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# The development of articular cartilage: I. The spatial and temporal patterns of collagen types

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ABSTRACT

Articular cartilage is both morphologically and biochemically heterogeneous. Its susceptibility to degenerative diseases such as arthritis and its limited repair capacity have made cartilage the focus of intense study; surprisingly, little is known of its development. Using a panel of specific antibodies, we have documented the temporal and spatial patterns of collagen types I, II, III, VI and X in the developing knee cartilage of the marsupial *Monodelphis domestica* from parturition to adulthood. Type I collagen was initially detected in the presumptive articular cartilage of the epiphyses in addition to the perichondrium. By 14 d postparturition, type I collagen was not detectable in the epiphyseal cartilage apart from insertion sites of ligaments and tendons of the joint. Similarly, type III collagen was detected at insertion sites of the major ligaments and tendons and within the perichondrium/periosteum but was never detected in the cartilage per se. Type II collagen was predictably distributed throughout the cartilage matrix and was also detected in the perichondrium. Type VI collagen was widely distributed throughout the cartilage matrix at parturition, but during development became restricted to a pericellular location particularly towards the presumptive articular cartilage, i.e. the epiphysis. Interestingly, generalised matrix immunopositivity was only retained in the hypertrophic cartilage of the secondary centre of ossification. After the formation of the secondary centre, type VI collagen became localised pericellularly in the deeper regions of the articular cartilage but was absent in the cartilage of the growth plate. Type X collagen showed a novel distribution pattern. In addition to being synthesised by hypertrophic chondrocytes, this collagen type was also expressed transiently by some cells at the presumptive articular surface. Furthermore, these surface chondrocytes also stained histochemically for alkaline phosphatase, suggesting that they were terminally differentiated. The fate of these terminally differentiated cells is unknown.

*Key words*: *Monodelphis domestica*; marsupials; knee joint; alkaline phosphatase.

## INTRODUCTION

Articular cartilage is a morphologically and biochemically heterogeneous tissue. The 2 major constituents of articular cartilage are collagen and proteoglycan which are present in a number of genetically distinct types with collagen type II and aggrecan being the most abundant (Miller, 1976; Martin et al. 1985; Eyre et al. 1987; Heinegard & Oldberg, 1989). In general terms, proteoglycans withstand compressive loading by nature of their ability to swell osmotically due to the negative charge of the sulphated glycosaminoglycans. Conversely, collagen provides the tissue with strength to withstand tensional forces. These mechanical properties can largely be ascribed to the major molecular species of aggrecan and collagen type II respectively. What is less well understood are the contributions of the minor collagen and proteoglycan species beyond their role in matrix integration and organisation. Furthermore, it is evident that some of these minor species form distinct spatial patterns within the tissue which can vary during development, growth, ageing and the onset of degenerative disease (Schmid & Linsenmyer, 1985; Kwan et al. 1989; Mendler et al. 1989; van der Rest et al. 1991; Hagiwara et al. 1993; Quarto et al. 1993). A detailed

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knowledge of how these patterns emerge during embryological development and how they may be related to the changes in pattern observed during degenerative disease (arthritis) where repair phenomena are elicited is central to our understanding of tissue homeostasis (Archer, 1994).

Collagen type II comprises 90–95% of the collagen present in adult articular cartilage (Eyre et al. 1987; Linsenmayer, 1991); it has also been located in fibrocartilage, the nucleus pulposus, notochord and vitreous humour (von der Mark, 1981; Ralphs et al. 1991). Type II collagen mRNA and protein have also been located transiently in other tissues during early development and prior to overt chondrogenesis such as ectoderm (Swiderski & Solursh, 1992) and heart (Cheah et al. 1991; Swiderski et al. 1994). Thus the expression of the major cartilage collagen is not necessarily associated with chondrogenesis nor, presumably, with a mechanically related function.

Type I collagen has been reported to be present at the onset of chondrogenesis but then becomes undetectable (von der Mark et al. 1976; Craig et al. 1987). Whilst it is generally thought that collagen type I is turned over early in chondrogenesis, more recent data suggest that it may simply be masked since immunodetectable type I collagen has been demonstrated in mature porcine articular cartilage after prolonged predigestion procedures (Wardale & Duance, 1993). In addition, during endochondral ossification, type II collagen is replaced by type I collagen which is secreted within the lacunae of hypertrophic chondrocytes (Leboy et al. 1988; Gallatto et al. 1994).

Type III collagen has been reported in the perichondrium of cartilaginous rudiments (Treilleux et al. 1992) and therefore may have a role in morphogenesis since disruption of the perichondrium can alter rudiment development in vitro (Rooney & Archer, 1992). Moreover, grossly dysplastic cartilage elements resulting from overexpression of bone morphogenetic protein 4 (BMP4) often lack a perichondrium (Francis-West et al. 1995, unpublished). Other collagens present in cartilage include types VI, IX, X and XI. Type VI collagen is a short fibrillar collagen preferentially located to the pericellular chondron of chondrocytes but is also synthesised by a number of other tissue types. It is believed that the function of collagen type VI may involve the stabilisation of fibrillar structures and linkage of matrix molecules (Amenta et al. 1988; Poole et al. 1988; Hagiwara et al. 1993; Quarto et al. 1993). Type IX collagen codistributes with type II collagen and appears to control fibril diameter and also may facilitate matrix integration both via its N-terminal domain which extends away from the fibril and its chondroitin sulphate chain (Eyre et al. 1987; van der Rest & Mayne, 1988; van der Rest et al. 1991). A similar fibril-controlling role has been proposed for type XI collagen which also codistributes with type II collagen (Mendler et al. 1989; Petit et al. 1993; Oxford et al. 1994).

Type X collagen is specific to cartilage and is developmentally regulated. It is synthesised by terminally differentiating chondrocytes, i.e. those undergoing hypertrophy (Kielty et al. 1985; Schmid & Linsenmayer, 1985). In the hypertrophic region of the epiphyseal plate in the maturing mammal, up to 18% of collagen synthesis may be type X (Grant et al. 1990), although this can be as much as 80% in the chick (Kielty et al. 1985). The biological function of type X collagen is not yet fully understood, but it is thought to facilitate the process of calcification possibly through matrical organisation changes due to its hexameric structure (Kwan et al. 1991).

Collagen is an important morphogenetic determinant of skeletogenesis. For example, Fitton-Jackson (1970) showed experimentally that if the collagen architecture of developing chick rudiments is disrupted through the application of collagenase and then the rudiments allowed to recover in culture, the subsequent morphology is dysplastic. Rudiments similarly treated with hyaluronidase show normal morphology. More recently, it has been shown that a number of skeletal dysplasias can be directly correlated with mutations in a number of collagen genes (Vandenberg, 1993; Brunner et al. 1994; Diab et al. 1994; Chan et al. 1995). However, we still know little of the cellular bases of these dysplasias primarily because of a paucity in our knowledge of the temporospatial patterns of these molecular species in early skeletogenesis and, importantly, during the development of articular cartilage. Consequently, we know little of the roles of these molecules during development.

The purpose of the present study was to establish the spatial and temporal patterns of a number of collagen types in developing articular cartilage of the knee joint. Many previous studies of skeletogenesis have utilised the embryonic chick as a model system. However, the chick has a fibrous articular surface and thus differs from mammalian hyaline articular cartilage (Craig et al. 1987). We have used the South American opossum, *Monodelphis domestica* as a model since like all marsupials, it is born at an early developmental stage and whilst its forelimbs are well developed at parturition, the hindlimbs are at the

initial stages of chondrogenesis (Archer et al. 1994, 1996). The model therefore provides an easily definable developmental series in addition to facilitating experimental studies.

