Journal of Cell Science 109, 327-334 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
JCS3294

Hyaluronan contributes to the enlargement of hypertrophic lacunae in the growth plate

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SUMMARY

Histochemical staining of the epiphysial growth plate revealed that free hyaluronan (i.e. available to the staining probe) was restricted to the zone of hypertrophy, where it was located in the pericellular space between the chondrocytes and the edge of the lacunae. Furthermore, the amount of hyaluronan staining was directly proportional to the size of the lacunae. Autoradiographic analysis of growth plates cultured with isotopically labeled glucosamine indicated that at least a portion of this hyaluronan was newly synthesized by the hypertrophic chondrocytes. Since hyaluronan can adsorb large amounts of water, it is possible that it exerted a hydrostatic pressure on the surrounding territorial matrix and thereby caused the expansion of hypertrophic lacunae. To assess this possibility, segments of the growth plate were placed in organ culture under different conditions. Under normal culture conditions, a band of hyaluronan staining migrated across the segments coinciding with the enlargement of lacunae in these regions, and the segments, as a whole, increased in size. In contrast, when the segments were cultured in the presence of hyaluronidase, which degraded the pericellular hyaluronan, the lacunae did not undergo enlargement and the overall size of the segments did not increase. These results suggest that the production of hyaluronan contributes to the enlargement of hypertrophic lacunae which is important for determining both the body's stature and proportions.

Key words: Hyaluronan, Chondrocyte, Growth plate, Zone of hypertrophy

INTRODUCTION

The epiphysial plate is responsible for the elongation of the long bones. To carry out this function, the plate, as a whole, grows by interstitial expansion while one end is continuously replaced by a calcified matrix. This interstitial expansion occurs through a combination of chondrocyte proliferation and enlargement of the lacunae that surround these cells (Breur et al., 1991: Hunziker et al., 1987; Ogden and Rosenberg, 1988). Indeed, the rate of long bone elongation can be estimated by multiplying the number of cells dividing per unit time by the height of the hypertrophic lacunae (Breur et al., 1991: Hunziker et al., 1987).

Breur et al. (1991) have shown that the volume change of the hypertrophic lacunae is a major determinant of the rate of longitudinal bone growth and that this contribution varies depending upon the species. In the case of growth plates from the tibiae of rodents, the lacunae increase in volume by five- to tenfold, while progressing from the zone of proliferation to the zone of hypertrophy (Buckwalter et al., 1986; Dean et al., 1989; Hunziker et al., 1987). The enlargement comes about as a result of expansion as opposed to removal of the territorial matrix, since image analysis of the growth plate has revealed that the ratio of territorial matrix per lacuna remains constant throughout the different regions of the growth plate (Alini et al., 1992; Hunziker et al., 1987). It is probable that this process is facilitated by collagenases and stromelysin which modify the matrix (Ballock et al., 1993; Brown et al., 1989; Dean et al., 1990). Clearly, the mechanism of lacunal expansion is of major importance in understanding the process of bone growth.

One mechanism responsible for lacunal expansion is that the hypertrophic chondrocytes themselves undergo enlargement, as has been demonstrated by numerous studies using both light and electron microscopy (Hunziker et al., 1983; Nuehring et al., 1991). Indeed, O'Keefe et al. (1989) have used a Coulter counter to analyze the size of freshly isolated chondrocytes and found that those from the hypertrophic zone were approximately 25% larger than those from the proliferating zone (658 versus 524 m^3, respectively). Thus, the chondrocytes themselves contribute to the process of hypertrophic expansion.

In this study, we will present evidence that the synthesis of hyaluronan also contributes to the enlargement of the lacunae. We have found that hypertrophic chondrocytes secrete large amounts of hyaluronan into the pericellular space, which we believe represents one of the driving forces behind lacunal expansion. Since hyaluronan has a large size and a high negative charge, it can adsorb large amounts of water and exert a swelling pressure on the surrounding tissue (Toole, 1981; Laurent and Fraser, 1992). This hyaluronan-induced swelling pressure causes the expansion of the lacunae, and in so doing plays a critical role in the expansion of the growth plate and the elongation of the long bones.

MATERIALS AND METHODS

Preparation of the proteoglycan probe

The proteoglycan probe (b-PG) used for the localization of hyaluronan was prepared by a previously described protocol (Green et al., 1988), which consists of extracting bovine nasal cartilage with 4 M guanidinium HCl, 0.5 M Na acetate, pH 5.8, briefly treating the dialyzed extract with trypsin to reduce the size, coupling the digest to biotin, and finally purifying the hyaluronan binding fraction by affinity chromatography. The resulting b-PG is composed of a mixture of the link protein and a fragment of the core proteoglycan, which binds to hyaluronan with high affinity and specificity (Green et al., 1988). However, it should be noted that this probe will bind to hyaluronan that is exposed or free and will not recognize that which is already bound by proteoglycan monomers or other protein.

Tissue preparation

The tibiae of 14- to 16 day-old rats (Charles River, Wilmington, MA) were dissected and fixed overnight with 3.7% formaldehyde in PBS at room temperature. After several washes with calcium- and magnesium-free phosphate buffered saline (PBS-CMF), the bones were decalcified with 20% EDTA for 7 days and then embedded in polyester wax (Gallard-Schlesinger, Carle Place, NY) according to the method of Kusakabe et al. (1984). Sections were cut on a cryostat at 0°C at a thickness of 4 μm and collected on slides coated with egg albumin. Sections were allowed to dry at 4°C and kept at 4°C until stained.

