Distribution of hyaluronan in the epiphysial growth plate: turnover by CD44-expressing osteoprogenitor cells

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SUMMARY
In the present study, we have examined the distribution of both hyaluronan and its receptor, CD44, during the process of endochondral ossification in the mouse tibia. Histochemical staining revealed that a large amount of hyaluronan was present in the lacunae located in the zone of hypertrophy, but it was greatly reduced or absent from the zone of erosion. In addition, hyaluronan was present in the cytoplasm of osteoprogenitor cells located in the zone of erosion. These cells also expressed CD44 on their surfaces, as revealed by double-label immunohistochemistry. These results suggested that the osteoprogenitor cells may use CD44 to bind and internalize hyaluronan, and subsequently degrade it with lysosomal enzymes. To test this possibility, we examined the human cell line, MG-63, which closely resembles osteoprogenitor cells. These cells produced several different forms of CD44, as determined by western blotting (85, 116 and 150 kDa). In addition, the binding of isotopically labeled hyaluronan to detergent extracts of these cells was blocked by a monoclonal antibody to CD44. Similarly, the degradation of hyaluronan by these cultured cells was also inhibited by a monoclonal antibody to CD44. To determine if these cells could remove hyaluronan from the growth plate, the cells were cultured directly on top of thin sections of the epiphysial region of long bone. After 16 hours, the sections were stained for hyaluronan. The MG-63 cells removed significant amounts of hyaluronan present in the zone of hypertrophy, and this effect was blocked by an excess of soluble hyaluronan and by a monoclonal antibody to CD44. Taken together, these results suggest that, under physiological conditions, osteoprogenitor cells in the zone of erosion can use a CD44-dependent mechanism to remove hyaluronan.

Key words: hyaluronan, CD44, chondrocyte, osteoblast

INTRODUCTION
During the process of endochondral ossification, a number of changes occur in the epiphysial growth plate (for review see Poole and Rosenberg, 1987). For example, chondrocytes increase their volume as they progress through the various layers of the growth plate, reaching up to 10 times their original size in the zone of hypertrophy (Buckwalter et al., 1986; Dean et al., 1989; Ballock et al., 1993). Alterations also occur in the matrix components of the growth plate. In the zone of hypertrophy, the chondrocytes secrete enzymes that degrade type II collagen, a major component of the cartilage (Brown et al., 1989; Dean et al., 1989; Alini et al., 1992; Ballock et al., 1993). Simultaneously, the amount and size of aggregated proteoglycan also decreases (Buckwalter et al., 1987; Poole et al., 1989; Matsui et al., 1991). These changes contribute to the enlargement of the lacunae and to a reorganization of the matrix components, which allows mineralization (Poole et al., 1989). In the zone of erosion, osteogenic cells migrate into the lacunae, attach to the surface of calcified cartilage and deposit bone matrix to form woven bone (for reviews see Reddi, 1981; Kahn et al., 1983).

Hyaluronan is another component of the extracellular matrix that undergoes changes in the cartilage growth plate. This was initially demonstrated by studies in which specific regions of the growth plate were isolated by micro-dissection and then analyzed biochemically (Matsui et al., 1991; Alini et al., 1992). The results of such studies have shown that the zone of hypertrophy contains a greater amount of hyaluronan per unit volume than either the zones of proliferation or maturation (Alini et al., 1992). However, the exact location of this hyaluronan and its turnover mechanism have not been addressed.

Previous studies have implicated CD44 in the turnover of hyaluronan (Culty et al., 1992; Underhill et al., 1993; Hua et al., 1993). CD44 defines a family of cell surface glycoproteins, some of which have the capacity to bind hyaluronan and are consequently termed the hyaluronan receptor (for review see Underhill, 1992). The extracellular domain of this cell surface protein is similar in structure to that of the link protein of cartilage and specifically binds to a six-sugar fragment of hyaluronan (Goldstein et al., 1989; Stamenkovic et al., 1989; Underhill, 1992). However, CD44 comes in a variety of different forms, which differ in their ability to bind hyaluronan (Stamenkovic et al., 1991; Lesley et al., 1992; Culty et al., 1994). In vitro studies of pulmonary macrophages and other
cells have shown that CD44 mediates the internalization of hyaluronan so that it can be eventually degraded by lysosomal enzymes (Culty et al., 1992). This process can be blocked by either antibodies to CD44 or by agents that block the action of acid hydrolases. Thus, CD44 appears to play a critical role in the degredation of hyaluronan.