## MATERIALS AND METHODS

### *Tissues*

Specimens from a developmental series of opossums were killed by chloroform overdose. The series ranged from pouch d 0 (day of parturition) until adulthood (from 8 mo up to 2 y). The hind limbs were dissected from the animals before being fixed either in 10% (v/v) formol saline or snap frozen in liquid nitrogen and stored at −80 °C.

Formol saline fixed specimens were processed for routine wax histology. Sections were cut in the longitudinal plane on a rotary microtome at 8 μm and stained with haematoxylin and eosin. A developmental sequence of the knee joint was examined and photographed (Ilford Plan F used at 50 ASA) on a Zeiss Photomicroscope III.

Frozen specimens were sectioned (8 μm) in the longitudinal plane through the proximodistal axis of the limb. A minimum of 2 specimens (4 limbs) per time point were used. Young specimens (parturition to d 30) were sectioned at −25 °C on a cryostat (Anglia Scientific, Cambridge, UK) and placed on 3-aminopropyltriethoxysiline (APES) coated slides (Maddox & Jenkins, 1987) and stored at −20 °C until required. Older specimens (2 mo to adult), were sectioned on a LKB PMV 2258 heavy duty cryomicrotome. Specimens were mounted on a chuck using carboxymethylcellulose as a supporting medium. When sections were to be cut, 2 layers of polyvinylpyriolidine (PVP) were thinly spread on the cutting face to which a piece of cigarette paper was applied to act as a support during sectioning. The 8 μm sections were placed on cooled slides previously coated with the adhesive, Durotak (Product No. 180–1197, National Adhesives and Resins Ltd, Slough, UK). Sections were then stored at −20 °C overnight (Aaron & Carter, 1987). Before use, all the sections were defrosted to room temperature and then fixed in 10% neutral buffered formol saline (5 min), washed in tap water and then PBS prior to immunolocalisation or alkaline phosphatase histochemistry.

### *Immunochemistry*

Monoclonal and polyclonal antibodies raised against collagens type I, II, III, VI and X were used (see Table). The procedure was carried out at ambient temperature in a humidified chamber. Phosphate buffered saline (PBS) was prepared at ×10 concentration (10 mM, pH 7.3) using Oxoid PBS tablets (Unipath, Basingstoke) and diluted before use. Suitable control slides (i.e. normal mouse immunoglobulins or normal rabbit immunoglobulins) were matched for protein or IgG concentration to the appropriate antibodies and used at all times.

Sections were fixed as above and then washed in PBS before being incubated in hyaluronidase/chondroitinase (1.5 units/0.25 $ml^{-1}$ respectively) (Sigma) for 30 min at room temperature. Slides were then washed in PBS (3 × 2 min) and incubated in an appropriate normal serum block (1:20 dilution) (Dakopatts, UK) for 20 min before application of the primary antibody (1 h). Slides were again washed in PBS (3 × 2 min) and then incubated with an appropriate fluorescein-conjugated secondary antibody (Dakopatts, UK) (1 h) raised against the primary antibody species. After incubation, slides were washed in PBS (3 × 2 min) and mounted in a glycerol based mountant containing the antifading agent 1,4-diazabicyclol [2.2.2] octane (DABCO; Sigma) (Johnson et al. 1982). Sections were viewed and photographed on a Zeiss Photomicroscope III with epifluorescence attachment and using HP5 (Ilford) film at 400 ASA.

### *Alkaline phosphatase histochemistry*

Incubation was carried out at room temperature with freshly prepared reagents following the naphthol AS-BI phosphate method of Burstone (1958).

Table. *Panel of primary antibodies used in this study*

| Antibody | Specificity | Host | Form | Dilution | Source | Reference |
|---|---|---|---|---|---|---|
| P1 | Collagen type I | Rabbit-antirat | Rabbit serum | 1:80 | Chemicon | Vialle-Presles et al. (1989) |
| CIICI | Collagen type II | Mouse-antichick | Supernatant | 1:5 | DSHB | Holmdahl et al. (1986) |
| P3 | Collagen type III | Rabbit-antirat | Rabbit serum | 1:80 | Chemicon | Vialle-Presles et al. (1989) |
| 5508a | Collagen type VI | Rabbit-antibovine | Rabbit serum | 1:100 | C. Kielty | Kielty et al. (1991) |
| MA3 | Collagen type X | Mouse-antichick | Ascites | 1:500 | A. Kwan | Kwan et al. (1989) |