Histochemical staining of hyaluronan

The sections were rehydrated in a graded series of ethanol and water mixtures and then incubated for 5 minutes in 10% H_2O_2 to block endogenous peroxidase activity, followed by washing in distilled water and PBS-CMF. For staining hyaluronan, the sections were incubated with 10 μg/ml of b-PG in 10% calf serum 90% PBS-CMF at room temperature for 1 hour. The control level of staining was determined by pre-digesting the sections with 50 ng of *Streptomyces* hyaluronidase (Sigma, St Louis, MO) in PBS-CMF for 1 hour at 37°C, prior to the addition of the b-PG. After two washes with PBS-CMF for 5 minutes each, the sections were incubated for 20 minutes at room temperature with horseradish peroxidase-labeled streptavidin (Kirkegaard & Perry, Gaithersburg, MD) diluted 1 to 250 in 10% calf serum, 90% PBS-CMF. After two additional washes in PBS-CMF, the sections were incubated in a peroxidase substrate consisting of 0.03% H_2O_2, 0.2 mg/ml 3-amino-9-ethylcarbazole in 0.05 M sodium acetate, pH 5.0, which gives rise to an intense red reaction product (Graham et al., 1965). After incubating the sections for 10 minutes, the sections were counter stained with Mayer's Hematoxylin (Accustain, Sigma) for 30 seconds, and the chromagens were preserved by coating with Crystal/mount (Biomeda, Foster City, CA). Finally, coverslips were attached by using Permount (Fisher, Columbia, MD).

Preparation of hyaluronan-gelatin

The hyaluronan-gelatin beads were prepared by mixing 20 mg of gelatin (Sigma) with 20 mg sodium hyaluronan (Lifecore, Minneapolis, MN) in 5 ml of 0.2 M NaCl. After stirring for more than 3 hours with heat to dissolve both hyaluronan and gelatin, the pH was adjusted to 4.5-4.7, and 4 mg of 3-ethyl-3-(3-dimethylaminopropyl) carbodiimide was added. The pH was maintained at 4.7 by adding 0.1 M HCl drop wise for 1 hour. The solution was then diluted with varying amount of 0.2 M NaCl and then mixed with equal volumes of 4% gelatin, 4% sucrose (final concentrations: 0, 1 and 2 mg/ml hyaluronan in 2% gelatin). Drops of each solution were placed on parafilm and allowed to solidify at 4°C overnight, before fixing with 3.7% formaldehyde for 2 hours. After several washes with PBS-CMF, the hyaluronan-gelatin beads were embedded in polyester wax and sectioned as described. Sections of this hyaluronan-gelatin were stained with b-PG as described above except that the incubation time with substrate was varied. The results of these experiments indicated that a 2 minute incubation with the substrate gave rise to a linear range of staining, as judged by image analysis (see inset in Fig. 2).

Quantitative analysis of pericellular hyaluronan

The growth plates from 16-day-old Sprague-Dawley rats (Charles River) were dissected from the tibiae and the phalanx. For quantitative analysis, the sections were incubated with the substrate for only two minutes and were not counter-stained. As described above, control experiments using hyaluronan coupled to gelatin revealed that these conditions gave rise to a linear range of staining densities with 1 and 2 mg/ml hyaluronan. The sections were examined by an Image Analysis system (Zeiss IBAS 2000, with a Kontron Aiag Processor interfaced to a Zeiss Axiophot Microscope equipped with a green filter). To make sure that the plane of the section passed through the central region of the lacunae, only those lacunae that contained nuclei were selected for analysis. For these lacunae, both the density of the staining as well as the total area were measured. Briefly, each lacuna was traced along the border of the territorial matrix and then the total area was measured. For measuring the staining density, the area of the lacuna was selected before the density was measured. Thus, the data of hyaluronan density represents the total density of hyaluronan in each section of the lacuna. For a typical lacuna, the standard deviations for the measurements of both area and density were in the order of 2 to 4%.

Culture of the epiphysial growth plate

The proximal ends of tibiae were dissected from 16-day-old rats and placed in a saline solution. After removing connective tissue and articular cartilage, the epiphysial growth plates were separated from the surrounding marrow cavity, bone trabeculae, and perichondrium under a dissecting microscope. The growth plates were cut longitudinally into small segments measuring approximately 1-2 mm wide and less than 0.5 mm thick. The segments were placed on transparent inserts (Millicell-CM, Millipore, Bedford, MA) and cultured in 24-well plates with 0.3 ml of medium (MEM supplemented with 10% FCS, antibiotics, and 50 μg/ml ascorbic acid). The medium was changed every other day and approximately 10 μl of the medium was added directly on top of the segments.

To investigate the role of hyaluronan, adjacent segments of the growth plate culture were cultured in the presence or absence of *Streptomyces* hyaluronidase (Sigma) and testicular hyaluronidase (type VI-S, Sigma). For these experiments, stock solutions of the enzymes were prepared in serum-containing medium and 10 μl of this was added directly on top of the segments (*Streptomyces* hyaluronidase, stock solution 500 units/ml, final concentration 17 units/ml: testicular hyaluronidase, stock solution 300 μg/ml, final concentration 10 μg/ml). To control for the possible effects of contaminating proteases, segments of the growth plate were cultured in medium containing both hyaluronidase and the protease inhibitor ovomucoid (Sigma, stock solution 1 mg/ml, final concentration 33 μg/ml). In addition segments were cultured in the presence of medium containing pronase E (Sigma, stock solution 750 ng/ml, final concentration 25 ng/ml).