In the present study, we have further examined the distribution of both hyaluronan and its receptor, CD44, during the process of endochondral ossification in the mouse tibia. The results of this study show that large amounts of hyaluronan surround the chondrocytes in the zone of hypertrophy. Furthermore, our results suggest that this hyaluronan is removed in the zone of erosion by osteoprogenitor cells that express CD44 on their surfaces. The dynamic nature of the synthesis and removal of hyaluronan from the growth plate may be an important aspect of bone development.

**MATERIALS AND METHODS**

**Preparation of biochemicals**

The proteoglycan probe (b-PG) used for the localization of hyaluronan was prepared by a previously described protocol (Green et al., 1988). This protocol consists of extracting bovine nasal cartilage with 4 M guanidinium HCl, 0.5 M sodium 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 consists 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).

KM-201 is a rat monoclonal antibody against the mouse CD44 that blocks its interaction with hyaluronan (Miyake et al., 1990; Culty et al., 1990). This antibody was purified from ascites fluid using a DEAE Affi-gel column (Bio-Rad, Richmond, CA) as described previously (Culty et al., 1992). In some cases, this antibody was coupled to biotin (b-KM-201-1) using sulfo-S-S Na-carboxymethyl} hexanoate (Pierce, Rockford, IL) as described by Updyke and Nicolson (1986).

K-3 is a mouse monoclonal antibody that recognizes hamster CD44, and inhibits the binding of hyaluronan to CD44 from several different species (Green et al., 1988; Culty et al., 1990). This antibody was purified from ascites fluid by chromatography on a Protein A-Sepharose column. BU-52 is a mouse monoclonal antibody against human CD44 and was purchased from the Binding Site (San Diego, CA). Control preparations of rat and mouse IgG were obtained from the Binding Site (San Diego, CA). Control preparations of rat and mouse IgG were obtained from the Binding Site (San Diego, CA).

**Immunocytochemistry**

The tibiae of 19-day-old mice (Charles River, Wilmington, MA) were dissected out 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 albumen. Sections were allowed to dry and kept at 4°C until staining. For immunohistochemistry, the sections were rehydrated in a graded series of ethanol and water mixtures and then incubated for 5 minutes in 10% H2O2 to block endogenous peroxidase activity, followed by washing in distilled water and PBS-CMF. For hyaluronan staining, 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) in PBS-CMF for 1 hour at 37°C prior to the addition of the b-PG. For CD44 staining, the sections were incubated with 10 µg/ml of b-KM-201 in 90% PBS-CMF, 10% calf serum for 90 minutes at 37°C. The control level of staining was determined by substituting the antibody with an equivalent amount of biotinylated rat-IgG. 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 90% PBS-CMF, 10% calf serum. After two additional washes in PBS-CMF, the sections were incubated in a peroxidase substrate consisting of 0.03% H2O2, 0.2 mg/ml 3-amin-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 (10 minutes for hyaluronan or 40 minutes for CD44), 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).

In some experiments, the sections were stained for both hyaluronan and CD44. In this case, the sections were first stained for CD44 using b-KM-201 in conjunction with peroxidase and 3-amin-9-ethylcarbazole to give a red reaction product. This was followed by an incubation with b-PG, which was detected by the sequential application of alkaline phosphatase-labeled streptavidin (Kirkegaard & Perry), and a substrate consisting of Fast Blue (Zymed, So. San Francisco, CA) that gives a blue reaction product. The sections were preserved with Crystal/Mount as described above.