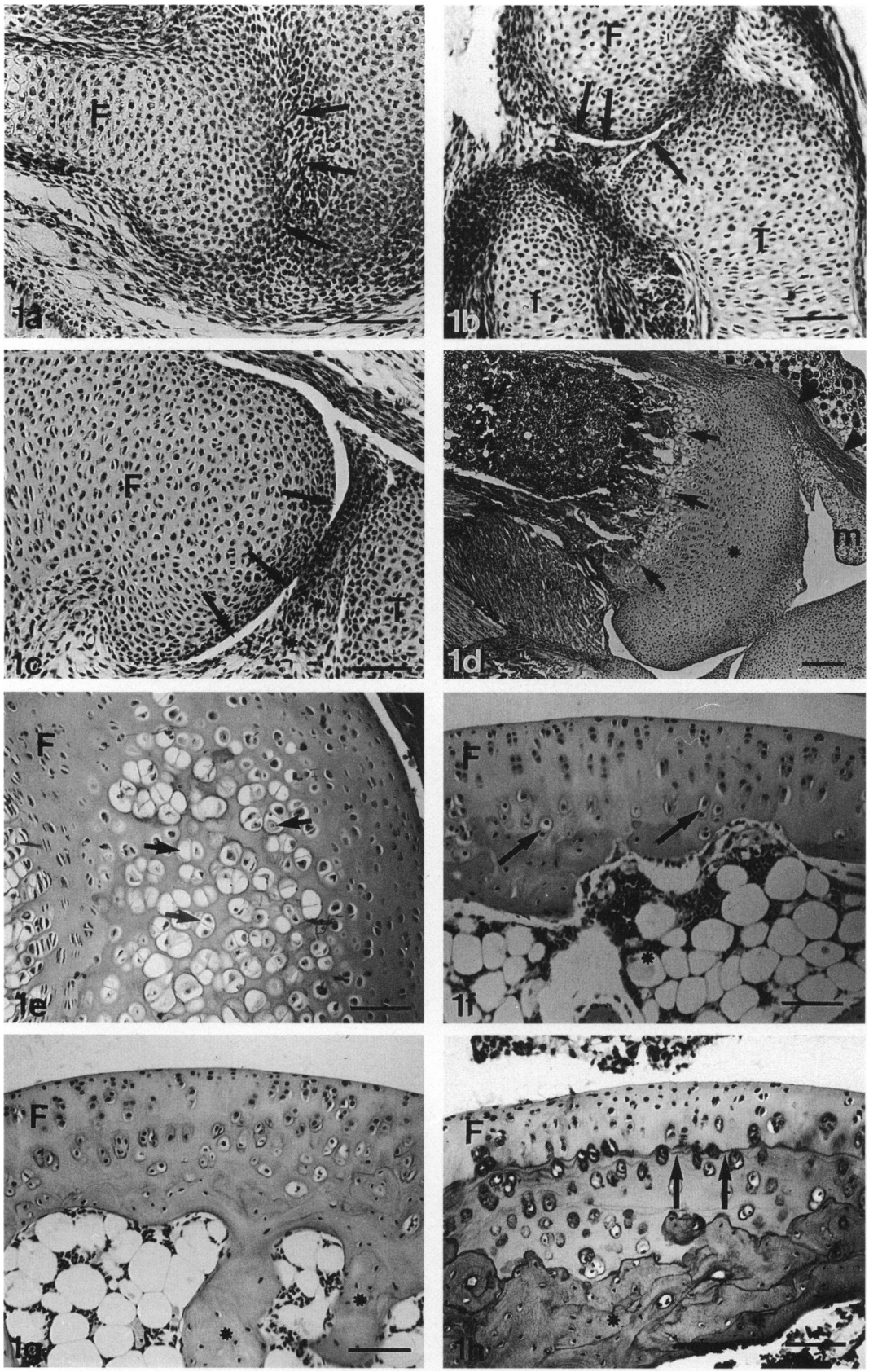


Fig. 1. Longitudinal wax sections (8 μm) of the developing knee joint of *Monodelphis domestica* stained with haematoxylin and eosin. F, femur; T, tibia; f, fibula. (*a*) Photomicrograph of a specimen at pouch d 1 showing the knee joint with no cavitation evident. The interzone of the joint is visible (arrows). Bar, 150 μm. (*b*) Section of a pouch d 5 specimen showing that partial cavitation of the knee joint has occurred (arrows). Note also the developing meniscus (asterisk). Bar, 150 μm. (*c*) Section of a pouch d 12 specimen. Cavitation is now complete

Sections were covered in PBS (pH 7.3) before being incubated in the detection medium for the required time (5 min ±30 s). The medium contained 500 µl 4% (w/v) new fuschin in 2 M HCl with 500 µl 4% (w/v) sodium nitrate in distilled water, mixed together and diluted to 40 ml using fresh 50 mM Tris buffer with 2 ml of 0.1 M HCl, adjusted to pH 9 and containing 0.025% (w/v) naphthol AS-BI phosphoric acid (Sigma). Control sections had 10 mM levamisole hydrochloride (Sigma) (an inhibitor of alkaline phosphatase) added to the incubation medium. When the reaction was complete, the sections were washed in tap water before being mounted in Aquamount (BDH).

## RESULTS

### *Histological observations*

At parturition, the femoral and tibial elements were still continuous showing no evidence of cavitation although an interzone could be delineated at the future joint line (Fig. 1*a*). By pouch d 5, the joint had partially cavitated and associated joint structures could be discerned, such as the meniscus (Fig. 1*b*). Further development showed that by pouch d 12, cavitation was complete (Fig. 1*c*). Concomitantly, both femoral and tibial epiphyses remained entirely chondrogenic and capsular, meniscal and major ligamentous structures could be resolved. By pouch d 30, endochondral ossification had advanced to the metaphyseal region, and the meniscal and capsular structures were well differentiated (Fig. 1*d*). At 2 mo postpartum, the chondrocytes at the centre of the epiphyses had hypertrophied, thus defining the secondary centre of ossification and the articular cartilage (Fig. 1*e*). By 4 mo, the articular cartilage showed features normally associated with the mature tissue, i.e. flattened surface articular chondrocytes, basal hypertrophic chondrocytes and a developing bony subchondral plate (Fig. 1*f*). A similar organisation was present at 6 mo postpartum; a notable difference is that the subchondral plate was then well developed and was continuous (Fig. 1*g*). Finally, in the adult tissue, the subchondral bone was highly ossified and there was a deep calcified cartilage zone with multiple 'tide marks' (Fig. 1*h*). Unlike some eutherian articular cartilages, there was little evidence of chondrocyte columns in the tangential zone.

### *Collagen type I*

At parturition, collagen type I was present throughout the mesenchyme of the developing limb bud including the ends of the developing epiphysis, the perichondrium and developing periosteum of the cartilage rudiment (Fig. 2*a*). By pouch d 5, immunopositivity was detected in the developing tendons, ligaments and meniscus in addition to the perichondrium and periosteum. This pattern of localisation continued until pouch d 14 when immunopositivity was also observed in the developing subperiosteal bone of the rudiment diaphysis (Fig. 2*b*). Concomitantly, collagen type I was not detected in the cartilage. With maturation of the tissue, immunopositivity within the mesenchyme of the limb bud became less pronounced. By pouch d 30, immunopositivity was located in the standard tissues associated with type I collagen, i.e. bone, tendon, ligaments and the meniscus (Fig. 2*c*).

At 2 mo, after the secondary centre of ossification has begun to form, localisation of collagen type I was observed in the bone, tendon, ligaments and meniscus. It was also present throughout the developing secondary centre of ossification. Some pericellular localisation was also observed in the cartilage, especially in the hypertrophic zones of the articular cartilage and the growth plate (Fig. 2*d*). This localisation pattern continued largely unchanged until adulthood.

### *Collagen type II*

At parturition, collagen type II was observed throughout the ECM of the developing rudiment and was also

(arrows) and the meniscus is observed in the joint cavity (asterisk). Bar, 150 µm. (*d*) Section of a pouch d 30 specimen. The mineralisation of the bone is advanced and the mineralising border has advanced to the metaphysis (arrows). The whole of the epiphysis of the femur, however, is still cartilaginous (asterisk) but the secondary centre of ossification has yet to form. Note also well differentiated meniscus (m) and patellar tendon (arrowheads). Bar, 200 µm. (*e*) Section of a 2-mo-old specimen. The secondary centre of ossification has begun to develop and hypertrophic chondrocytes can be seen in the centre of the cartilaginous epiphysis (arrows). Mineralisation is not present. Bar, 100 µm. (*f*) Section of a 4-mo-old specimen. The articular cartilage shows features associated with the mature tissue, i.e. flattened surface cells, rounded mid zone cells and deep hypertrophic chondrocytes (arrows). The secondary centre is ossified (asterisk). Bar, 100 µm. (*g*) Section of a 6-mo-old specimen. The articular cartilage is very similar to the adult tissue. However, large bony areas are still present within the secondary centre of ossification (asterisk). Bar, 100 µm. (*h*) Section of an adult specimen. The articular cartilage is fully developed as is the secondary centre of ossification. Tide marks can be clearly seen in the tissue (arrows) and the subchondral bone forms a continuous supporting plate (asterisk). Bar, 100 µm.