After the culture period, the segments of growth plates were fixed with 3.7% formaldehyde for 1 hour, embedded in polyester wax, followed by sectioning and staining for hyaluronan.

Autoradiography

After culturing segments of the growth plate in normal medium for one day, the medium was changed to one containing 10 μCi/ml of [^{3}H]glucosamine (NEN, Boston, MA). Sixteen hours later, the segments were washed in CMF-PBS, and then fixed for 1 hour in 70% ethanol. After decalcification (20% EDTA for 24 hours), these segments were embedded in polyester wax and cut into 5 μm sections. The sections were rehydrated and some were digested with *Streptomyces* hyaluronidase (1 unit/ml in PBS-CMF, 1 hour). Both hyaluronidase digested and non-digested sections were coated with

autoradiographic emulsion (NTB-2, Kodak, Rochester, NY) and exposed for 4 weeks at 4°C. The sections were then developed, according to the protocol from Kodak, counter stained with Toluidine Blue (Clark, 1984), dehydrated, and covered with Permount.

Proteolytic assay

The amount of proteolytic activity present in the hyaluronidase preparation was determined by the technique of Tomarelli et al. (1949). Both *Streptomyces* hyaluronidase (Sigma) and a serial dilution of pronase E (Sigma) were dissolved in culture medium with 10% fetal calf serum to mimic the culture conditions. Aliquots of the enzymes (0.1 ml) were mixed with equal volumes of a solution of azoalbumin (25 mg/ml, Sigma) and incubated at 37°C for 24 hours. A solution of 5% trichloroacetic acid (0.8 ml) was added to the samples which were incubated on ice for 1 minute and centrifuged for 5 minutes. The supernatants were mixed with equal volumes of 0.5 N NaOH and the OD_{440} was determined.

Cell proliferation assay

Segments of the growth plate were cultured, as described above, for two days and then a solution of bromodeoxyuridine (BrdU) was added to the medium (Cell Proliferation Kit, Amersham, Arlington Heights, IL). After 16 hours, the segments were fixed, processed for histology and stained for incorporated BrdU according to the protocol from Amersham. The number of labeled nuclei were counted under the light microscope and compared to the total number of nuclei per optical field (20×).

RESULTS

Distribution of hyaluronan in the tibial growth plate

The distribution of hyaluronan in the growth plate was examined using a histochemical probe derived from cartilage proteoglycan, termed b-PG (Green et al., 1988). This probe binds with high affinity and specificity to hyaluronan, however, it will recognize only hyaluronan that is exposed and not that which is tightly complexed with proteins (Green et al., 1988). Fig. 1A of the rat epiphysial plate shows that hyaluronan staining was present in the hypertrophic zone (labeled h) but not in the resting, proliferating or maturing zones (labeled r, p and m, respectively). The amount of hyaluronan was greatly reduced or absent from the epiphysial growth plate at the zone of erosion. A similar pattern of hyaluronan expression was also found in all other growth plates that we examined (developing vertebra, occipital bones, phalanges). This distribution confirms our earlier histochemical study (Pavasant et al., 1994) and that of Alini et al. (1992) who used direct biochemical analysis to show that the zone of hypertrophy had a higher content of hyaluronan than other zones. At higher magnification (Fig. 1B), the hyaluronan is shown to occupy the region between hypertrophic chondrocytes and their territorial matrix. Histochemical staining with Toluidine Blue revealed that most of the sulfated proteoglycans were associated with the territorial matrix and not the pericellular zone (data not shown). Thus, the pericellular hyaluronan is clearly distinct from that present in the territorial matrix where it is complexed with other proteins of cartilage and thus is unavailable to the staining probe.

It appears, from Fig. 1, that the intensity of hyaluronan staining increases with the size of the lacunae. To test this possibility, the intensity of hyaluronan staining was determined using an image analysis system which gives semi-quantitative results. For this, we established a staining protocol that gave rise to a linear staining with 1 to 2 mg/ml of hyaluronan coupled to 2% gelatin (see inset in Fig. 2). To make sure that the plane of section passed through the central region of the lacunae, only those that contained nuclei were selected for analysis. As shown in Fig. 2 (closed circles), there was a direct linear correlation between the intensity of hyaluronan staining and the total area of the lacunae as determined by image analysis (r^2 = 0.919). A similar type of relationship was obtained with growth plates from the phalanges (Fig. 2, open circles) even though the lacunae tended to be smaller and contained less hyaluronan. However, it is clear that these two groups of data displayed the same type of linear relationship.

These results suggest that as the chondrocytes progress toward the final stages of maturation, they deposit more pericellular hyaluronan than in earlier stages. Based on these observation, we postulate that greater amounts of hyaluronan result in higher osmotic pressures on the surrounding territorial matrix and are responsible for the formation of larger lacunae.