**Cell culture conditions**

The MG-63 cell line (ATCC, Rockville, MD) was originally derived from osteosarcoma of the femur of a 14-year-old male by Heremans et al. (1978). This cell line was grown in 90% MEM, 10% fetal calf serum supplemented with penicillin-streptomycin. Cells were routinely passaged every 5 days in 100 mm culture plates.

**Western blotting**

MG-63 cells were released from the culture plates by flushing with a Pasteur pipette following a 10 minute incubation with 0.02% EDTA in PBS-CMF. The resulting cell suspension was centrifuged and the pellet was extracted in Laemmli sample buffer without β-mercaptoethanol. Samples were subjected to electrophoresis on a 7.5% polyacrylamide gel along with prestained high molecular mass standards (Bio-Rad). The proteins were transferred to a sheet of Immobilon-NC (Millipore, Bedford, MA) using a Trans-blot cell (Idea, Carvalis, OR) at 0.9 amp for 30 minutes. The nitrocellulose sheet was incubated in 5% nonfat milk for 1 hour and then stained for CD44 using BU-52 monoclonal antibody that was diluted 1 to 5,000 with 90% PBS-CMF, 10% calf serum, 0.05% Tween-20. Following a one hour incubation with BU-52, the nitrocellulose sheet was washed and incubated for 30 minutes in a solution of peroxidase-labeled anti-mouse IgG (Kirkegaard and Perry), which was diluted 1 to 500 in the same buffer. Finally, the sheet was incubated in a substrate for peroxidase consisting of H2O2 and 3-amin-9-ethyl carbazole.

**Hyaluronan binding assay**

MG-63 cells were released from the culture plates with 0.02% EDTA in PBS-CMF as described above. After centrifugation, the cell pellet was extracted with 0.1% sodium deoxycholate, 0.5 M NaCl, 0.02 M Tris, pH 8.0 (DOC buffer) and homogenized with a Polytron. Samples of the extract were incubated for 15 minutes in the presence or...
Hyaluronan in the growth plate

abundance of antibodies (100 µg/ml). Next, 1 µg of [3H]hyaluronan was added to each sample in a final volume of 250 µl. After 20 minutes of shaking at room temperature, an equal volume of saturated (NH4)2SO4 was added along with a small amount of 5% nonfat milk as a carrier. Following centrifugation (9,000 g, 5 minutes), the pellets were washed with 50% saturated (NH4)2SO4, resuspended in water and processed for scintillation counting. The background level of binding was determined by including a large excess of nonlabeled hyaluronan (100 µg) in some samples. The results are expressed in terms of specific binding (after subtraction of background) per µg of protein present in the cell layer, which was determined by the BCA method (Pierce).

Hyaluronan degradation assay

MG-63 cells were subcultured into a 12-multiwell plate (106 cells/well) and allowed to grow overnight. The next day, the medium was changed to 95% MEM, 5% fetal calf serum. Cells were cultured in the presence and absence of either K-3 antibody or mouse IgG (100 µg/ml) for 15 minutes prior to the addition of [3H]hyaluronan (2 µg/ml). After 40 hours, the medium was collected, digested with protease and the hyaluronan degradation was determined by centrifugation of the medium in Centricon 30 Microconcentrators (Amicon, Beverly, MA) as described previously (Culty et al., 1992). The fragments of hyaluronan that passed through the filter were collected and processed for scintillation counting. The background level of degradation was determined by incubating [3H]hyaluronan in the absence of cells under the same conditions. The BCA method (Pierce) was used to determine the amount of protein in the cell layer following extraction with DOC buffer.

Culture of MG-63 cells on the growth plate

MG-63 cells were cultured directly on sections of the tibia growth plate. For this experiment, polyester wax sections were dewaxed thorough a graded series of ethanol and water solutions, and incubated in CMF-PBS for 10 minutes. After the sections were dried, circles were drawn around each section with a PAP PEN (the Binding Site) to make a hydrophobic barrier for the culture medium. The MG-63 cells were trypsinized, centrifuged and resuspended in medium (90% MEM, 10% fetal calf serum) at a concentration of approximately 2×106 cells/ml. The cell suspension was gently added to the sections and the slides were placed inside a 100 mm culture plate. At 4 hours after incubation (37°C in a 5% CO2 atmosphere), and 20 minutes prior to the addition of [3H]hyaluronan (1000 for 10 minutes to release the MG-63 cells and then stained for hyaluronan using the b-PG probe. In some experiments, exogenous hyaluronan (1 mg/ml) or the K-3 monoclonal antibody (50 µl of ascites fluid/ml) was mixed with the cell suspension prior to addition to the sections.