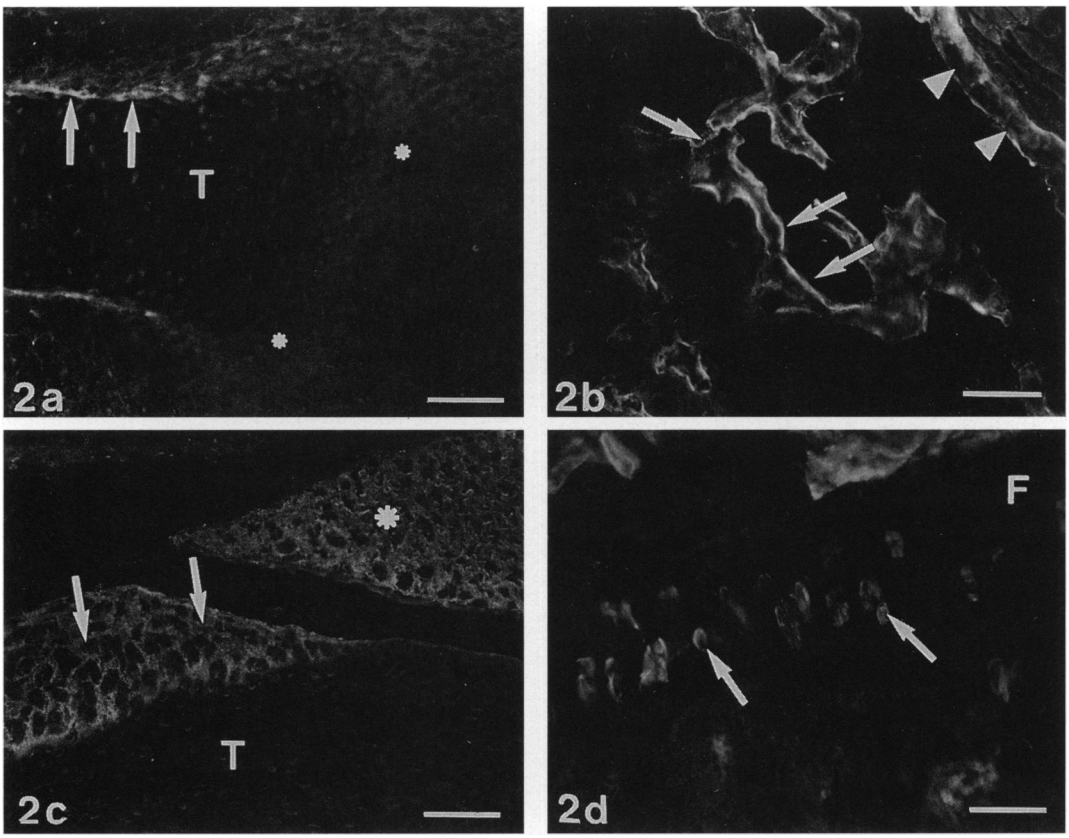


Fig. 2. Longitudinal cryosections (8 μm) of the developing knee joint of *Monodelphis domestica* showing localisation of collagen type I. F, femur; T, tibia. (*a*) Section of a pouch d 1 specimen. The photomicrograph shows the localisation of type I collagen in the perichondrium of the developing rudiments (arrows). Weak labelling is also detected within the epiphysis and in the surrounding matrix (asterisks). Bar, 75 μm. (*b*) Section of a pouch d 19 specimen. The photomicrograph shows the localisation of type I collagen in the periosteum and subperiosteal bone (arrowheads) and endochondral bone of the rudiments (arrows). Bar, 50 μm. (*c*) Section of a pouch d 30 specimen. Type I collagen is localised in the cruciate ligament (arrows) and meniscus (asterisk) of the joint. Bar, 50 μm. (*d*) Photomicrograph of a section from an adult specimen. Cellular and pericellular localisation of type I collagen is seen in the articular cartilage (arrows). This label appears to be concentrated in the deeper zones of the tissue. Bar, 50 μm.

detected in the perichondrium (Fig. 3*a*). By pouch d 5, stronger immunopositivity was observed towards the epiphyseal ends of the rudiment and in the diaphyseal hypertrophic zone (Fig. 3*b*). Strong immunopositivity was also observed in the perichondrium. This pattern of localisation continued up until pouch d 30, although with endochondral ossification, the hypertrophic zone became restricted towards the epiphyseal ends.

At 2 mo, with the formation of the secondary centre of ossification, collagen type II was present throughout the ECM of the articular cartilage and the growth plate. It was also present throughout the matrix of the secondary centre, as this had not fully formed (Fig. 3*c*). By adulthood, type II collagen was no longer present in the secondary centre of ossification, although it was still present throughout the ECM of the articular cartilage and the growth plate. Weak immunolabelling was also present in the basal mineralising zone (Fig. 3*d*).

*Collagen type III*

At parturition and in the neonate, collagen type III was present in the perichondrium and periosteum of the rudiment and in the surrounding mesenchyme (Fig. 4*a*). By pouch d 5, collagen type III was detected in the major tendons, ligaments and meniscus as well as the perichondrium (Fig. 4*b*). This labelling was weak but continued throughout development until adulthood, when labelling was not detected in the cartilage, but the surrounding tissues of the joint, i.e. tendons and ligaments, were immunopositive (Fig. 4*c*).

*Collagen type VI*

Collagen type VI was detected throughout the mesenchyme of the neonatal limb bud but was not detected in the ectoderm. Type VI collagen was also

