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HYPERTROPHIC CHONDROCYTES ARE SURROUNDED BY A CONDENSED LAYER OF HYALURONAN

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Abstract

Histochemical staining of the epiphysial growth plate revealed that freely-exposed hyaluronan (i.e., available to a staining probe) was present in the pericellular region of hypertrophic chondrocytes. Indeed, the distribution of this hyaluronan was very similar to that of type X collagen, a marker for the hypertrophic zone. In this region, the free hyaluronan was located pericellularly between the hypertrophic chondrocyte and the surrounding cartilage matrix. To further evaluate the contribution that this pericellular hyaluronan makes to the expansion of the hypertrophic lacunae, we examined the morphology of the growth plate following fast-freezing and cold fixation which preserves the *in situ* morphology of the tissue. Under these conditions, the pericellular hyaluronan was apparent as a thin ring surrounding the hypertrophic chondrocytes. However, in regions of the growth plate in which the integrity of the chondrocyte or the cartilage matrix had been compromised, then the hyaluronan appeared to be expanded. These results suggest, but do not prove, that under normal conditions the pericellular hyaluronan is highly compressed and acts as a seal or gasket between the expanding chondrocyte and the surrounding cartilage matrix. Furthermore, when the pressure inside the lacunae is released, the pericellular hyaluronan may be able to undergo expansion.

Key Words: Chondrocytes, extracellular matrix, fast-freezing, hyaluronan, hypertrophic, *in situ* morphology.

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Introduction

One of the major functions of hyaluronan is simply to occupy space in the extracellular matrix (Toole, 1981; Laurent and Fraser, 1992). Since individual molecules of hyaluronan are hydrophilic, have a high-negative charge and can have a high molecular weight, they can adsorb large amounts of water and exert a swelling pressure on surrounding tissues. The presence of hyaluronan in tissues is responsible for maintaining spaces between individual cells and at the same time allows the diffusion of low molecular weight materials. Such spaces are prominent in embryonic tissues and can be demonstrated by the fact that treatment with hyaluronidase causes the condensation of the mesenchymal cells (Solursh *et al*., 1979; Morriss-Kay *et al*., 1986). In addition, the production of hyaluronan has been implicated in a number of morphological phenomena involving tissue swelling. For example, the expansion and contraction of the cornea prior to the invasion of mesenchymal cells is correlated with the appearance and subsequent removal of hyaluronan (Toole and Trelstad, 1971). Similarly, the production of hyaluronan has been implicated in the closure of the neural tube (Schoenwolf and Fisher, 1983), and the fusion of the palate during embryonic development (Wilk *et al*., 1978). And finally, the expansion of the follicular cells that surround the oocyte immediately prior to ovulation is due to a dramatic up-regulation of hyaluronan synthesis at this time (Salustri *et al*., 1992). Thus, in many cases the swelling pressure provided by hyaluronan is one of the driving forces behind tissue expansion.

In previous studies, we have postulated that hyaluronan plays a similar role in the expansion of the epiphysial growth plate based on the following observations (Pavasant *et al*., 1996a,b). First, hyaluronan was expressed at the correct time and place. Histochemical staining of the growth plate with a staining probe derived from cartilage proteoglycan and link protein, showed that free hyaluronan was associated with the hypertrophic zone but not with other zones of the growth plate (Pavasant *et al*., 1994). This hyaluronan was located in the pericellular space between the hypertrophic chondrocyte and the cartilage matrix and

may be considered to be part of the chondron which consists of the chondrocyte and associated pericellular microenvironment (Lee *et al*., 1997). Furthermore, image analysis showed that the amount of hyaluronan staining was directly correlated with the size of the lacunae (Pavasant *et al*., 1996a). Secondly, factors such as insulin-like growth factor which promote the differentiation of chondrocytes in organ cultures of the growth plate as well as parathyroid hormone and transforming growth factor $\beta 1$ which inhibit this differentiation, had a parallel effect on the production of hyaluronan by the chondrocytes entering the hypertrophic phase (Pavasant *et al*., 1996b). And finally, when organ cultures of the growth plate were treated with hyaluronidase to degrade the pericellular hyaluronidase, the expansion of the lacunae was inhibited (Pavasant *et al*., 1996a). We interpreted these results to mean that hyaluronan contributed to the expansion of the hypertrophic lacunae and consequently was important for the expansion of the growth plate as a whole.