RESULTS

Distribution of hyaluronan and CD44 in the tibial growth plate

In initial experiments, we examined the distribution of hyaluronan in the growth plates of tibiae from young mice. Following fixation and decalcification, the epiphyseal regions of the tibiae were sectioned and stained with the b-PG probe. This probe is derived from the core and link protein regions of cartilage and binds specifically to hyaluronan. However, it is important to note that this probe may not have access to hyaluronan that is already complexed with proteins or other components. Thus, the b-PG probe will only bind to exposed hyaluronan. Fig. 1A shows a section of the growth plate that has been stained with b-PG, while Fig. 1B shows the background level of staining obtained by predigesting the section with Streptomyces hyaluronidase. As shown in Fig. 1A, positive staining was present in the hypertrophic zone (labeled h) and in the lower part of the zone of maturation (labeled m), but was absent from the zone of proliferation (labeled p). Hyaluronan was greatly reduced on the surface of calcified cartilage in the erosion zone (labeled e) and on the surface of calcified matrix in the ossification zone (labeled o). A similar pattern of hyaluronan expression was also found in the tibiae of younger mice and in the developing vertebrae and occipital bones (data not shown).

A higher magnification view of the growth plate (Fig. 1C) shows that most of the exposed hyaluronan was present in the territorial matrix of hypertrophied chondrocytes. In addition, cells were present in the zone of erosion that have hyaluronan on their surfaces and in their cytoplasm (see Fig. 1D). These cells had a spindle-shaped or round morphology, adhered to the surface of the cartilage matrix, and were closely associated with the walls of invading blood vessels. On the basis of their morphology and location, we believe that these are osteoprogenitor cells, which will eventually differentiate into osteoblasts.

We then examined the distribution of CD44, the hyaluronan receptor, in the growth plate. Following staining with a monoclonal antibody against mouse CD44 (b-KM-201), positive staining was apparent on the surfaces of cells in the zones of erosion and ossification (see Fig. 2A). The background level of staining, obtained with a non-specific antibody is shown in Fig. 2B. As shown in Fig. 2A, most of the positively stained cells were located next to the erosion front. In addition, some osteocytes also showed positive staining (data not show), consistent with the work of Hughes et al. (1994). Smaller amounts of CD44 were also associated with some, but not all, osteoblasts in the ossification zone (labeled o). These osteoblasts expressed lower levels of CD44 than the cells in the zone of erosion, as judged by the intensity of staining. In addition, within the woven bone, spindle-shaped osteogenic cells stained more intensely for CD44 than those that were cuboidal (data not shown). This may reflect the fact that the expression of CD44 is altered as a function of osteoblast maturation (Hughes et al., 1994).

To determine if the CD44 positive cells in the zone of erosion also contain hyaluronan in their cytoplasm, the sections were examined using a double-labeling technique in which hyaluronan was stained in blue and CD44 was stained in red (Fig. 2C,D). As shown in Fig. 2D, cells with a spindle-shaped morphology closely opposed to the cartilage septum stained positively for both CD44 and hyaluronan. It appears that the disappearance of hyaluronan from the cartilage in the zone of erosion corresponds to the presence of osteoprogenitor cells that express CD44 and contain hyaluronan in their cytoplasm (see Figs 1D, 2D). These results suggest that osteoprogenitor cells could potentially use a CD44-dependent mechanism to internalize and degrade the hyaluronan.