Hypertrophic chondrocytes synthesize hyaluronan

The pericellular hyaluronan detected around hypertrophic

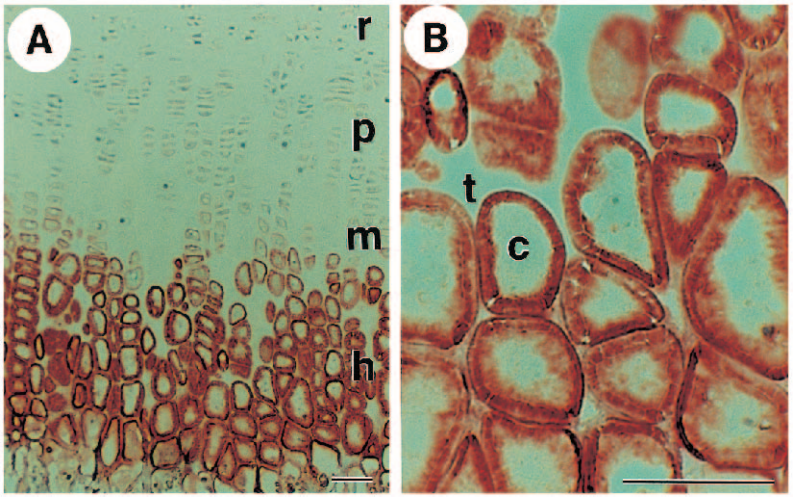

Fig. 1. Distribution of hyaluronan in the rat growth plate. The tibial growth plate from a 16-day-old rat was sectioned and stained for hyaluronan. (A) A low magnification view of the rat growth plate shows that hyaluronan staining (red) is present in the hypertrophic zone (h), but not in the resting (r), proliferating (p), or maturation (m) zones. Control experiments in which the sections were initially incubated with *Streptomyces* hyaluronidase did not show positive staining. (B) A high magnification view shows that hyaluronan is located in the pericellular region between the hypertrophic chondrocytes (c) and the territorial matrix (t). Bars, 50 μm.

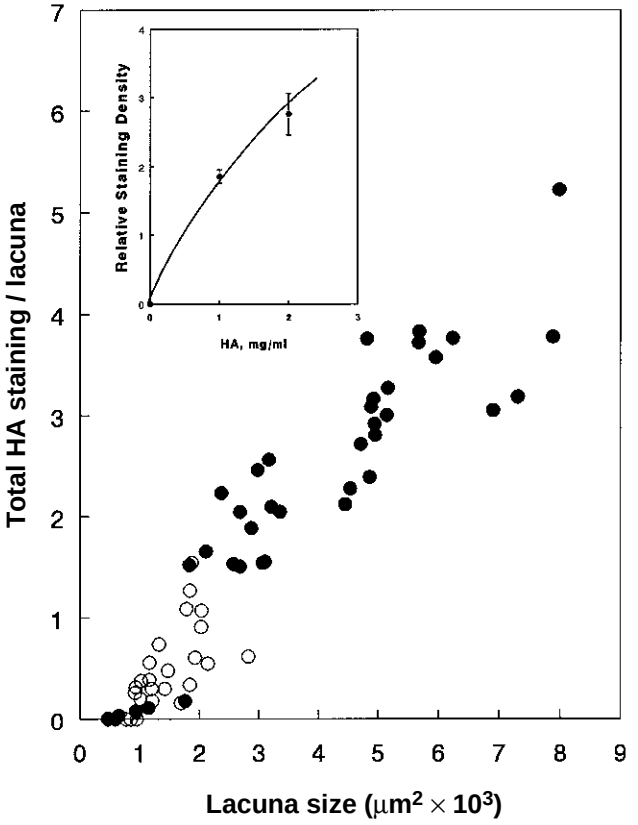

Fig. 2. A scatter plot of hyaluronan staining in each lacuna relative to its total area. Each data point represents a separate lacuna in the hypertrophic zone that was traced and measured. Only lacunae containing chondrocytes with clearly discernible nuclei were selected for analysis (see Materials and Methods). Scatter plot of tibial hypertrophic lacunae (●) revealed a linear correlation between the hyaluronan staining and the area of the lacunae (r^2=0.919). A similar type of linear relationship was observed with the lacunae from the phalanges (○). The inset shows the staining density of sections of gelatin beads containing 0, 1 and 2 mg/ml hyaluronan.

chondrocytes could either be newly synthesized or preexisting hyaluronan that was exposed by cleavage of the aggrecan and collagen in the territorial matrix. To distinguish between these possibilities, we examined the incorporation of [^{3}H]glucosamine by the hypertrophic chondrocytes. Segments of growth plate were cultured for two days and then [^{3}H]glucosamine was added to the medium for 16 hours. The segments were sectioned, incubated in the presence and absence of *Streptomyces* hyaluronidase to digest the hyaluronan, and then coated with an autoradiographic emulsion. A comparison of the dark field images shown in Fig. 3A and B reveals the presence of hyaluronidase-sensitive grains in the pericellular region of hypertrophic lacunae. These results suggests that at least a portion of this pericellular hyaluronan is newly synthesized by the hypertrophic chondrocytes and is consistent with reports that hypertrophic chondrocytes are metabolically active cells with prominent rough endoplasmic reticulum and Golgi complexes (Brighton et al., 1973; Buckwalter et al., 1986).

Digestion of hyaluronan inhibits the enlargement of lacunae

To further investigate the possible role of hyaluronan in the enlargement of the lacunae, we examined the effects of hyaluronidase on organ cultures of the epiphysial plate. For this, growth plates were dissected from the tibiae of 14- to 16-day-old Sprague-Dawley rats, cut into segments approximately 0.5 mm thick, and cultured on the surfaces of polycarbonate membrane inserts. In control cultures shown in Fig. 4A-C, the size of the segments increased over the 5 day culture period as judged by phase contrast microscopy (9% and 18% increases over initial size on days 3 and 5, respectively). However, as shown in Fig. 4D-F, when *Streptomyces* hyaluronidase, which specifically degrades hyaluronan (Ohya and Kaneko, 1970), was added to the medium, the segments did not expand (–4% and –3% decrease compared to the initial size on days 3 and 5, respectively). A similar type of inhibition was obtained with testicular hyaluronidase (data not shown), which has a somewhat wider substrate specificity, in that it can digest both hyaluronan and chondroitin sulfate (Meyer et al., 1960).