The present study was undertaken to further evaluate the role of pericellular hyaluronan in the expansion of the growth plate by examining its contribution to the volume of the hypertrophic zone of cartilage. For this, we analyzed the appearance of pericellular hyaluronan in its native form by using a fast-freezing technique that avoids many of the artifacts associated with conventional fixation and maintains the original morphology of the tissue (Hunziker and Schenk, 1984; Akisaka *et al*., 1987). The results of these studies suggest that in its native state, the pericellular hyaluronan is in a highly compressed form. Presumably, the pericellular hyaluronan acts as a spring or gasket between the expanding chondrocyte and the surrounding cartilage matrix (Toole *et al*., 1984). At the zone of erosion, when the physical constraints of the cartilage matrix are released, the pericellular hyaluronan expands dramatically. This compressed layer of hyaluronan may account for a number of phenomena concerning the epiphysial growth plate.

Materials and Methods

Histochemical staining for hyaluronan and type X collagen

The epiphysial plate from the metatarsal bone of a fetal calf (kindly donated by Dr. Gary J. Gibson, Henry Ford Hospital, Detroit, MI) was fixed in formalin, embedded in paraffin and sectioned using routine procedures. The sections were rehydrated in a graded series of solutions of alcohol and water. For hyaluronan staining, the sections were incubated for one hour in a solution of b-PG, a biotinylated preparation of proteoglycan {10 μg/ml of b-PG in PBS (phosphate buffer saline) containing 10% calf serum}. The b-PG preparation binds to hyaluronan with high affinity and specificity (Green *et al*., 1988). This was followed with peroxidase coupled to streptavidin (Kiregaard and Perry, Gaithersburg, MD) diluted 1:500 in PBS containing 10% calf serum. After rinsing in distilled water, the sections were incubated for approximately 30 minutes in a peroxidase substrate consisting of 0.03% H_2O_2 and 0.2 mg/ml 3-amino-9-ethyl carbazole, that gives rise to an intense red reaction product (Graham *et al*., 1965). For type X collagen staining, the sections were pre-incubated with testicular hyaluronidase (1 mg/ml, Sigma type V, St. Louis, MO), and then incubated for 1 hour with mouse serum containing the anti-type X collagen diluted 1:100 in PBS containing 10% calf serum. This antibody (donated by Dr. Gary J. Gibson) was raised against type X collagen from deer antlers and does not cross react with other types of collagens (Gibson *et al*., 1996). This was followed by a one hour incubation in a solution of peroxidase labeled goat anti-mouse IgG (Kiregaard and Perry) diluted 1:250, and finally a substrate consisting of H_2O_2 and 3-amino-9-ethyl carbazole. In each case, the sections were counter-stained with Mayer's hematoxylin, and the chromogens were preserved with Crystal/Mount (Biomeda, Foster City, CA).

In the case of the rat tibia, the knee joint of an 18-day-old rat was fixed by immersion in formaldehyde, decalcified in 20% ethylenediaminetetraacetic acid (EDTA), and embedded in polyester wax (Kusakabe *et al*., 1984). The section was stained for hyaluronan using the b-PG reagent and counterstained with hematoxylin, as described above. To determine the area occupied by the pericellular hyaluronan in the hypertrophic zone, the appropriate regions were cut from a photograph with a scalpel and weighed.

Fast-freezing and fixation of the growth plate

The knee joint of an 18-day-old rat was quickly dissected in order to expose the end of the tibia. Cross-sectional cuts were then made along the secondary ossification center in the epiphysis and at the end of the bony shaft of the diaphysis, thereby isolating the epiphysial plate. This was cut in half length-wise and the freshly exposed surface of the epiphysial plate was placed face up on the specimen mount of a cryo-fixation unit (Model CFG651, Electron Microscopy Sciences, Fort Washington, PA). Upon activation of the unit, the specimen was rapidly driven against a metal anvil that previously had been cooled with liquid nitrogen, resulting in the rapid freezing of the tissue. The specimens were then stored in liquid nitrogen.

For freeze-substitution fixation, the samples were immersed in a mixture of osmium tetroxide (1%), glutaraldehyde (3%) and uranyl acetate (1%) in methanol at -80°C for 20 hours (Glikey and Staehlin, 1986). After this, the samples were gradually warmed to room temperature. At this point, the specimens were embedded in Spurr resin (EMS, Fort Washington, PA), semi-thin sections (1 μm) were taken and stained with toluidine blue. The area occupied by the pericellular hyaluronan was determined by cutting

