joint region would represent a localized area of weakness to shear forces, thus effectively forming a hinge. It is envisaged that shear forces are generated by the musculature that becomes functional at this time (Provine, 1978) and we suggest that the resulting mechanical forces play a crucial role in the eventual separation of the cartilaginous elements. Indeed, other workers have shown that paralysis at these stages leads to joint fusion, albeit of a secondary nature (Ruano-Gil, Nardi-Vilardaga & Tejedo-Mateu, 1978).

At stage 28, collagen type II and KSPG are distributed across the presumptive metatarsophalangeal joint and other joints. Therefore it can be argued that cartilage differentiates as a continuous rod in the digit region, which is subsequently divided into separate elements by joint formation. A similar sequence of events has been shown in the developing newt limb (Smith, 1978). In addition, this particular mechanism of skeletal development would support the recently proposed mechanism of condensation formation in tetrapod limbs (Shubin & Alberch, 1986). Presumably, the precise locations of joints are specified when the pattern of skeletal elements is laid down in the limb (Wolpert, 1969).

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