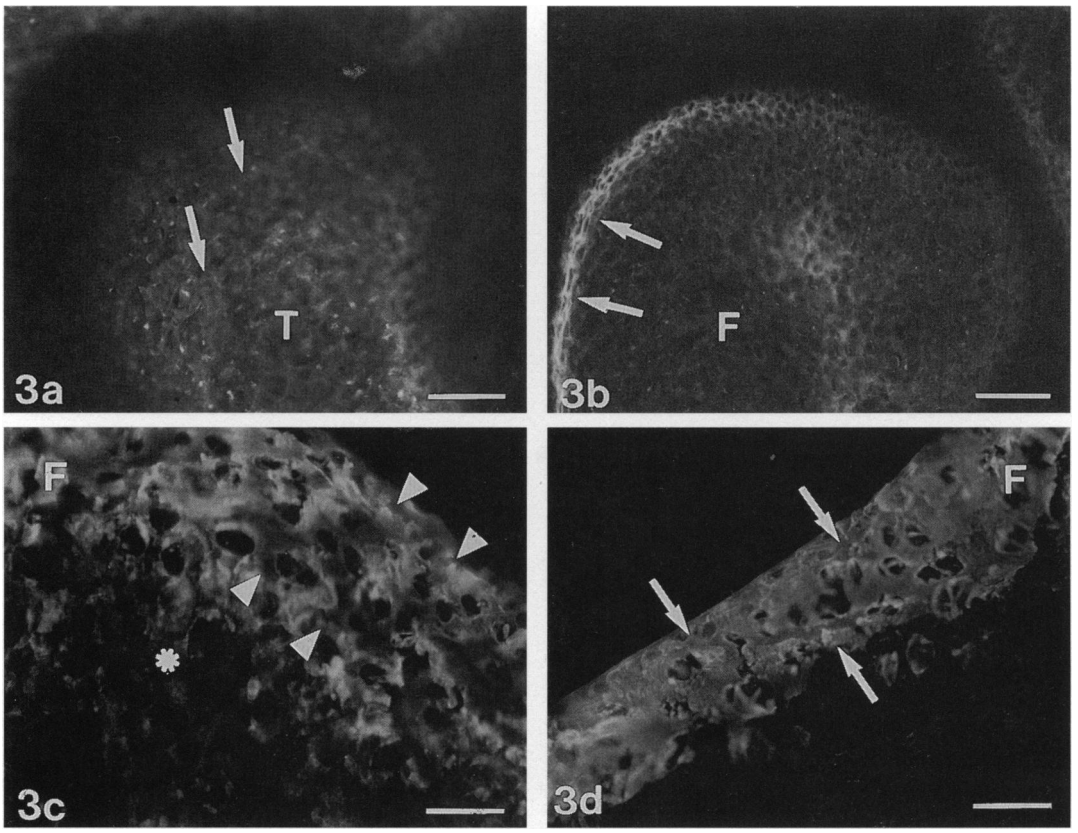


Fig. 3. Cryosections (8 μm) through the longitudinal axis of the developing knee joint of *Monodelphis domestica* showing localisation of collagen type II. F, femur; T, tibia. (*a*) Section of a pouch d 2 specimen. Type II collagen is localised throughout the extracellular matrix of the developing rudiments (arrows). Bar, 50 μm. (*b*) Section of a pouch d 5 specimen. Type II collagen remains localised throughout the extracellular matrix of the developing rudiments. Note the higher intensity of label in the perichondrium/periosteum (arrows). Bar, 75 μm. (*c*) Section of a 2-mo-old specimen showing the distribution of type II collagen throughout the extracellular matrix of the developing articular cartilage (arrowheads) in addition to the developing secondary centre of ossification (asterisk). Bar, 75 μm. (*d*) Section of an adult specimen showing the localisation of type II collagen throughout the extracellular matrix of the articular cartilage (arrows). Bar, 75 μm.

localised throughout the ECM of the rudiment cartilage and was prominent pericellularly (Fig. 5*a*). By pouch d 8, immunopositivity was strong in the hypertrophic zone matrix but much weaker in the remaining cartilage matrix. From pouch d 10, immunopositivity became more pericellular particularly in the hypertrophic zone and towards the epiphyseal ends of the rudiment. However, from pouch d 14, matrix localisation was only observed in the hypertrophic zone, cellular and pericellular localisation being present throughout the remaining cartilage. Additionally, pericellular localisation was more prominent towards the articular surface of the joint (Fig. 5*b*). At this time, collagen type VI was still detected throughout other structures of the limb including, tendons, ligaments and muscle and connective tissue. This pattern of localisation continued until pouch d 30.

At 2 mo, after the secondary centre of ossification had begun to form, pericellular localisation was observed throughout the articular cartilage and was especially intense in the deeper zones. This pattern of immunolocalisation continued until adulthood (Fig. 5*c*).

### *Collagen type X*

In the neonate, type X collagen was detected in the matrix of the central hypertrophic portion of the developing rudiment and, interestingly, was also detected in the periosteum (Fig. 6*a*). With the progression of osteogenesis, type X collagen localisation was observed in the hypertrophic matrix of the mineralising border up until pouch d 30 (Fig. 6*b*). Interestingly, from pouch d 6 until pouch d 30 some immunopositivity was detected at the articular surface of the joint (Fig. 6*c*).

After 2 m, immunopositivity was present in the hypertrophic cells of the growth plate, and of the forming secondary centre of ossification (Fig. 6*d*). By adulthood, localisation was no longer observed in the secondary centre of ossification, but was still present

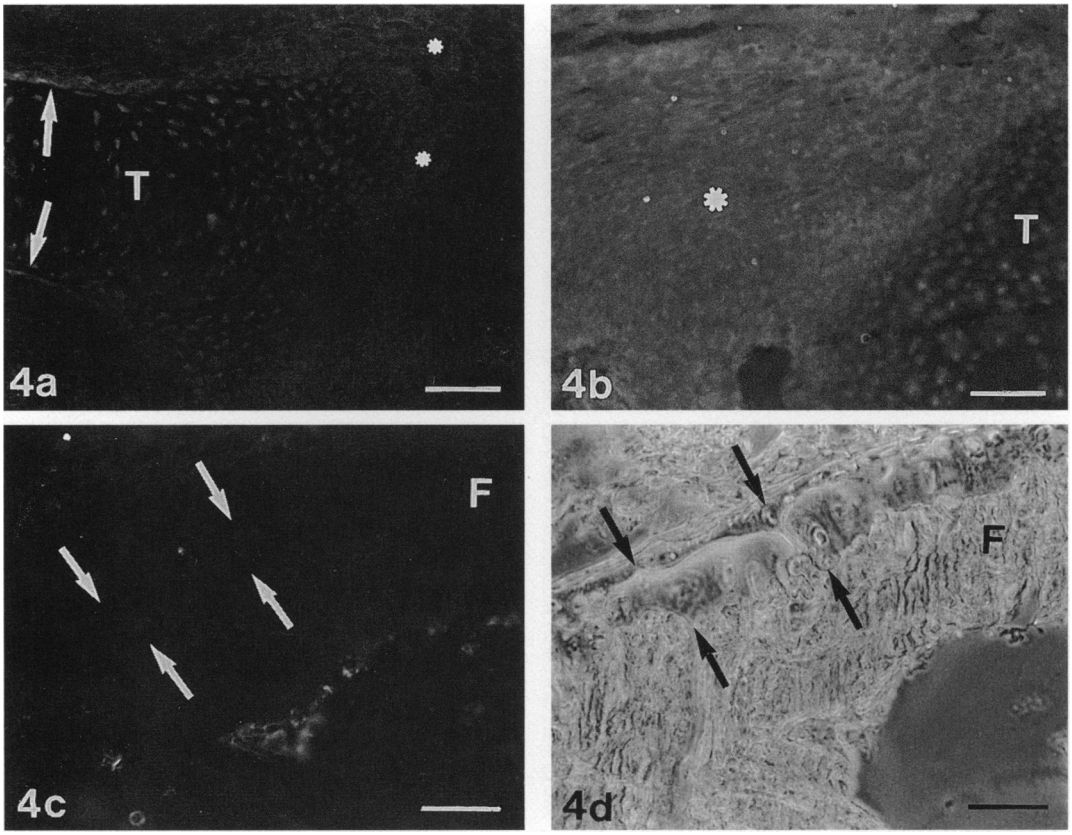


Fig. 4. Cryosections (8 μm) through the longitudinal axis of the developing knee joint of *Monodelphis domestica* showing localisation of collagen type III. F, femur; T, tibia. (*a*) Section of pouch d 1 specimen showing the distribution of type III collagen in the developing rudiments of the neonate. Immunopositivity is observed in the perichondrium (arrows) and more weakly in the surrounding mesenchymal tissues (asterisks). Bar, 75 μm. (*b*) Section of a pouch d 10 specimen. Type III collagen can be detected in the cruciate ligament of the developing joint (asterisk). Bar, 50 μm. (*c*) Section of an adult specimen. Type III collagen is absent from the matrix of the articular cartilage (arrows) but can be detected in the subchondral plate. Bar, 50 μm. (*d*) Phase contrast image of joint shown in (*c*). Arrows delineate the extent and depth of the articular cartilage. Bar, 50 μm.

pericellularly in the deep hypertrophic cells of the articular cartilage and growth plate (Fig. 6*e*).

*Alkaline phosphatase*

In the neonate, alkaline phosphatase activity was present in the diaphyseal periosteum of the cartilage rudiment (Fig. 7*a*). At this stage, it was also observed in the mesenchyme directly subjacent to the dermis (Fig. 7*b*). By pouch d 5, alkaline phosphatase activity was detected in the hypertrophic cells of the central diaphysis in addition to the periosteum (Fig. 7*c*). Some activity also occurred at the epiphyseal ends in the developing articular surface cartilage within the joint, thus showing a similar distribution to type X collagen at this time (Fig. 7*d*). Enzyme activity was still present in the mesenchyme directly below the limb bud epithelium. From pouch d 10, alkaline phosphatase activity was present in the deep hypertrophic cells and in the developing subperiosteal bone of the diaphysis, in addition to the periosteum (Fig. 7*e*). There no longer appeared to be any positivity at the epiphysis (thus contrasting the type X collagen distribution which persisted until d 30), although weak activity was present at the sites of tendon and ligament attachment (Fig. 7*f*). This pattern of positivity continued until pouch d 30.