Histological analysis of the control segments (Fig. 5A-C)

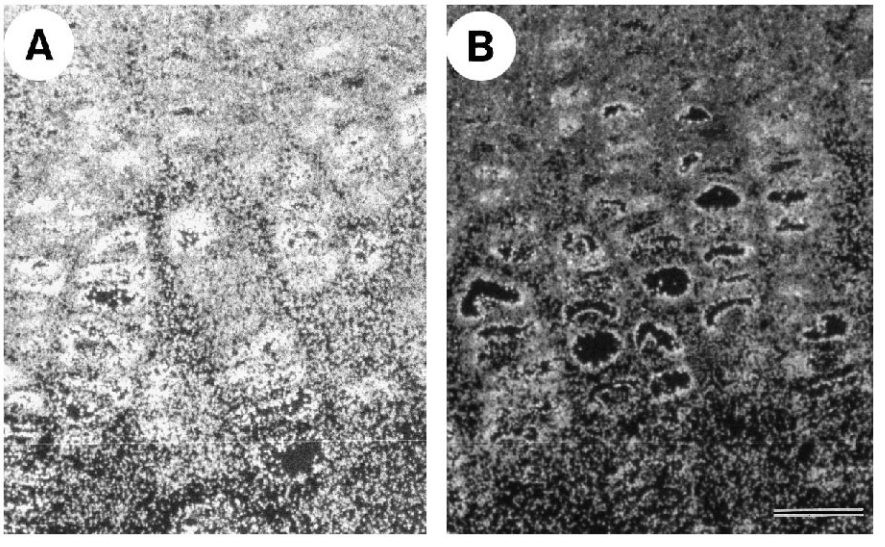

Fig. 3. Incorporation of [^{3}H]glucosamine in cultured growth plate. A segment of the growth plate was cultured with [^{3}H]glucosamine for 16 hours and then analyzed by autoradiography. (A) A dark field view of a section of the growth plate shows the presence of grains in the hypertrophic lacunae. (B) An adjacent section that was incubated with *Streptomyces* hyaluronidase shows a reduction of the number of grains present in the hypertrophic lacunae. This suggests that newly synthesized hyaluronan is present in the lacunae. Bar, 50 μm.

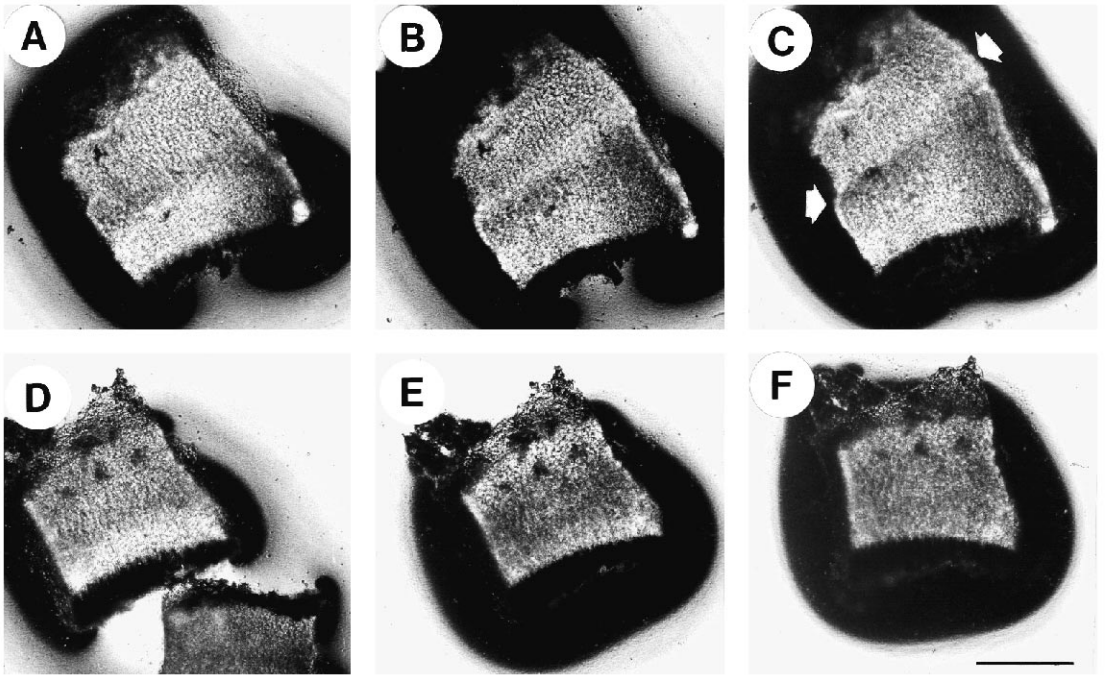

Fig. 4. Phase contrast views of growth plate segments cultured in the absence and presence of hyaluronidase. (A,B,C) A segment of the growth plate which was cultured in control medium for 0, 3 and 5 days, respectively, increased in size during this period. The arrows in C indicate regions where the growth is most apparent. The areas of the segment were 0.817, 0.894 and 0.968 mm^2 on days 0, 3 and 5, respectively. These figures underestimate the total increase in volume since the segments also grew in the perpendicular direction. (D,E,F) An adjacent segment which was cultured in medium containing *Streptomyces* hyaluronidase (10-15 units/ml) for 0, 3 and 5 days, respectively, did not increase in size. The areas of the segment were 0.506, 0.484 and 0.490 mm^2 on days 0, 3 and 5, respectively. Bar, 500 μm.