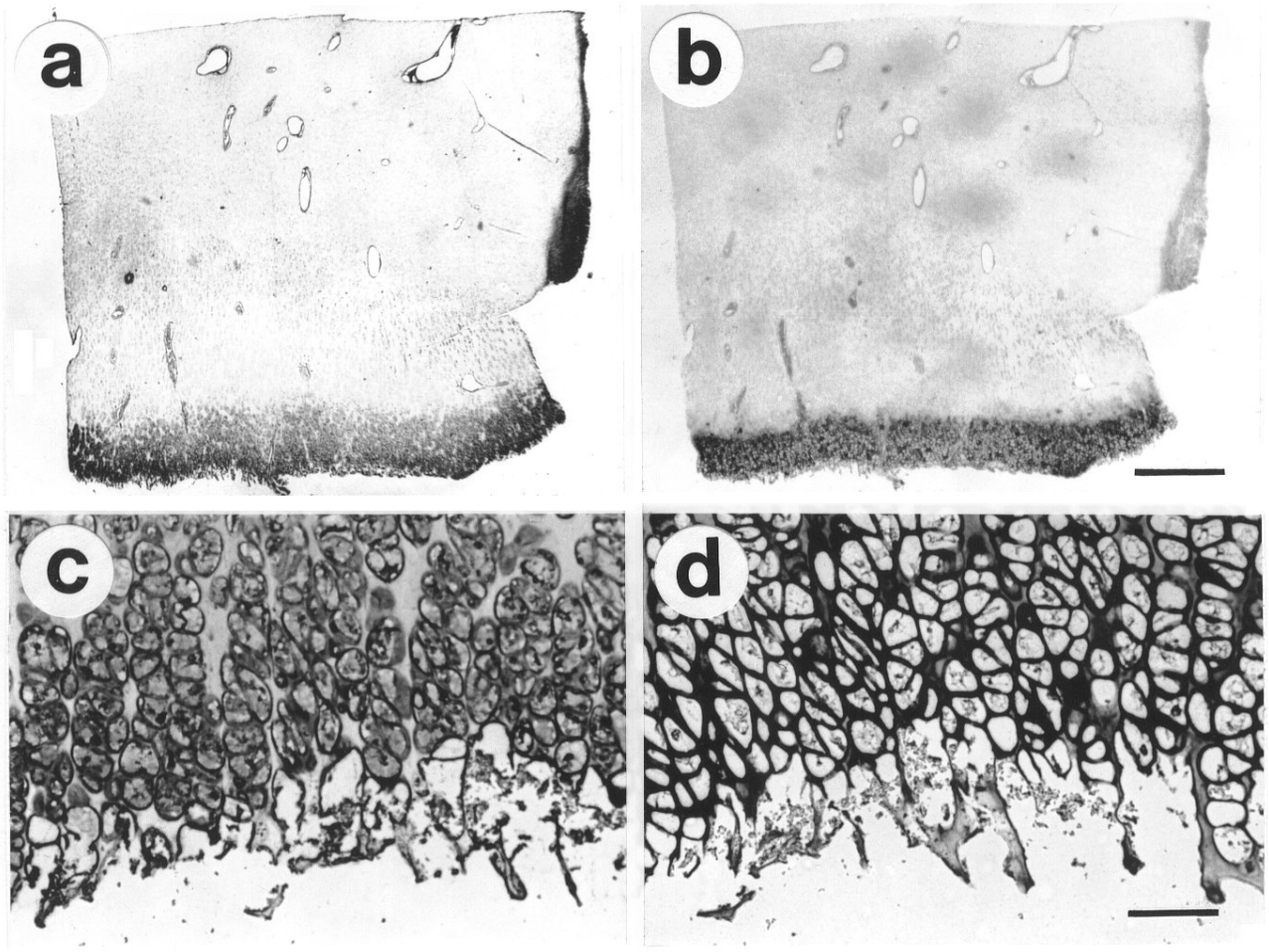

Figure 1. The distribution of exposed hyaluronan and type X collagen in the growth plate. The metatarsal growth plate from a fetal cow was fixed in formalin, embedded in paraffin and then processed for histology. Adjacent sections were stained for hyaluronan with b-PG (a and c) and for type X collagen using mouse antibodies (b and d). (**a** and **b**) Low magnification views of free hyaluronan and type X collagen staining, respectively, show that both of these agents are associated with the hypertrophic zone. The faint ovals in Figure 1b do not represent specific staining. (**c** and **d**) High magnification views of the hypertrophic zones demonstrate that the free hyaluronan is located within the individual lacuna spaces, while the type X collagen is present in the cartilage matrix. Bars = 1,000 μm (b; Fig. 1a is the same magnification as Fig. 1b) and 100 μm (d; Fig. 1c is the same magnification as Fig. 1d).

the appropriate regions from a photograph as described above.

Results

The association of hyaluronan with hypertrophic chondrocytes was initially suggested by histochemical staining of the growth plate with a reagent consisting of a biotinylated fragment of the aggrecan molecule and the link protein for cartilage (Green *et al*., 1988). This reagent, termed b-PG, binds to hyaluronan with high affinity and specificity (Green *et al*., 1988), however, it does so only when the molecule is freely exposed and available to it (Green *et al*., 1988). Thus, the hyaluronan present in the cartilage matrix does not stain because it is already complexed with proteins and/or the b-PG reagent is unable to penetrate into the tissue.