At 2 mo, alkaline phosphatase activity was present in the cells of the mineralising border of both the articular cartilage and the growth plate. Enzyme activity was also observed throughout the developing secondary centre of ossification and was present in deep, columnar hypertrophic cells of the growth plate, although it was not observed in the cartilage matrix. Enzyme activity was also observed at the periosteal cuff (Fig. 7*g*). The above pattern of localisation continued until adulthood with only a few localised alterations. These changes were that alkaline phosphatase activity was no longer present throughout the secondary centre of ossification but localised at the

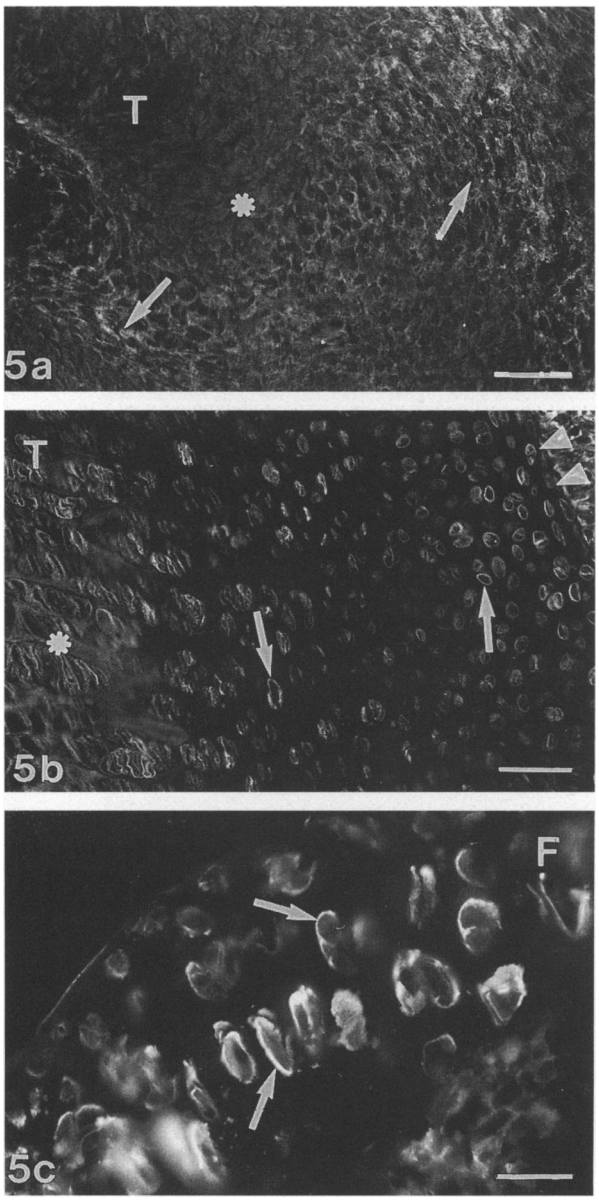


Fig. 5. Cryosections (8 μm) taken throughout the development of the knee joint in *Monodelphis domestica* showing immunolocalisation of type VI collagen. F, femur; T, tibia. (*a*) Section of a pouch d 1 specimen. The photomicrograph shows the distribution of type VI collagen in the matrix of the developing rudiment (asterisk) and surrounding mesenchyme (arrows). Bar, 50 μm. (*b*) Section of a pouch d 30 specimen. Pericellular localisation of type VI collagen can be seen in the developing epiphysis (arrows). This pattern appears to be concentrated towards the epiphyseal boundaries (arrowheads). Immunopositivity can also be observed in the matrix of the hypertrophic region (asterisk). Bar, 50 μm. (*c*) Section of an adult specimen. The photomicrograph shows pericellular localisation of type VI collagen in the articular cartilage (arrows). This localisation pattern appears to be more intense in the deeper zones. No immunopositivity is observed in the interterritorial matrix. Bar, 20 μm.

surface of the bone, and activity was present in the pericellular matrix of the deep hypertrophic cells of the growth plate and articular cartilage (Fig. 7*h*).

## DISCUSSION

The histology of joint formation in *Monodelphis* closely resembles that described for a variety of joints in eutherian mammals (Haines, 1947; Garner & O'Rahilly, 1968). It is not germane to this study to detail small differences or variations that do exist, apart from being able to state that the use of *Monodelphis* as a model for joint and articular cartilage studies appears justified. Furthermore, in adult *Monodelphis domestica* articular cartilage, the distribution patterns of the different collagens are consistent with those seen in other eutherian mammals, further supporting the use of this marsupial as a model for skeletal development (Archer et al. 1996).

The presence of type I collagen in developing cartilaginous rudiments is well established (Miller, 1976; von der Mark et al. 1976; Craig et al. 1987); its continued synthesis or its persistence into adult articular or even growth plate cartilage has been more contentious. However, some recent publications have identified de novo synthesis of type I collagen notably in hypertrophic chondrocytes of the chick prior to true endochondral ossification (LeBoy et al. 1989; Gallotto et al. 1994) and in those hypertrophic chondrocytes involved directly in endochondral ossification from an early stage of skeletogenesis as in mammals (Carter et al. 1992). In the context of articular cartilage, Wardale & Duance (1993) have recently demonstrated type I collagen as a constitutive component in mature porcine articular cartilage. They demonstrated strong immunolocalisation in the matrix of the articular surface and a pericellular localisation around the hypertrophic chondrocytes. However, this pattern of localisation was only obtained after extensive enzyme digestion. It has yet to be determined at what stage this collagen is elaborated during the formation of articular cartilage and if it is constitutively synthesised. Our data suggest that collagen type I may be constitutively synthesised since we detected both cellular and pericellular labelling mainly in the basal hypertrophic chondrocytes, but also in some chondrocytes within the transitional zone of the tissue.

Type III collagen was detected in the perichondrium and developing periosteum and the insertion points of tendons and ligaments and is consistent with other reports (Miller, 1976; von der Mark et al. 1976). Treilleux et al. (1992) have investigated the expression and deposition of collagens type I, II and III by in situ hybridisation of mRNA and immunolocalisation of protein in developing human femoral heads. Although only 2 time points were analysed, surface articular

