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A role for hyaluronan in joint development*

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INTRODUCTION

The final phase in the development of diarthrodial joints, apart from growth, is cavitation and thus the preparation of the joint for functional use. Factors such as cell death (Mitrovic, 1971, 1972) and vascular influences (Mitrovic, 1974) have been implicated as having a primary role in the cavitation process. None of these proposed mechanisms, however, can wholly explain how the joint space is formed against the forces generated by the growth of the opposing elements.

Hyaluronan, a common connective tissue component, has been shown to influence cell adhesion, migration (Turley, Bowman & Kytryk, 1985) and cytodifferentiation *in vitro* (Turley, 1982; Toole, 1972, 1981) and because of these properties has been postulated to have a role in morphogenesis. A number of developing systems have been described in which there is a close temporal and spatial correlation between hyaluronan synthesis and cell movement. Its removal, in contrast, has been associated with cytodifferentiation, for example in newt limb regeneration (Toole & Gross, 1971), chick embryo cornea development (Toole & Trelstad, 1971) and limb bud chondrogenesis (Toole, 1972; Oster, Murray & Maini, 1985). In cartilage, hyaluronan is found as a part of large proteoglycan aggregates (Hascall, 1977) and in the pericellular matrices (Goldberg & Toole, 1984; Knudson & Toole, 1985, 1987).

Various mechanisms have been proposed for the formation of the space in diarthrodial joints. Andersen & Brorasmussen (1961) suggested that cavitation was related to the deposition of large quantities of intercellular metachromatic substance (extracellular matrix) and that this caused a pronounced loosening of the tissue, forcing the cells apart. Munaron (1954) suggested that hyaluronan was present in the intermediate layer of the joint interzone on the basis of the loss of metachromasia following hyaluronidase digestion. Its presence here was discounted by Andersen (1962) on the basis that the hyaluronidase Munaron used was unspecific and also decreased chondroitin sulphates A and C (Meyer, Davidson, Linker & Hoffman, 1956). Indirect evidence for the role of hyaluronan in establishing and maintaining spaces during chick embryonic development includes the presence of hyaluronan in newly forming spaces underlying the ectoderm, into which neural crest cells migrate during craniofacial development (Pratt, Larsen & Johnston, 1975), and the marked loss of intercellular space after injecting embryos, *in ovo*, with hyaluronidase (Fisher & Solursh, 1977).

Conventional histochemical and immunocytochemical localisation methods have

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not met with much success because of the highly conserved nature of hyaluronan. In an attempt to define more clearly a possible role for HA in joint formation, we have employed a biotinylated hyaluronan-binding region link-protein complex, as described by Ripellino, Klinger, Margolis & Margolis (1985) and Ripellino, Bailo, Margolis & Margolis (1988) as a biochemical probe to locate hyaluronan in the developing third metatarsophalangeal joint (MTPJ) of the embryonic chick.

MATERIALS AND METHODS

Preparation of probe

A mixture of hyaluronic acid-binding region and link protein (binding proteins) was prepared by trypsin digestion of pig laryngeal cartilage proteoglycan aggregates as described by Bonnet, Dunham & Hardingham (1985), and their purity was analysed by gel electrophoresis (Fairbanks, Steck & Wallach, 1971). The proteins were complexed with hyaluronic acid and subsequently biotinylated using biotinyl *N*,*N*-hydroxysuccinimide ester (Ripellino *et al.* 1985) in this way the functional domains of the binding proteins are protected from biotinylation. The biotinylated proteins were separated from hyaluronic acid by HPLC on a TSK-3000 column eluted with 4 M-guanidine HCl/50 mM acetate buffer, pH 5·8, containing 1 mM-EDTA. Biotinylated binding region and link protein were pooled, dialysed against 50 mM acetate buffer (pH 5·8), dialysed further against distilled water, lypophilised and stored at −70 °C.

Tissue preparation

Stage 36–42 chick embryo legs (Hamburger & Hamilton, 1951) were removed by amputation through the upper femur and immediately frozen in liquid nitrogen. The legs were cut at this level to prevent any muscular contraction during amputation affecting the third MTP joint. The frozen feet were removed by cutting through the metatarsals and then mounted in Tissue-Tek O.C.T. Compound 4583 (Miles Scientific, Stoke Poges, UK). Ten micrometre sections were cut on a Reichert–Jung 2800 Frigocut E and mounted on subbed slides. The subbing medium used was that described by Boyd (1955), and comprised 1% w/v gelatin (BDH Chemicals, Poole, England) and 0·1% w/v chromium potassium sulphate in millipore-filtered distilled water. All sections were stored in a dust-free box at −20 °C until required for staining.