As illustrated in Figure 1a, when the growth plate from a fetal calf was stained with the b-PG reagent, freely-exposed hyaluronan was clearly apparent in the lower margin of the tissue. Indeed, at low magnification the distribution of this hyaluronan was indistinguishable from that of type X collagen, which is shown in Figure 1b and is a widely accepted marker for the hypertrophic zone (Schmid *et al*.,

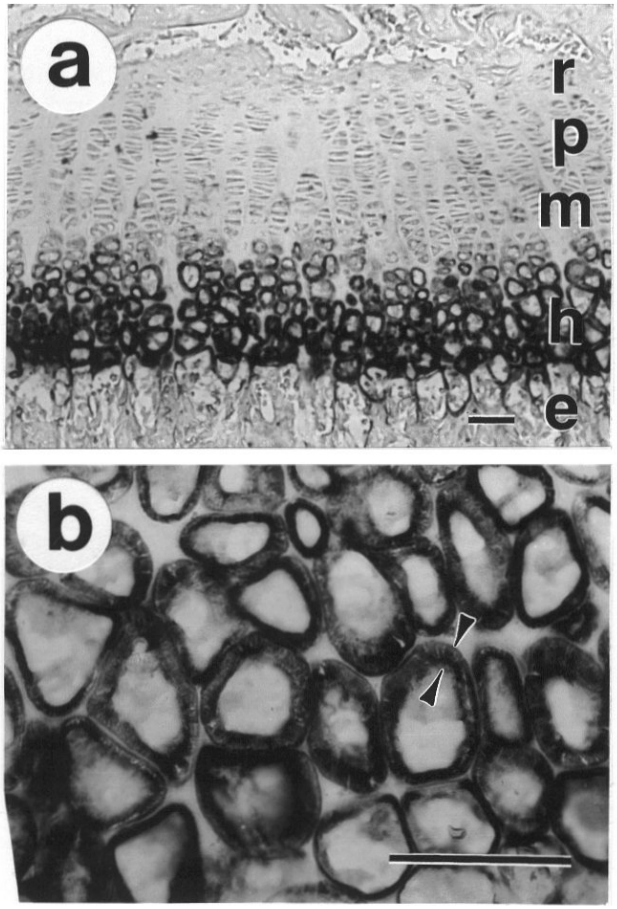

Figure 2. The distribution of free or exposed hyaluronan in the growth plate. The tibia from an immature rat was fixed by immersion in formaldehyde, decalcified in EDTA, embedded in polyester wax (Kusakabe *et al*., 1984) and processed for histology. The resulting sections were stained for free (exposed) hyaluronan using the b-PG probe followed by counter-staining in Mayer's hematoxylin. The sections were photographed with a blue filter. The dark regions correspond to hyaluronan staining while lighter regions demonstrate the background. (**a**) A low magnification view of the entire growth plate shows that specific hyaluronan staining is present in the hypertrophic zone (h) but not in the resting, proliferation or maturing zones (r, p and m). The hyaluronan staining disappeared in the zone of erosion (e). (**b**) A high magnification view of the hypertrophic zone shows that the exposed hyaluronan is located in the pericellular region between the chondrocyte and the surrounding cartilage matrix (see arrowheads). In this section, the pericellular hyaluronan occupies approximately 57% of the tissue volume. Bars = 50 μm.

1990). However, at higher magnification (Fig. 1c), it was apparent that most of the hyaluronan staining was located within the lacuna space whereas the type X collagen was predominantly in the matrix (Fig. 1d). Thus, the presence of freely-exposed hyaluronan is a marker for hypertrophic chondrocytes.

The distribution of hyaluronan is more clearly demonstrated in Figure 2 of a growth plate taken from the tibia of an immature rat following routine fixation with formaldehyde. Again, at low magnification shown in Figure 2a, large amounts of staining were present in the hypertrophic zone (labeled h), while little staining was apparent in the resting, proliferating, maturing or erosion zones (labeled r, p, m and e). The higher magnification view shown in Figure 2b demonstrates that the hyaluronan is located pericellularly between the hypertrophic chondrocytes and the surrounding matrix of cartilage. Clearly, there is a greater amount of exposed hyaluronan in the pericellular matrix as compared to cartilage matrix that does not stain. In this particular case, the hyaluronan occupies approximately 57% of the volume in the hypertrophic zone. However, as will be discussed later, this figure is probably high due to the expansion of the pericellular hyaluronan following the death of the chondrocyte. Similar staining patterns were obtained with all other growth plates that we examined.

Based on these and similar observations, we proposed that the pericellular hyaluronan contributes to the expansion of the growth plate. Presumably, the hyaluronan accumulates in the tissue and exerts a swelling pressure on the surrounding tissue causing it to expand. This possibility was consistent with our observation that the amount of this pericellular hyaluronan was closely correlated with the size of the lacunae space in the cartilage and the fact that treatment of organ cultures of the growth plate inhibited the expansion of the plate (Pavasant *et al*., 1996a).