showed that during the 5 day culture period, a band of hyaluronan staining progressed from the zone of hypertrophy towards the zone of proliferation. At the same time, the lacunae in these regions underwent expansion, as determined by image analysis (Fig. 6A-C). The size of the lacunae in the lower part of the zone of proliferation (region 1 in Fig. 5D) began to enlarge after 3 days in culture, while the upper part of the zone of proliferation (region 2 in Fig. 5D) enlarged after 5 days in culture. These results suggest that the chondrocytes in the control segments were undergoing a maturation process similar to that which occurred in vivo, except that the hypertrophic zone was not replaced by bone so that the tissue, as a whole, expanded.

It is interesting to note that during the culture period, hyaluronan was lost from the older hypertrophic regions of the segments (see Fig. 5B and C). The absence of hyaluronan in these regions was confirmed by staining with Toluidine Blue (data not shown) which also demonstrated that it was not being masked by proteins. Presumably, the hyaluronan from the older hypertrophic lacunae was released into the culture medium. These results also suggest that once the lacunae had expanded, the continued presence of hyaluronan was not required to maintain their size.

In contrast to the above, when the segments were cultured in the presence of *Streptomyces* hyaluronidase for 3 to 5 days, no free hyaluronan was detected (Fig. 5E-F) nor was any expansion of the lacunae apparent (Fig. 6D-F). Histochemical staining of the sections with Toluidine Blue revealed that the hyaluronidase also caused the release of proteoglycans from the territorial matrix (data not shown). While it is possible that the removal of the hyaluronan and proteoglycan from the territorial matrix could alter its properties so that it could not undergo expansion, the more likely possibility is that pericellular hyaluronan is required for the enlargement of the lacunae.

To determine if the effect of hyaluronidase was due to the proteases that contaminate the preparation, two sets of control experiments were carried out. In the first set of experiments, the effects of the protease inhibitor, ovomucoid, were examined (Caputo et al., 1980). In the presence of ovomucoid, no protease activity was detectable in the preparation of *Streptomyces* hyaluronidase (see Materials and Methods). When ovomucoid was added to the culture system, it had no obvious effect on the hyaluronidase-induced inhibition of lacunal enlargement (data not shown). Similarly, the synthetic matrix metalloprotease inhibitor (SC44463) did not inhibit the effect of hyaluronidase (data not shown). In the second set of control experiments, segments of growth plate were cultured in the presence of pronase E, which had approximately 25-fold more protease activity than that present in the preparation of *Streptomyces* hyaluronidase. Again, the addition of excessive amounts of proteolytic activity had no detectable effect on the normal expansion of the lacunae in the cultured segments of growth plate. These experiments suggest that proteases present in the hyaluronidase preparation were not responsible for the inhibition of lacunal expansion.

Hyaluronidase treatment does not effect proliferation of growth plate chondrocytes

To examine the possibility that hyaluronidase has a toxic effect that inhibits the enlargement of lacunae, segments of the growth plate were labeled with 5-bromo-2′-deoxyuridine. Both control and hyaluronidase treated growth plates were cultured for 2 days. At the end of this period, 5-bromo-2′-deoxyuridine was added into the medium and the cultures were incubated for an additional 16 hours. In both cases, the proportion of chondrocytes incorporating 5-bromo-2′-deoxyuridine in the resting and proliferating zones were approximately the same,