Staining procedure

The sections were fixed for 30 minutes with a 3% formaldehyde, 0·5% cetylpyridinium chloride (CPC, Sigma Chemical Co. Ltd, Poole), and 30 mM sodium chloride in 0·1 mM phosphate buffer (PB), pH 7·4. All reagents were prepared using millipore-filtered distilled water, filtered again, pH adjusted and used at room temperature. After fixation, the sections were washed briefly (3 × 5 minutes) in 0·1 M phosphate buffer (PB) and incubated with blocking serum (1% w/v bovine serum albumin for 30 minutes) (Sigma). The sections were again washed (PB, 3 × 10 minutes) and incubated for two hours with 50 μg/ml biotinylated binding region proteins. The sections were left washing in phosphate buffer overnight. Incubation with a 1:400 dilution of avidin-peroxidase (Sigma) was followed by a further wash (PB, 3 × 10 minutes) before the application of a peroxidase mouse anti-peroxidase antibody reagent (PAP) (20 minutes). The probe was visualised, either by the application of a diamino-benzidine (DAB) or with a 3-amino-9-ethylcarbazole (AEC) reaction product. After rinsing, sections to be stained with the latter were covered with the substrate solution (aqueous hydrogen peroxide and AEC in *N*,*N*-dimethylformamide)

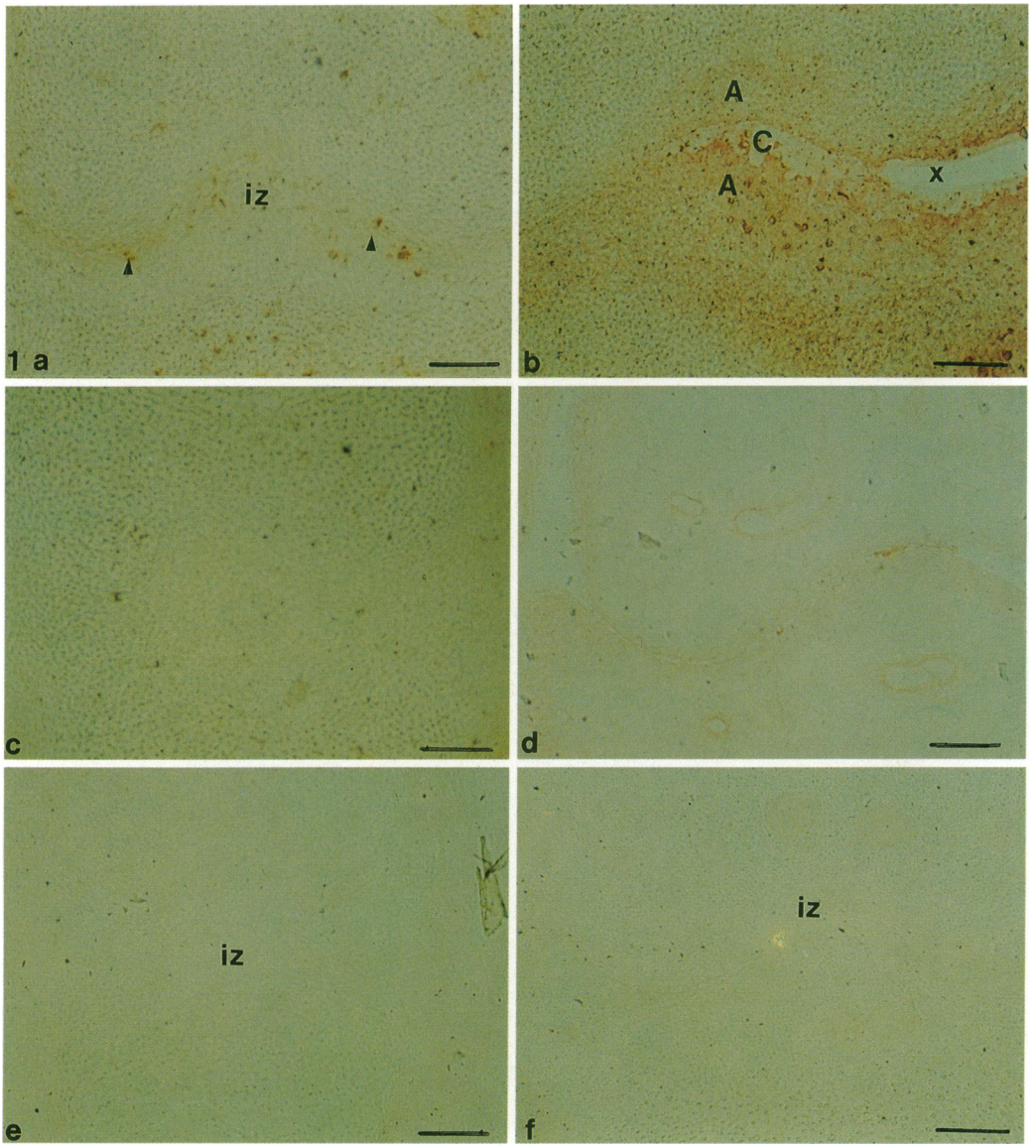

Fig. 1 (a-f).