To determine the extent of hyaluronan's contribution to the expansion process, we wanted to determine the volume that it occupied in the hypertrophic zone. However, the traditional fixation of cartilage with formalin does not necessarily reflect the true morphology of the growth plate, since shrinkage artifacts may be quite substantial in these cases. To circumvent these fixation artifacts, we employed the process of rapid-freezing, which has been shown to accurately preserve the structure of the epiphysial plate (Hunziker and Schenk, 1984; Akisaka *et al*., 1987). For this, we used a freshly prepared section of the tibia from a young rat (similar to that shown in Fig. 2). Following the quick-freezing, the samples were fixed, embedded in plastic resin, thin sectioned and then stained with toluidine blue, which allows differentiation of the hyaluronan from the more darkly staining cartilage matrix as well as other tissue.

Figure 3a shows a low magnification view of a well-preserved section of the rat growth plate. It is apparent that as the epiphysial chondrocytes differentiate and pass

through the various zones, they increase in size, while the intensity of the toluidine blue staining of the cytoplasm decreases. This decrease in staining intensity suggests that protein concentration within the chondrocytes decreases as the chondrocytes undergo hypertrophy.

Figure 3b, a higher magnification view of the hypertrophic zone, shows that the chondrocytes fill most of the lacuna spaces, as has been demonstrated previously (Hunziker and Schenk, 1984). The pericellular layer of hyaluronan was apparent as a thin band located between the chondrocyte and the darker staining cartilage matrix (indicated by arrowheads). In this particular case, the pericellular layer of hyaluronan represented approximately 17% of the tissue volume, which was a much smaller fraction than that of the 57% present in the formalin fixed tissue shown in Figure 2b. It was also interesting to note that the pericellular layer was surrounded by a dark staining layer of cartilage which may represent a compact layer of chondroitin sulfate. In addition, vesicles were apparent in the cytoplasm of the hypertrophic chondrocytes (indicated by the arrows), but were absent from the proliferating chondrocytes (data not shown).

In general, the pericellular layer of hyaluronan was restricted to a thin layer surrounding the hypertrophic chondrocytes. However, in some cases, the pericellular hyaluronan appeared to be expanded. An example of this is shown in Figure 3c, in which the lateral wall of a lacunae in the zone of erosion has been broken and the hypertrophic chondrocyte appears to be extending into an adjacent space. In this lacuna, the pericellular layer of hyaluronan (indicated by arrowheads) is much thicker than in adjacent lacunae, and the staining density of the hyaluronan is reduced. These results suggest that prior to entering the zone of erosion, the pericellular hyaluronan is under pressure created by the expansion of the hypertrophic chondrocyte. However, when the lacuna enters the zone of erosion and the restraining pressure of the cartilage wall is broken, this allows the pericellular hyaluronan to expand, and this may in some cases, partially push the chondrocyte from the space as is suggested by Figure 3c. This could account for the empty lacunae that have been observed in the zone of erosion (Farnum and Wilsman, 1989).

The ability of the pericellular hyaluronan to undergo expansion was also suggested by the morphology of lacunae that had suffered mechanical damage. For example, Figure 3d shows a region of the hypertrophic zone near the freshly cut edge of the growth plate in which many of the lacunae with damaged walls contained expanded pericellular hyaluronan (indicated by the stars). Another example of this is shown in Figure 3e of regions located somewhat distant from the fast-freezing zone, where the cells suffered from the formation of ice crystals. Presumably in these regions, the cell membranes have been disrupted by the formation of ice crystals before the freezing was complete. Again, the pericellular hyaluronan is much thicker (as indicated by the arrowheads) than adjacent regions not suffering from damage.

Taken together these results suggest, but do not prove, that *in situ*, the pericellular hyaluronan is under pressure and is compressed into a thin layer between the expanding chondrocyte and the resisting cartilage matrix.

Discussion

The results of the present study suggest, but do not prove, that the layer of pericellular hyaluronan that surrounds the hypertrophic chondrocytes is in a compressed state. The existence of this layer of hyaluronan was initially demonstrated by histochemical staining of the growth plate following conventional fixation with formaldehyde. Large amounts of free hyaluronan were apparent in the hypertrophic zone, located between the chondrocyte and cartilage matrix. As we have demonstrated in this study, the distribution of this pericellular hyaluronan parallels that of type X collagen, another marker for hypertrophic zone (Schmid *et al*., 1990).