Expression of CD44 by MG-63 cells

In view of the preceding results, it would be useful to characterize further the osteoprogenitor cells present in the zone of erosion. Unfortunately, these cells are difficult to obtain from this region. For this reason, we examined the MG-63 cell line, which was originally derived from a human osteosarcoma and is considered to be representative of mesenchymal osteoprog-
enitor cells. Indeed, the MG-63 cells possess many of the characteristics of osteoprogenitor cells, in that under appropriate conditions they will undergo morphological changes, synthesize type I collagen and alkaline phosphatase, and secrete osteocalcin and osteonectin (Franceschi et al., 1985, 1988; Mahonen et al., 1990, 1991; Pirskanen et al., 1991; Andrianarivo et al., 1992).

Western blot analysis of the MG-63 cells revealed that they expressed several different isoforms of CD44, a major isoform of approximately 85 kDa and two minor isoforms of approximately 116 and 150 kDa (see Fig. 3). Furthermore, the CD44 expressed by the cells has the capacity to bind hyaluronan, in contrast to other isoforms of CD44 that lack this property (Culty et al., 1994). Indeed, binding assays with detergent extracts of these cells showed that they could bind significant amounts of [3H]hyaluronan (see Fig. 4). The specificity of the binding of hyaluronan to CD44 was confirmed by the fact that it was partially blocked by the K-3 monoclonal antibody, which is directed against hamster CD44, but will also block binding of hyaluronan to human CD44 (Culty et al., 1990).

Fig. 1. The distribution of exposed hyaluronan in the growth plate of a long bone. The tibia was dissected from a 19-day-old mouse, fixed in formalin, decalcified, embedded in polyester wax and sectioned on a cryostat. The sections were stained for hyaluronan using the b-PG probe (red) and counter-stained with hematoxylin (blue). (A) A low magnification view of the growth plate shows the different zones: r, resting; p, proliferating; m, maturing; h, hypertrophic; e, erosion; and o, ossification. Clearly, most of the exposed hyaluronan is present in the hypertrophic zone. (B) An equivalent section was pretreated with Streptomyces hyaluronidase (1 hour, 37°C) to show the background level of staining. (C) A higher magnification view of the zone of hypertrophy shows that most of the hyaluronan is associated with an amorphous material in the lacunae immediately surrounding the chondrocytes. Most of this hyaluronan is lost in the zone of erosion. (D) A high magnification view of the erosion zone shows the presence of hyaluronan on the surface and in the cytoplasm of osteoprogenitor cells, which are indicated by the arrows. These spindle-shaped cells are adjacent to an invading blood vessel, the lumen of which is indicated by the stars. (Note: the endothelial cells are not clearly visible in this photograph.) Bars: 50 µm in B and C (A is the same magnification as B); 10 µm in D.
The MG-63 cells were then examined for their ability to degrade hyaluronan. For this, the cells were cultured in the presence of [³H]hyaluronan and the resulting fragments of hyaluronan were collected by passage through Centricon 30 Microconcentrators and quantified as described in Materials and Methods. As shown in Fig. 5, the MG-63 cells were able to degrade significant amounts of hyaluronan and this degradation was partially blocked by the K-3 monoclonal antibody. The fact that the K-3 monoclonal antibody was somewhat more efficient in the binding assay (80% inhibition) than in the degradation assay (45% inhibition) may reflect the differences in the conditions of the two assays. The binding assay was carried out at a single time point on detergent extracts of the cells in which the antibody has ready access to all antigenic sites. In contrast, in the degradation assay, intact cells were incubated with the antibody and labeled hyaluronan for a period of 40 hours. During this period, the antibody may not have access to all of the antigenic sites on the intact cells. Despite these differences, the results indicate that CD44 is at least partially involved in the process of hyaluronan degradation.