Hyaluronan localisations in Stages 37-42; third MTPJ.

(a) Stage 37 MTPJ (DAB reaction product). Limited staining (arrowed) is seen in the interzone of the joint (iz). Bar, 100μm. (b) Stage 41 MTPJ (AEC reaction product). Hyaluronan is found in the articular surfaces (A) and in the cavity forming between these (C). A tear is seen at X; there is no reaction product here but hyaluronan is localised beyond this up to the articular surfaces. Bar, 100μm.

Controls for hyaluronan localisations (all stained with AEC).

(c) Stage 37 MTPJ incubated with non-biotinylated binding region. No staining is seen. Bar, 100μm. (d) Stage 42 joint. Treated with hyaluronidase after fixation. Hyaluronan has been removed from the cavities and, although reduced, is still apparent in the joint region and surrounding soft tissues. Bar, 140μm. (e) Stage 38 MTPJ, treated with hyaluronidase prior to fixation. All the hyaluronan has been removed. Joint interzone (iz). Bar, 140μm. (f) Stage 41. Section pretreated with phosphate buffer prior to fixation. Staining intensity is reduced but the pattern seen is much the same as in the test sections. Joint interzone (iz). Bar, 140μm.

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as described in the supplier's instructions (RNN.34 mouse universal immunocytochemical staining system kit supplied by Amersham International plc, Amersham, UK). The sections were incubated with substrate until an acceptable colour intensity was achieved (approximately 10 minutes). An alternative staining procedure involved incubating the sections with *Streptomyces* avidin-gold, after washing, followed by silver enhancement (supplied as a kit and used as in makers' instruction; Janssen Pharmaceuticals, Wantage, UK). After staining, the sections were well rinsed with tap water, mounted in 9:1 glycerol/phosphate buffer and photographed.

Controls

Processing of control sections was performed as above except that either the biotinylated binding region was omitted, or 50 μg/ml non-biotinylated binding region was used. Further controls were performed by incubating sections with 5 units *Streptomyces* hyaluronidase (Sigma) in 0·1 M sodium acetate (BDH), pH 5, in the presence of protease inhibitors (100 mM-6-aminocaproic acid, 10 mM-EDTA and 5 mM benzamidine HCl, all supplied by Sigma) for 5 hours at 37 °C in a humidified chamber, before being processed as before. It was found that hyaluronidase treatment after fixation only removed a proportion of the staining. Therefore, most sections were incubated with hyaluronidase prior to fixation. A parallel run was performed substituting phosphate buffer for the enzyme, to ensure than any loss of staining was not due to non-specific causes, such as leaching. Treatment with chondroitinase ABC (Sigma, 0·25 IU/ml, 30 minutes at 37 °C) made no difference to the staining pattern.

RESULTS

Hyaluronan was first localised in the central region of the joint at Stage 37 (Fig. 1a), twelve hours prior to the onset of cavitation, but was not clearly visible here until Stage 38, when it was restricted to the developing articular surfaces and intermediate region of the joint (Fig. 2a). Interestingly, using frozen sections, we found that cavitation was observable later than that seen in wax-embedded material (Craig, Bentley & Archer, 1987). Presumably, this is due to shrinkage which occurs during the processing of wax-embedded material. Between the later stages studied (38–42; Figs. 1b, 2) the pattern of localisation remained constant. Hyaluronan was restricted to the articular surfaces, perichondrium, developing synovium, meniscus and soft tissues (not shown). Hyaluronan was also associated with the developing cavities and it persisted as these enlarged. The cavities first appeared laterally at Stage 38 and became apparent in the medial joint interzone at Stage 41 (Figs 1b, 2b). Hyaluronan was also found around the invading vascular canals within the round cell zone of the cartilaginous epiphyses (Stage 41) (Fig. 2b). The staining was more intense in the developing synovial tissue bordering the developing cavity than in the tissue surrounding the cartilage *anlage* (Fig. 2d). Hyaluronan was absent in the rounded cell zone of the cartilage *anlage*, although it was localised in the flattened and hypertrophic regions.