The evidence that this layer of hyaluronan was compressed in its native state was suggested by several morphological features of tissues processed by fast-freezing, which avoids many of the artifacts associated with conventional fixation and preserves the *in situ* morphology of the tissue. Following fast-freezing, only a thin layer of hyaluronan was apparent around the hypertrophic chondrocytes and accounted for only 17% of the tissue volume in the hypertrophic zone. In contrast, in sections of the growth plate that had been processed by conventional formaldehyde fixation, the pericellular layer of hyaluronan was much larger, corresponding to ~57% of the tissue volume. We believe that the discrepancy between these two values may be attributed to the fact that *in situ*, the pericellular hyaluronan is highly compressed, as reflected in the fast-frozen tissue, whereas following formaldehyde fixation, the cell membrane is disrupted so that the layer of hyaluronan expands to fill more of the lacuna space. This could also account for the fact that in certain regions of the fast-frozen tissue such as near the cut edge or where ice damage was apparent, the pericellular layer of hyaluronan appeared to be much thicker and the staining density of the hyaluronan was reduced. These regions correspond to those in which the chondrocytes would be likely to experience mechanical trauma resulting in the rupture of the membrane. This could, in turn, allow the expansion of the pericellular hyaluronan so that it occupies a greater volume of the tissue. A similar situation may occur naturally in the growth plate at the zone of erosion. In this location, the lateral walls of the hypertrophic lacunae are broken and as

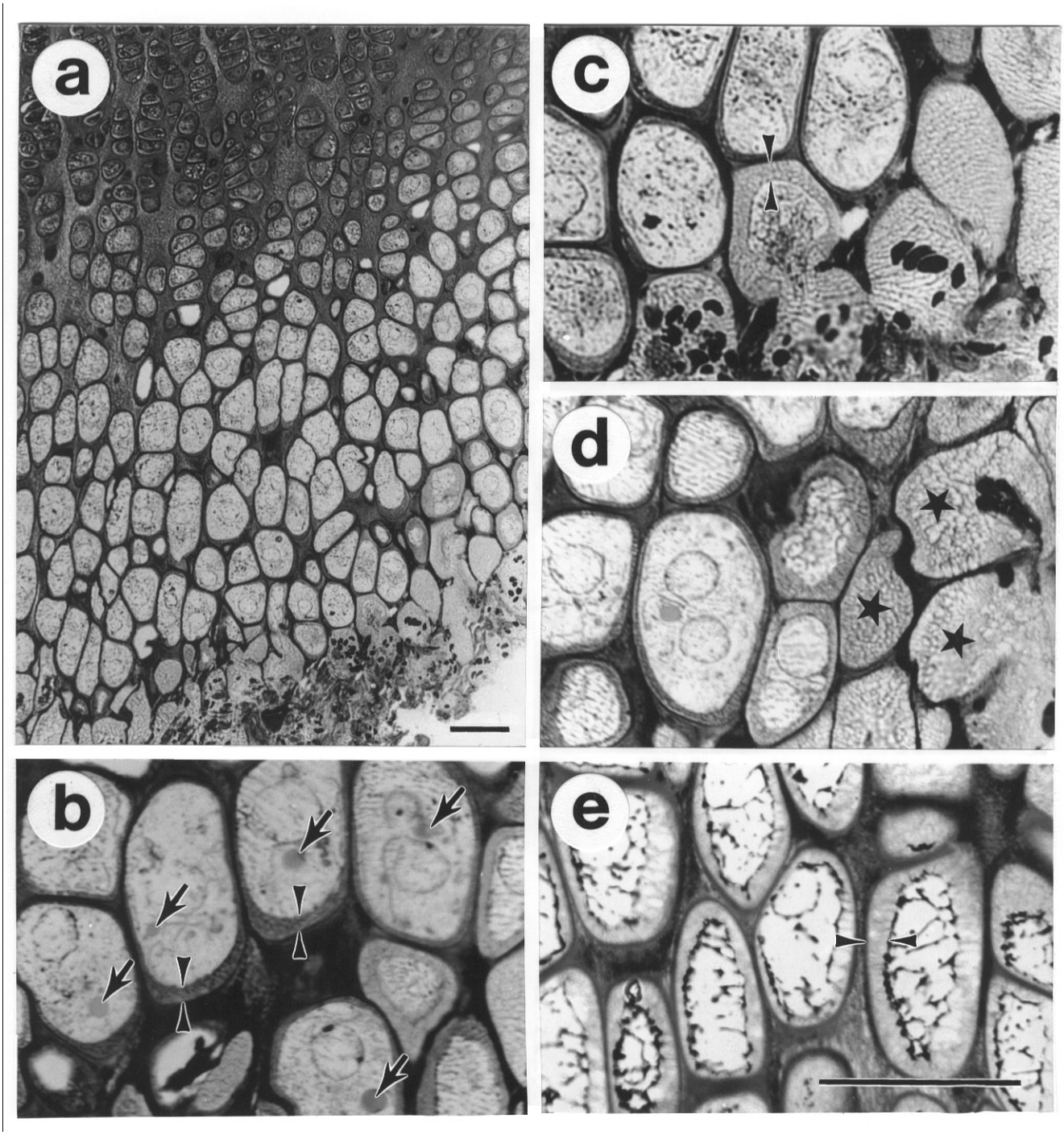

Figure 3 (caption on facing page)