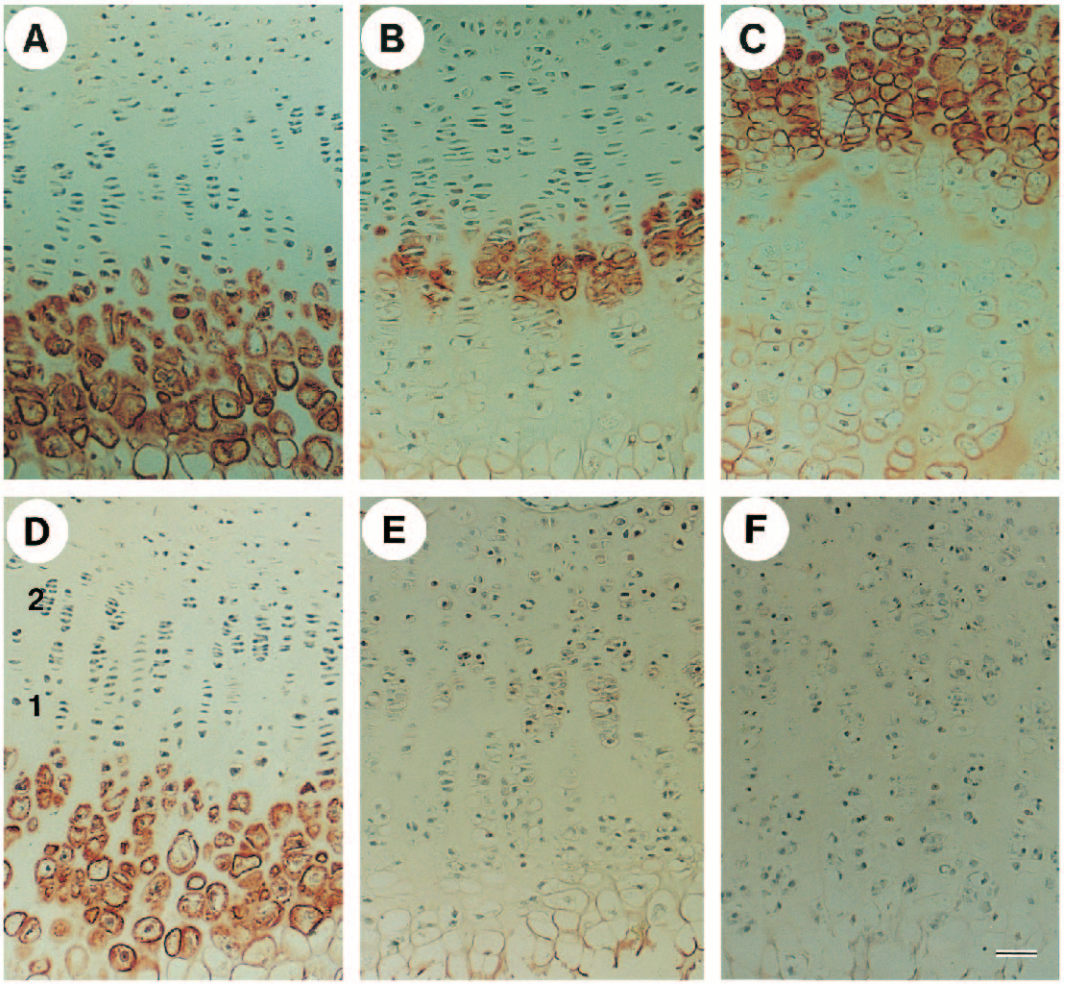

Fig. 5. Distribution of hyaluronan in segments of the growth plate cultured under control conditions and in the presence of hyaluronidase. (A,B,C) Untreated (control) segments of the growth plate which had been cultured for 0, 3 and 5 days, respectively, show that the expression of hyaluronan (red) progressed from the original zone of hypertrophy towards the zone of proliferation. This progression of hyaluronan staining corresponded to an increase in the size of the lacunae in these regions. (D,E,F) Segments of the growth plate cultured in the presence of *Streptomyces* hyaluronidase for 0, 3 and 5 days, respectively, show the absence of hyaluronan (E and F) and the lack of lacunal expansion. The regions labeled 1 and 2 in D represent the lower and upper portions of the zone of proliferation, respectively. Bar, 50 μm.

being 12±4% for the control and 11±3% for the hyaluronidase treated segments. Clearly, the hyaluronidase did not adversely effect the viability of the chondrocytes.

The most likely explanation for these results is that the accumulation of hyaluronan around the hypertrophic chondrocytes caused the expansion of the lacunae and the degradation of this hyaluronan by hyaluronidase prevented this expansion.

DISCUSSION

We propose that the synthesis of hyaluronan contributes to the expansion of the lacunae in the growth plate. More specifically, as the epiphysial chondrocytes mature, they secrete free hyaluronan into the pericellular space and this hyaluronan exerts a hydrostatic pressure on the surrounding territorial matrix, causing the lacunae to expand. In this fashion the synthesis of hyaluronan is partially responsible for the interstitial growth of the epiphysial plate which, in turn, determines the rate of bone elongation.

This hypothesis is supported by several lines of evidence. First, histochemical staining has revealed that free hyaluronan in the growth plate is restricted to the pericellular region of hypertrophic lacunae. Secondly, image analysis has indicated that the amount of this hyaluronan is directly proportional to the size of the lacunae. Thirdly, autoradiography of growth plates cultured with [^{3}H]glucosamine revealed that at least a portion of this pericellular hyaluronan is newly synthesized by the hypertrophic chondrocytes. And finally, hyaluronidase treatment of organ cultures of the growth plate blocked both the accumulation of pericellular hyaluronan and the expansion of the lacunae. Taken together, these results suggest that hyaluronan is required for the enlargement of the hypertrophic lacunae.

The hyaluronan-mediated expansion occurs in a number of other tissues as well (Toole, 1981; Toole et al., 1984). Perhaps the best characterized example of this occurs during the embryonic development of the cornea in which the production of hyaluronan causes the stroma to swell immediately prior to the migration of mesenchymal cells (Toole and Trelstad, 1971). Another example of this occurs during ovulation when the production of hyaluronan results in an explosive increase in the volume of the cumulus cells surrounding the oocyte (Salustri et al., 1992). Similarly, the swelling pressure of hyaluronan has also been implicated in the closure of the neural tube (Schoenwolf and Fisher, 1983) and the formation of the palate (Wilk et al., 1978). It is probable that the production of hyaluronan represents a generalized mechanism for morphogenic changes.