Staining was not apparent in no-substrate (not shown) and non-binding region controls (Fig. 1c). The reaction product was removed in the joint cavities by hyaluronidase treatment but, in prefixed tissue, staining was still apparent in the articular surfaces (Fig. 1d). However, if sections were treated with enzyme before fixation, all the staining was abolished (Fig. 1e). The pattern of staining, although greatly reduced, was similar to test sections when pretreated with phosphate buffer prior to fixing (Fig. 1f).

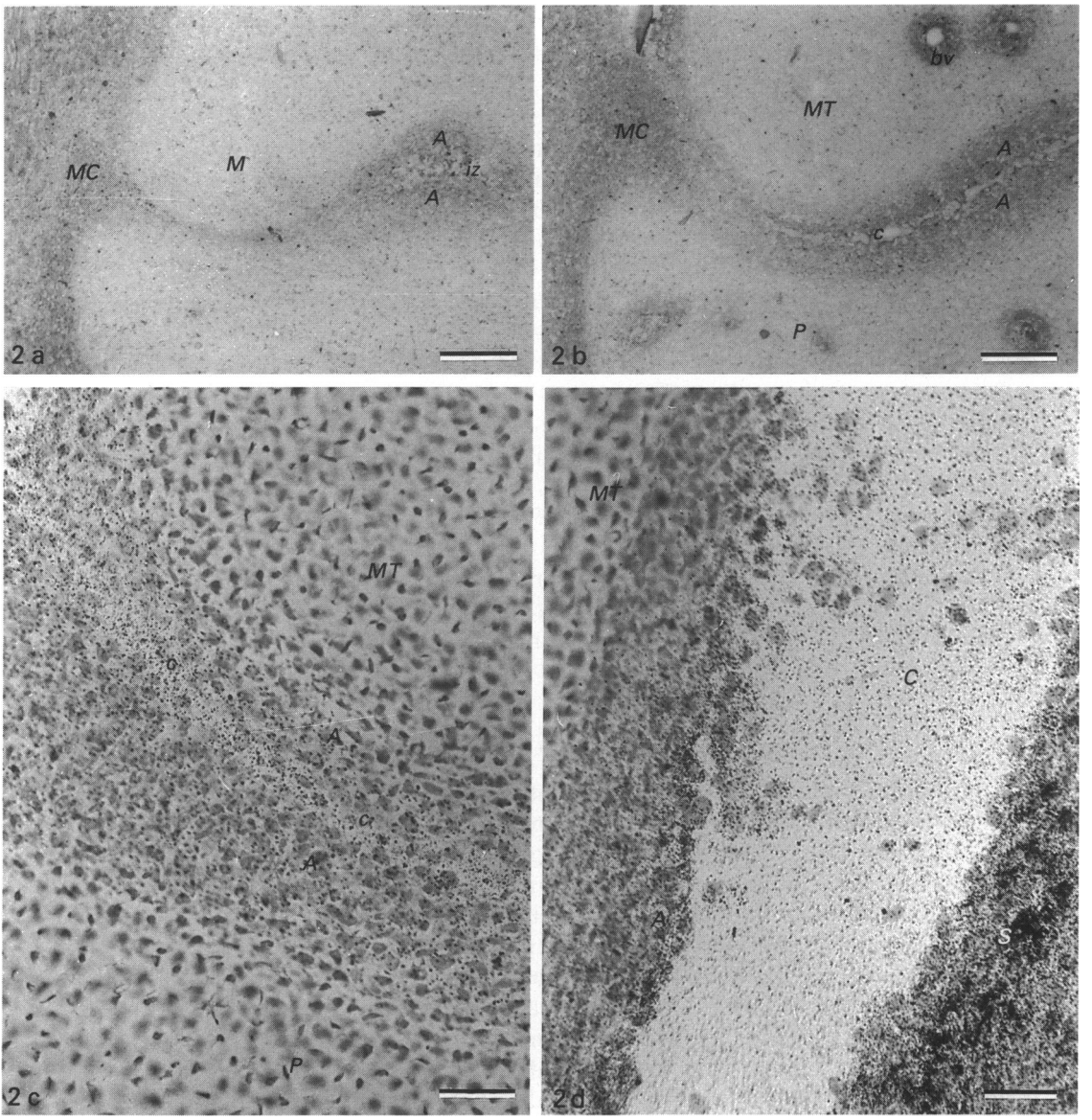

Fig 2(a–d). (a) Stage 38 MTPJ (AEC reaction product). Hyaluronan is localised in the articular surfaces (*A*), intermediate layer of the interzone (*iz*), developing meniscus and capsule (*MC*). There is no obvious staining in the cartilage matrix (*M*) (rounded cell zone). Bar, 100 μm. (b) Stage 41 MTPJ (AEC reaction product). Hyaluronan is found surrounding the blood vessels (*bv*), in the developing meniscus and capsule (*MC*) as well as in the articular surfaces (*A*) and enlarging clefts (*C*). Again, no staining is seen in the rounded cell zone of the metatarsal (*MT*) and phalangeal (*P*) elements. Bar, 100 μm. (c) Stage 41 MTPJ. Hyaluronan localisation enhanced with silver. The staining can be seen in the clefts (*c*) forming between the articular surfaces (*A*) and in the developing meniscus and capsule between the metatarsal (*MT*) and phalengeal (*P*) elements. Bar, 20 μm. (d) Stage 42. Hyaluronan, seen here in the enlarging cavity (*C*) and laterally in the joint, adjacent to the metatarsus (*MT*). Enhanced with silver. The staining in the articular surface (*A*) is less intense than that of the early (presumptive) synovium (*S*). There is no staining in the matrix of the metatarsus. Bar, 20 μm.

DISCUSSION

The results presented here provide histochemical evidence for the presence of free binding sites on the hyaluronan in the articular surfaces and joint cavities of the developing chick limb. Hyaluronan was first identified in the interzone at Stage 37, concomitant with the first signs of cavitation, and was maintained in these spaces as the joint formed. This probe shows that hyaluronan is indeed present in the 'intercellular metachromatic substance' described by Andersen (1961). The absence of staining in the rounded cell zone of the cartilage suggests three possibilities. Firstly, that there is no hyaluronan present; secondly, that hyaluronan is saturated with proteoglycan monomer; therefore, no sites are available for binding the biotinylated probe; thirdly, it may be that CPC precipitation of HA may block available sites of binding as suggested by Scott (1989). However, why this should occur in the rounded cell zone and not in the flattened or hypertrophic cell zones, where binding does occur, is unclear. Given the known biochemical properties of cartilage, the second possibility seems most likely. The presence of labelling around the developing vascular canals (areas of matrix degradation) in the rounded cell zone, suggests this possibility.