a result the pressure inside the lacunae is suddenly released. Presumably, this accounts for the expansion of the pericellular hyaluronan that is observed in this region. Similarly, this would account for the diminished staining intensity with toluidine blue that was apparent in this hyaluronan as compared to that in adjacent intact hypertrophic lacunae. Taken together, these results suggest, but do not prove, that *in situ*, the pericellular layer of hyaluronan is compressed. This is in keeping with the physical properties of hyaluronan such that it can behave much like a spring (Toole *et al*., 1984) or a gasket between the chondrocyte and the cartilage.

The existence of this compressed layer of hyaluronan surrounding hypertrophic chondrocytes may account

Figure 3 (on previous page). The distribution of pericellular hyaluronan in fast-frozen sections of the growth plate. The tibial growth plate from an immature rat similar to that shown in Figure 2, was processed by fast-freezing, cold fixation followed by embedding in Spurr resin. Sections approximately 1 μm thick were obtained and stained with toluidine blue. With this technique, the pericellular hyaluronan is readily distinguished from the surrounding cartilage matrix. (**a**) A low magnification view of the growth plate shows that the chondrocytes in the proliferating zone towards the top are smaller and darker staining than those in the hypertrophic zone towards the bottom. (**b**) A higher magnification view of the hypertrophic zone, shows that the chondrocytes occupy most of the lacuna space and the pericellular hyaluronan (between the arrowheads) is present as a thin rim between the chondrocyte and the cartilage matrix. In this section, the pericellular hyaluronan accounts for approximately 17% of the tissue volume. In addition, the hypertrophic chondrocytes were observed to contain cytoplasmic vesicles (arrows) that were absent from cells in other zones. (**c**) A section showing one of the terminal hypertrophic lacunae next to the zone of erosion. In this case, the lateral wall of the lacunae has been broken, and the pericellular hyaluronan has expanded (between the arrowheads). In addition, the staining density of this pericellular hyaluronan was less than that present in adjacent intact lacunae. (**d**) A high magnification view of a section taken from the freshly cut edge of the growth plate. In this region, the damaged lacunae (stars) have expanded pericellular hyaluronan and in some cases, densely stained chondrocytes. (**e**) A high magnification view of a region of the growth plate that shows evidence of freeze-damage to the cells. Again, the pericellular layer of hyaluronan (indicated by arrowheads) has expanded and occupies a greater volume of the tissue than in regions with no evidence of freeze-damage. Bars = 50 μm (a and e); (b), (c), and (d) are at the same magnification as (e).

for a number of observations concerning the growth plate. For example, it can account for the poor morphology of this tissue following conventional fixation with formaldehyde. It is likely that this fixative compromises the integrity of membrane of the hypertrophic chondrocytes, allowing the pericellular layer of hyaluronan to expand and disrupt the morphology of the cell. Such a situation could account for the fact that pretreating the growth plate with hyaluronidase to degrade the pericellular hyaluronan prior to fixation improves the integrity of the plasma membrane (Hunziker *et al*., 1983). Similarly, treatment of the growth plate with salts of ruthenium also improves the final morphology (Hunziker *et al*., 1982, 1983). In this case, the ruthenium salts appear to form a complex with the hyaluronan and prevent it from expanding. A final phenomenon that may be due to the compressed layer of hyaluronan is the fact that many of the lacunae located in the zone of erosion appear to be empty (Farnum and Wilsman, 1989). In fact, these lacunae may be filled by expanded hyaluronan that have partially pushed the chondrocyte out of the terminal lacunae. It is also possible that during this process, the hypertrophic chondrocyte may be damaged and this contributes to the mortality of the chondrocytes in passing from the zone of hypertrophy to the zone of erosion.