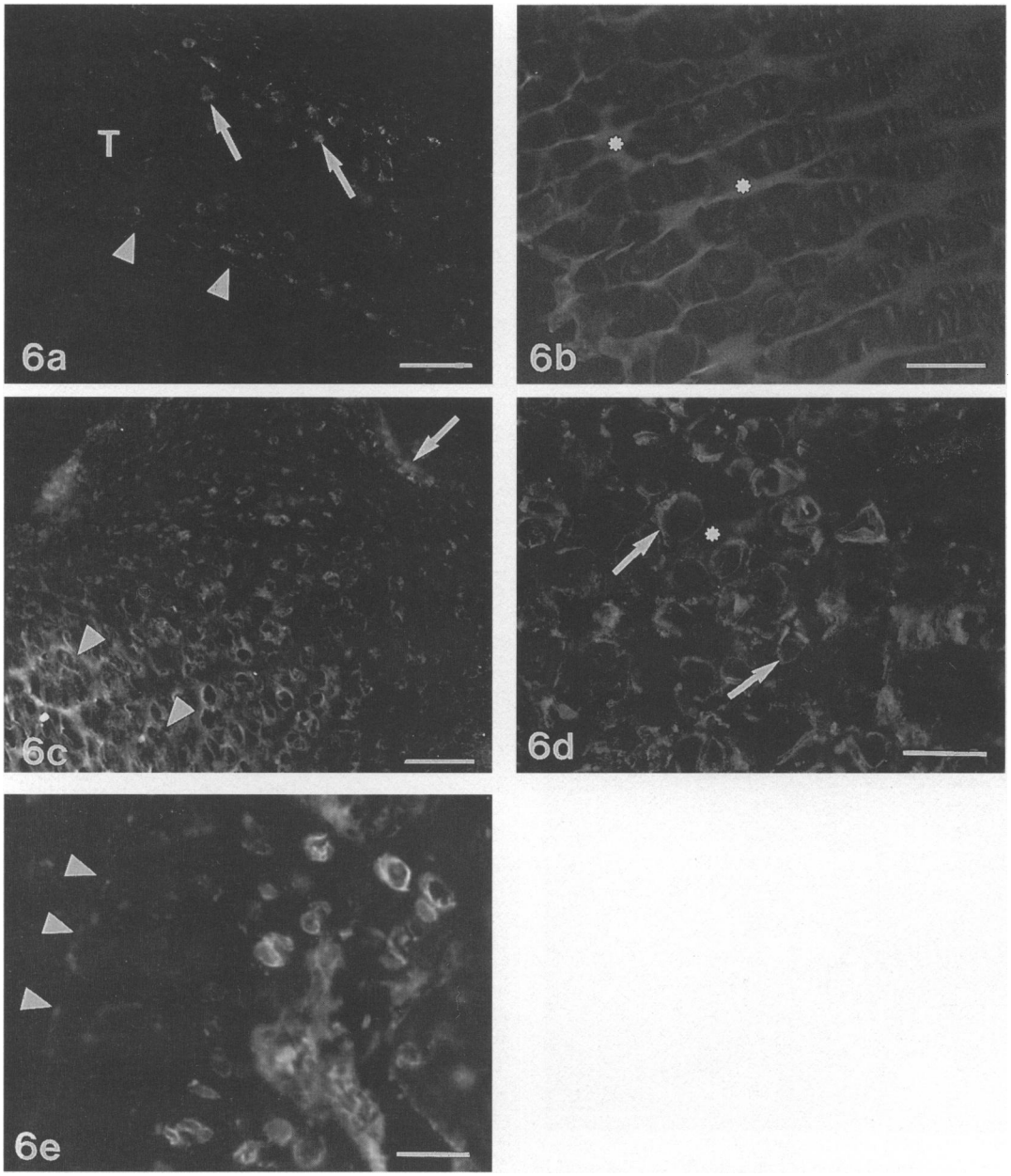


Fig. 6. Cryosections (8 μm) through the longitudinal axis of the developing knee joint of *Monodelphis domestica* showing localisation of collagen type II. T, tibia. (*a*) Section of a pouch d 1 specimen. The photomicrograph shows the distribution pattern of type X collagen in the hypertrophic cells of the middiaphysis of the developing tibia (arrows) including the periosteum (arrowheads). Bar, 50 μm. (*b*) Section of a pouch d 30 specimen. Weak type X collagen labelling can now be seen in the matrix of the hypertrophic region (asterisks). Bar, 30 μm. (*c*) Section of a pouch d 10 specimen. The photomicrograph shows the localisation pattern of type X collagen in the matrix of the hypertrophic region of the developing tibia (arrowheads). Additionally it shows positive labelling of type X collagen at the articular surface (arrow). Bar, 50 μm. (*d*) Section of a 2-mo-old specimen. Type X collagen is distributed throughout the secondary centre of ossification. Labelling is seen both pericellularly (arrows) and in the matrix (asterisk). Bar, 20 μm. (*e*) Section of an adult specimen. Type X collagen is now restricted to pericellular labelling within the basal calcified cartilage zone. Arrowheads denote articular surface. Bar, 20 μm.

chondrocytes synthesised initially only collagen types I and III (at 22 wk gestation) and then types I, II and III (newborn). In other studies, we have found type III collagen extensively distributed in the articular cartilage of adult human proximal interphalangeal joints (A. R. Lewis, personal communication). The absence of type III collagen from *Monodelphis* articular cartilage except from around ligament and tendon insertions suggests that there is interspecies variation in articular cartilage development and, therefore, a degree of functional redundancy may exist between different collagen types particularly perhaps between type III and type I.

Recent studies have shown that collagen type II has 2 spliced variants II A and II B (Ryan & Sandell, 1990; Nah & Upholt, 1991). These 2 variants show