The swelling pressure in the cartilage extracellular matrix is mainly due to its constituent negatively charged polymers, principally hyaluronan and chondroitin sulphates. Hyaluronan is a powerful hydrophilic polymer, its voluminous structure presumably being due to inherent chain stiffness produced by hydrogen bonding between the sugar residues (Laurent & Fraser, 1986). It is capable of osmotic swelling and deswelling by a factor equal to or greater than five (Oster *et al.* 1985). The collagen meshwork in the articular surfaces and cartilage matrix proper will restrict the swelling properties of hyaluronan and associated proteoglycans in these regions. In contrast, the central region of the joint interzone is sparse in collagen and other proteoglycans (Craig *et al.* 1987). The hyaluronan may, therefore, have the ability to realise its full swelling potential. Thus, we have two parts of the joint which exhibit differential swelling potentials. This in turn may suggest a simple mechanism whereby the joint cavity is formed. Free hyaluronan becomes available in the joint interzone concomitant with the first signs of cavitation. Hyaluronan has been described as a weakly adhesive molecule. Correlative changes in hyaluronan content and resistance to detachment have been found in a number of cell lines (Culp, Rollins, Buniel & Hitri, 1978; Barnhart, Cox & Kraemer, 1979). Exogenous hyaluronan has also been shown to facilitate cell detachment both intercellularly and from the artificial substratum (Abatangelo, Cortivo, Martelli & Vecchia, 1982). If it were to decrease intercellular interactions, the tissue of the collagen-deplete presumptive cavity (intermediate region of the interzone) would become an area of tensile weakness and rupture would be more likely to occur in this region. The swelling pressure of the hyaluronan could physically separate the cells, increase and maintain the cavity's volume and prevent secondary fusion across the joint space, thus protecting the newly formed articular surfaces from damage caused by the movement of the embryo *in ovo*.

SUMMARY

Hyaluronan, a common connective tissue component, influences cell adhesion, migration and cytodifferentiation *in vitro*, and because of these properties has been postulated to have a role in morphogenesis. Its role in the development of cartilage-associated structures, such as joints, has yet to be defined.

Using a biotinylated hyaluronan-binding region-link protein complex, free

hyaluronan binding sites have been localised in the joint region concomitant with the first signs of cavitation (Stage 37), whereafter it is localised in the joint space and is maintained here as this enlarges. The application of our results is discussed in the context of a primary role for hyaluronan in joint cavity formation.

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