Fig. 2. Expression of CD44 in the growth plate of long bone. (A) A low magnification view of the growth plate stained for CD44 with b-KM-201 (red) followed by counter-staining with hematoxylin (blue). CD44 is present on the surfaces of cells in the zones of erosion and ossification, but not in other regions of the growth plate. The staining was most intense on osteogenic cells next to hypertrophic cartilage. (B) An equivalent section was processed with a biotinylated rat IgG to show the control level of staining. (C) A low magnification view of the growth plate stained for both CD44 (red) and for hyaluronan (blue) is shown. (D) A higher magnification view of the zone of erosion from a section doubly stained for CD44 and hyaluronan, shows an osteoprogenitor cell (indicated by the arrow) that express CD44 and is closely associated with hyaluronan. (Note; the nuclei are not visible in these doubly stained sections.) The lumen of an invading blood vessel is indicated by a star. Bars: 50 μm in B (A, B and C are the same magnification); 10 μm in D.
Release of hyaluronan from the hypertrophic zone by MG-63 cells

In a final series of experiments, we investigated the action of MG-63 on the hyaluronan of the hypertrophic zone. MG-63 cells were cultured on decalcified sections of mouse long bone for 16 hours, and then released from the section by treatment with distilled water, followed by Triton X-100. The sections were then stained for hyaluronan, using b-PG. As shown in Fig. 6B, the amount of hyaluronan in the zone of hypertrophy was significantly reduced by the presence of the MG-63 cells in comparison to control sections (Fig. 6A). One possible explanation for these results is that the MG-63 cells secrete proteases that degrade the proteins that hold the hyaluronan in place. The degradation of these proteins would then cause the release of the hyaluronan into the medium. To test this possibility, exogenous hyaluronan was added to the medium of one of the cultures. As shown in Fig. 6C, this added hyaluronan prevented the MG-63-induced loss of hyaluronan from the hypertrophic lacunae. This result suggests that the loss of hyaluronan was not due to the action of secreted proteases, since this phenomenon should not be blocked by the addition of exogenous hyaluronan.

A more likely explanation is that the MG-63 cells use a CD44-dependent mechanism to remove the hyaluronan, in which they initially bind the hyaluronan of the hypertrophic zone and subsequently internalize it for degradation by lysosomal enzymes. This possibility is supported by the fact that addition of the K-3 antibody also blocked the ability of these cells to remove hyaluronan from the section (Fig. 6D). Taken together, these results suggest that, under normal conditions, osteoprogenitor cells can remove hyaluronan in the zone of erosion.

DISCUSSION

The results of the present study indicate that hyaluronan changes in a dynamic fashion as cells progress through the different zones of the growth plate. Our histochemical analysis has revealed the presence of large amounts of hyaluronan sur-
ronding hypertrophic chondrocytes. These results are in keeping with the work of Alini et al. (1992), who analyzed sequential sections of the bovine growth plate using microchemical techniques. The results of their analysis revealed that the amount of hyaluronan, relative to hydroxyproline, was higher in the hypertrophic zone than in either the maturing or the proliferating zones. Presumably, as the chondrocytes mature, they begin to produce a larger pericellular coat of hyaluronan. Indeed, the chondrocytes in the zone of hypertrophy have an elaborate Golgi apparatus and endoplasmic reticulum, and numerous mitochondria, indicative of high metabolic activity (Buckwalter et al., 1986). In addition, several studies have demonstrated that cultured chondrocytes produce a large pericellular coat of hyaluronan, which is capable of excluding particles such as formalin-fixed erythrocytes (Goldberg and Toole, 1984). This pericellular coat is composed of both hyaluronan and aggrecan and is attached to the surfaces of the chondrocytes through a member of the hyaladherin family of hyaluronan binding proteins (Knudson, 1993; Lee et al., 1993). Presumably, this pericellular coat on the chondrocytes cultured in vitro is similar to that present in lacunae in the zone of hypertrophy in vivo.