While the functional role of the pericellular hyaluronan remains something of a mystery, it is clearly important for the differentiation of chondrocytes, as suggested by the fact that treatment of segments of the growth plate in organ culture with hyaluronidase inhibited the subsequent expansion of the maturing chondrocytes (Pavasant *et al*., 1996a). Initially, we proposed that this hyaluronan contributed to the expansion of the hypertrophic lacunae. However, based upon the results of the fast-freeze fixation, we now believe that the contribution of pericellular hyaluronan to the expansion of the growth plate is relatively small. Clearly, the vast majority of the expansion is due to the expansion of the hypertrophic chondrocytes themselves which compresses the pericellular layer of hyaluronan. Another possible function of the pericellular hyaluronan is that it acts like a spring or gasket to regulate the pressure between the membrane of the chondrocyte and the matrix. In this fashion, it could protect the chondrocyte from damage by preventing direct contact with the cartilage. And finally, the pericellular hyaluronan may be important for regulating the passage of nutrients into and out of the chondrocytes. It is possible that in organ cultures of the growth plate, removal of the pericellular hyaluronan by hyaluronidase alters the diffusion of O_2 and other nutrients such that the maturation of the chondrocytes is inhibited. Along these lines, earlier studies have shown that the concentration of O_2 alters the expansion of organ cultures of the growth plate (Brighton *et al*., 1969). In conclusion, pericellular hyaluronan appears to be compressed by the expansion of the hypertrophic chondrocytes and is vital to the maturation of epiphysial chondrocytes. However, the larger question of how the hypertrophic chondrocytes themselves are able to expand remains to be addressed.

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Discussion with Reviewers

C.B. Knudson: What matrix structure or components allow for the clear line of demarcation between the HA-enriched pericellular matrix zone and the territorial matrix? In Figure 3b, there are pairs of arrowheads, and the lower one of each pair points to this line of demarcation.

Authors: Since it stains darkly with toluidine blue, we suspect that this is a compressed layer of chondroitin sulfate that is associated aggrecan, however, we have no proof that this is the case.

C.B. Knudson: Could the seal or gasket of pericellular hyaluronan function to prevent debris from dead or dying chondrocytes from entering the cartilage?

Authors: While it could perform this function, we do not believe that this is the case because in some sections it is possible to see cells from the bone marrow that have invaded the terminal layer of lacunae (Pavasant *et al*., 1994).

C.B. Knudson: In Figure 1b, there are faint ovals of staining for type X collagen within the section in addition to the intense staining at the lower margin. Do you have any comments about this staining for type X that is not within the hypertrophic zone?
Authors: The faint ovals are simply artifacts of photography and do not represent specific staining.

C.E. Farnum: The results would be strengthened if the same kind of cytochemistry could be done on the material fixed by freeze substitution. Would this be possible using a more hydrophilic plastic, rather than Spurr resin?
Authors: So far, all our attempted to do b-PG histochemistry on freeze substitution sections have not given satisfactory results. We believe that the b-PG probe is so large that it is unable to penetrated Spurr or other types of resins.

C.E. Farnum: One of the important phenomena that correlates with differential growth in different growth plates is that the degree of hypertrophy of chondrocytes is variable: the faster the rate of growth the greater the degree of chondrocytic hypertrophy. Do your results address the mechanism of this phenomenon?
Authors: In previous studies using image analysis, we have found that the amount of hyaluronan present in each lacunae was directly proportional to the size of the lacunae (Pavasant *et al*., 1996a,b). This relationship held for growth plates from different sources that expanded at different rates. However, it should be emphasized that these measurements were done on formalin-fixed sections in which the pericellular hyaluronan has already undergone expansion.

C.E. Farnum: Are you hypothesizing that the hyaluronan is produced by the hypertrophic chondrocytes as a part of the enlargement process, and, if so how might you be able to demonstrate this? What are the definitive criteria to demonstrate active hyaluronan production of tissue sections?
Authors: We are indeed suggesting that the production of hyaluronan is part of the enlargement process, albeit much smaller than that due to the expansion of the chondrocytes themselves. In a previous publication (Pavasant *et al*., 1996a), we demonstrated that hyaluronan was actively produced by hypertrophic chondrocytes as demonstrated by autoradiography of sections of the growth plate that had been incubated with isotopically labeled glucosamine. We have also shown that treatment of organ cultures of the growth plate with hyaluronidase inhibited the expansion of the hypertrophic lacunae. We now interpret these results to mean that the production of hyaluronan is required for the expansion process.

C.E. Farnum: How do your results relate to the observation that terminal hypertrophic chondrocytes may die by apoptosis?
Authors: While the present study does not directly address the fate of the terminal chondrocytes, it is possible that if the lateral walls of the terminal chondrocytes suddenly bursts open, this could physically damage the hypertrophic chondrocyte. This, in addition to apoptosis, could lead to chondrocyte death.

B.A. Vertel: What do you think defines the outer border of the pericellular hyaluronan rim so sharply?
Authors: We believe that this outer rim is simply a compressed layer of chondroitin sulfate.

B.A. Vertel: How might the highly compressed hyaluronan pericellular rim protect the hypertrophic chondrocyte from damage, and why might this cell require such protection?
Authors: This is an important question. We speculate that when the hypertrophic chondrocytes undergo expansion, their plasma membranes push on the surrounding cartilage tissue. The function of the pericellular hyaluronan would be to act as a cushion and spread the pressure evenly across the cartilage matrix. If the plasma membrane were pushing directly on the cartilage itself, then it may be more susceptible to damage.