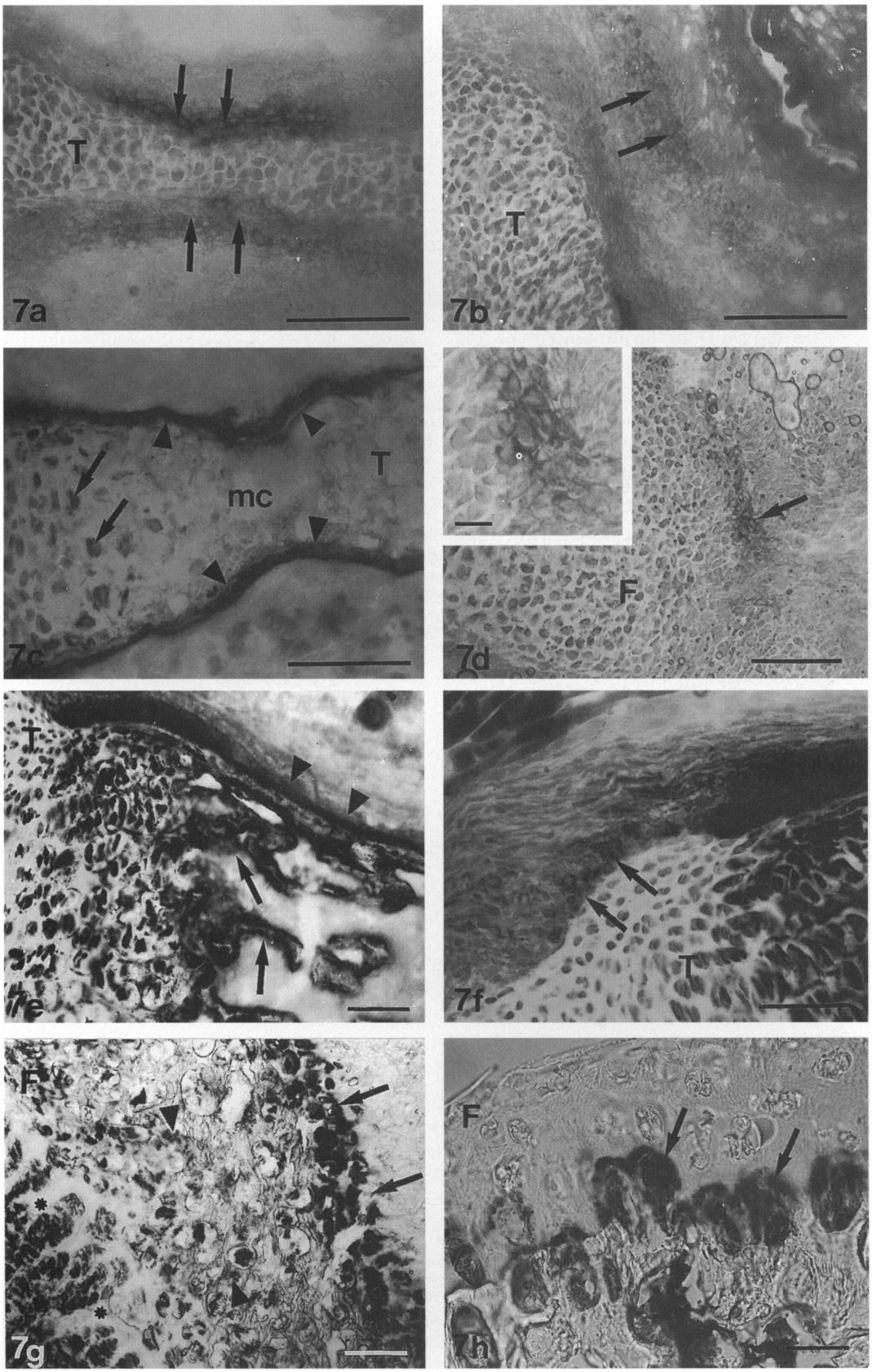


Fig. 7. Cryosections (8 μm) through the longitudinal axis of the developing knee joint of *Monodelphis domestica* showing localisation of alkaline phosphatase activity. F, femur; T, tibia. (*a*) Section of a pouch d 1 specimen. Localisation of alkaline phosphatase is observed in the periosteum of the tibia (arrows). Bar, 150 μm. (*b*) Section of a pouch d 1 specimen. Alkaline phosphatase activity is present in the mesenchyme directly below the dermis (arrows). Bar, 150 μm. (*c*) Section of a pouch d 5 specimen showing alkaline phosphatase activity in

differential distributions during development with type IIB mRNA being expressed by overt chondrocytes and type IIA mRNA being expressed by perichondral cells and prechondrocytes (Nah & Upholt, 1991; Sandell et al. 1991, 1994). In the chick, more recent work has demonstrated type IIA procollagen within the interzone (Nalin et al. 1995). Our data also show that type II collagen is present in both the interzone and perichondrium and we are currently making probes to the alternatively spliced variants of *Monodelphis* type II collagen which will be used in attempts to determine whether articular surface chondrocytes are derived from the joint interzone, the hyaline cartilage epiphyses or a combination of both.

Type VI collagen was found throughout the mesenchyme of the neonatal limb bud including the ECM of the developing cartilage rudiments. Some intense pericellular localisation was already present in the neonatal rudiment at this stage, and, following development, the pericellular localisation became more pronounced whilst the general intensity of the matrix localisation decreased. With continued development (from pouch d 14), the pericellular localisation concentrated towards the surface of the joint. This concentration seems to result in a paucity of localisation in the midepiphyseal region of the tissue. Interestingly, it is this area in which the secondary centre of ossification forms at 2 mo, and the lack of the type VI collagen may be related to this future event. Also, the growth plate which is delineated by the secondary centre of ossification is immunonegative for type VI collagen consistent with other data using the porcine epiphyseal plate (Wardale & Duance, 1993). It has been suggested that type VI collagen removal is a prerequisite for growth and remodelling (Keene et al. 1988; Sloan et al. 1993) and its absence from the growth plate may be related to this function allowing continued growth of the long bones.

New evidence is now appearing for the existence of differentially spliced variants of type VI collagen and for molecules with different chain combinations being expressed during different stages of development and in normal and diseased tissue (Timple & Chu, 1994). Therefore, distinct variants of type VI collagen could be expressed at different stages of chondrocyte differentiation and in different environments (e.g. hypertrophic cartilage), which could lead to alterations in interactions with other cells and macromolecules. To date, there are no published data of the differential expression of these variants in articular cartilage.

Type X collagen is localised classically, i.e. within the matrix of hypertrophic cartilage (Kielty et al. 1985) and is associated with the terminal differentiation of the chondrocytes. This localisation remained until adulthood since there is no closure of the growth plate. Like type X collagen, alkaline phosphatase has also been linked to the mineralising process (Beertsen & van den Bos, 1992) and showed a similar pattern of localisation to type X throughout the development of the bone rudiment. Unexpectedly, type X collagen and alkaline phosphatase also appeared to be localised towards the surface of the developing joint both, presumably, synthesised by presumptive articular chondrocytes. Whilst we have no idea of the significance of this observation, further studies are warranted to elucidate the fate of these cells. However, the ability of mature surface articular chondrocytes to initiate type X collagen synthesis has been documented by us in culture suggesting that all subpopulations of articular chondrocytes have the ability to terminally differentiate (Stephens et al. 1992).

With the development of the secondary centre of ossification, it is interesting to note that in the initial stages of formation, all of the above collagens, except type III are present. This situation is only transitory; however, it may indicate the importance of different collagen types at different stages of development and in different tissues and how the production of one type of collagen can be superseded by another, for example, type II by type I in endochondral bone ossification or alternatively type I collagen by type II in the early developing rudiments.

The distribution of the collagen types investigated in this study were located in a predictable pattern compared to other studies. However, as no complete developmental series of the distribution of collagen

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the bone underlying the periosteum (arrowheads) and hypertrophic cells of the middiaphysis of the developing tibia (arrows). mc, early marrow cavity. Bar, 150 μm. (*d*) Section of a pouch d 5 specimen. Alkaline phosphatase activity is localised towards the boundary of the rudiment at the developing joint line (arrow). Bar, 100 μm. Inset: higher power micrograph showing pericellular alkaline phosphatase activity. Bar, 20 μm. (*e*) Section of a pouch d 19 specimen showing alkaline phosphatase activity in the subperiosteal bone (arrowheads) and in the endochondral bone (arrows). Bar, 100 μm. (*f*) Section of a pouch d 26 specimen. Alkaline phosphatase activity is present at the attachment zone of a ligament (arrows). Bar, 100 μm (*g*) Section of a 2-mo-old specimen showing localisation of alkaline phosphatase activity at the mineralising border of the articular cartilage (arrows). Alkaline phosphatase activity is also present in the mature cells of the epiphyseal plate (asterisks) and throughout the developing secondary centre of ossification (arrowheads). Bar, 50 μm. (*h*) Section of an adult specimen. Alkaline phosphatase activity is observed in the cells and matrix of the calcified cartilage deep zone and its underlying bone (arrows). Bar, 20 μm.

types has previously been reported, these expectations are gathered from a number of different studies (von der Mark et al. 1976; von der Mark, 1981; Schmid & Linsenmayer, 1985; Poole et al. 1988; Wardale & Duance, 1993). This 'normal' pattern of collagen distribution confirms that *Monodelphis* is an appropriate model with which to study skeletal development. The results also demonstrate a general continuity of the different collagen types and, presumably their functional roles across species. However, as noted earlier, some functional redundancy is likely to exist.

Further work on the collagens will concentrate on the recently discovered alternatively spliced variants of type II and type VI collagen. It will be interesting to discover if these forms are developmentally regulated in this model as they appear to be in other species and how this may be related to specific developmental processes (Nah & Upholt, 1991; Sandell et al. 1991).

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