It should be noted that when growth plates are fixed in the

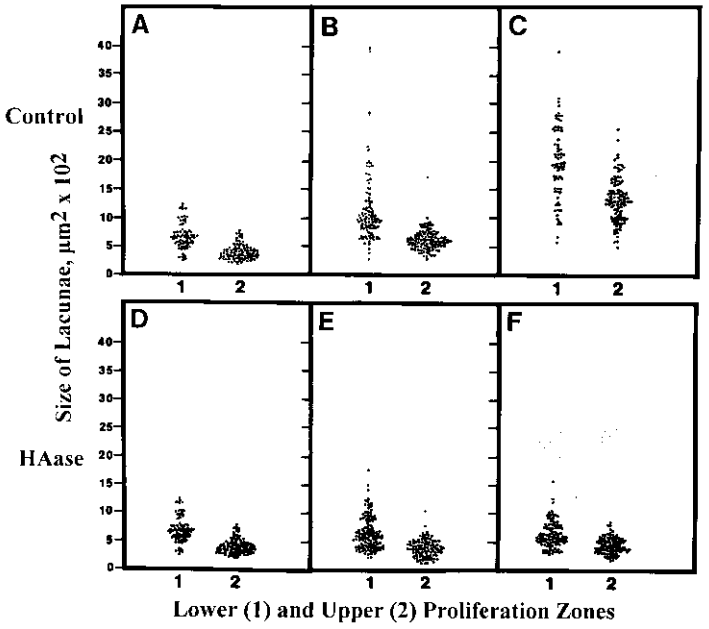

Fig. 6. Scatter graph showing the size of lacunae in segments of the growth plate cultured under different conditions. Histological sections of cultured growth plates were stained with Toluidine Blue and the lower and upper portions of the zone of proliferation (regions 1 and 2, shown in Fig. 5D) were located by their distance from the original hypertrophic zone. (A,B,C) The area of lacunae in regions 1 and 2 of control growth plate segments are shown for days 0, 3 and 5, respectively. The lacunae in the lower part of the proliferation zone (region 1) begin to increase in size more rapidly than those in the upper part (region 2). (Mean areas: region 1, 653, 1194, 1,960; region 2, 380, 610, 1,319 μm^2 on days 0, 3 and 5, respectively) (D,E,F) The area of lacunae in segments of the growth plate cultured in the presence of *Streptomyces* hyaluronidase are shown for days 0, 3 and 5, respectively. The values did not increase significantly over this period. Means areas: region 1, 653, 652, 631; region 2, 380, 390, 466 μm^2 on days 0, 3 and 5, respectively.

presence of ruthenium hexamine trichloride, the hypertrophic chondrocytes fill most of the lacunae and very little pericellular space is apparent. However, ruthenium is a strong cation and can form an ionic complex with hyaluronan, causing it to collapse (Underhill, 1982). This, in turn, could induce the pericellular hyaluronan to shrink and pull the chondrocytes to the edge of the lacunae so that they are stretched beyond their normal boundary. Such a situation could account for the large voids found in the cytoplasm of these cells following treatment with ruthenium salts (Hunziker et al., 1983; Nuehring et al., 1991). However, when the growth plate is fixed in the absence of ruthenium, the hypertrophic chondrocytes are smaller, the cytoplasmic voids are absent and large pericellular spaces between the membranes of the chondrocytes and the walls of the lacunae are apparent (Hunziker et al., 1983; Nuehring et al., 1991). We believe that this is more representative of the actual state of the hypertrophic lacunae and that the pericellular spaces are filled with hyaluronan.

In addition to hyaluronan production and the growth of the chondrocytes themselves, other factors are clearly involved in the expansion of the epiphysial growth plate. For example, the rate of chondrocyte proliferation is a major factor involved in determining the rate of bone elongation. In addition, hypertrophic chondrocytes have been shown to secrete a variety of proteases and collagenases which are probably important in the remodeling of the surrounding territorial matrix, so that expansion can occur (Alini et al., 1992; Brown, 1989; Hunziker et al., 1987; Mikuni-Takagaki and Cheng, 1987).

The hyaluronan produced by the hypertrophic chondrocytes may also play a role in determining the fate of these cells. We speculate that the swelling pressure created by the hyaluronan causes the rupture of the lateral walls of the lacunae in the zone of erosion. If this process occurs suddenly, then the plasma membrane of the chondrocyte could be damaged and result in cell death. The hyaluronan remaining in the lacunae would then be removed by osteoprogenitor cells that migrate into the zone of erosion. Previous studies indicate that the osteoprogenitor cells first bind and internalize the hyaluronan through a CD44-dependent mechanism and subsequently degrade it through the action of lysosomal enzymes (Pavasant et al., 1994).

The role of hyaluronan in the expansion of the epiphysial growth plate has a number of implications. First, the amount of hyaluronan synthesized may determine the eventual size of the hypertrophic lacunae which, in turn, is a major factor in controlling the rate of bone growth (Breur et al., 1991). Thus, the rate of hyaluronan synthesis may ultimately influence both an individual's stature and proportions. Secondly, the production of hyaluronan by the hypertrophic chondrocytes must be under tight control to assure that the expansion of the growth plates is appropriate for the different regions of the body. Along these lines, in preliminary studies, we have found that the production of hyaluronan is influenced by factors which control the rate of bone elongation, such as growth hormone and TGF-β (Liberman et al., 1992; Suzuki, 1992). And finally, a failure of the chondrocytes to produce the appropriate amount of hyaluronan could result in abnormalities in the expansion of the hypertrophic lacunae, such as those that occur in achondroplasia and metatropic dwarfism (Boden et al., 1987; Maynard et al., 1981; Sannasgala and Johnson, 1990). Clearly, the factors that control the production of hyaluronan by the hypertrophic chondrocytes deserve further consideration.

The authors thank Dr Martine Culty for her help in carrying out this study and preparing this manuscript. This investigation was supported by United States Health Services Grant HD 26758 to Charles Underhill.

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(Received 20 July 1995 - Accepted 30 October 1995)