Fig. 6. The release of hyaluronan from the hypertrophic zone of the growth plate by MG-63 cells. MG-63 cells were cultured for 16 hours on rehydrated sections of long bone in 90% MEM, 10% FCS supplemented with the various agents to be tested. The sections were incubated in distilled water followed by 0.5% Triton X-100 to release the MG-63 cells. Sections were then stained for hyaluronan using the b-PG probe and counter-stained with hematoxylin. (A) A control section incubated in the absence of cells for 16 hours. (B) A similar section incubated with MG-63 cells for a similar length of time shows a significant loss of hyaluronan. (C) In the presence of exogenously added hyaluronan (1 mg/ml) the removal of hyaluronan from the growth plate by the MG-63 cells was reduced. (D) The addition of the K-3 antibody (50 µl of ascites fluid/ml, approximately 5 mg/ml of antibody) also inhibited the removal of hyaluronan by the MG-63 cells. Bar, 50 µm.
The production of the pericellular coat of hyaluronan by the hypertrophic chondrocytes may play a critical role in the expansion of the growth plate. Hyaluronan is an extremely large molecule that is capable of absorbing large amounts of water. This property allows it to exert a hydrostatic pressure on the surrounding tissue, leading to expansion (Toole, 1981). For example, in the case of the preovulatory follicle, the hormone-induced production of hyaluronan by the granulosa cells results in a rapid expansion of the cumulus (Salustri et al., 1992). It is possible that a similar situation occurs in the hypertrophic zone of the epiphyseal growth plate. The chondrocytes in this region may produce hyaluronan, which exerts a hydrostatic pressure on the surrounding tissue resulting in an expansion of the lacunae in this region. Presumably this process occurs in conjunction with the secretion of various proteases and collagenases, which are important in the remodeling of the surrounding cartilage matrix (Poole and Rosenberg, 1987; Dean et al., 1989). If this is the case, then the production of hyaluronan may be the driving force behind the expansion of the hypertrophic zone.

Several lines of evidence also suggest that the hyaluronan is removed by osteoprogenitor cells in the zone of erosion using a CD44-dependent mechanism. In this mechanism the hyaluronan is initially bound to the cell surface through CD44 and is subsequently internalized and then degraded by lysosomal enzymes. First, the osteoprogenitor cells in this region express CD44, as revealed by immunohistochemistry. Secondly, double-label staining showed that these CD44-positive osteoprogenitor cells also had hyaluronan present on their surfaces and in their cytoplasm. This suggests that these cells had both bound and internalized the hyaluronan. Thirdly, the human osteoblast-like cell line, MG-63 (which has characteristics of osteoprogenitor cells), was also able to remove hyaluronan when cultured on top of sections of the epiphyseal growth plate. Similar results were obtained when the cells were cultured in vitro in the presence of isotopically labeled hyaluronan. In both cases, the degradation was partially blocked by a monoclonal antibody directed against CD44, which demonstrates the involvement of this molecule. In addition, the removal of hyaluronan from the growth plate by MG-63 cells was also blocked by the addition of a large excess of hyaluronan to the culture medium. Presumably, the exogenously added hyaluronan competed with the hyaluronan present in the section of the growth plate for binding to CD44. This experiment confirmed that the removal of hyaluronan by the MG-63 cells was not due to the secretion of proteases that would degrade matrix proteins responsible for stabilizing hyaluronan in the sections.

It is interesting to note that the MG-63 cells released the hyaluronan from the sections at a much faster rate than would be expected from the in vitro degradation assay. The MG-63 cells released the majority of the hyaluronan from the sections after only 16 hours, while in the degradation assay only a small fraction of the isotopically labeled hyaluronan was degraded after 40 hours. It is possible that the cells can more rapidly bind and internalize hyaluronan when they are in direct physical contact with it than when soluble hyaluronan is added to the medium. However, it is also possible that the disappearance of the hyaluronan from the section may reflect only its internalization rather than its degradation, such that when the MG-63 cells were lysed at the end of the incubation period they may have contained intact hyaluronan. In other words, while the hyaluronan from the sections may be rapidly bound and internalized by the MG-63 cells, the complete degradation of hyaluronan may require more extended periods of time.

CD44-mediated removal of hyaluronan occurs in a number of other cases. For example, during the development of the lungs, there is a correlation between the influx of CD44 positive macrophages and the removal of hyaluronan. Indeed, the postnatal decrease in the level of lung hyaluronan is blocked by injection of antibodies directed against CD44 (Ueberhill et al., 1993). Similarly, during the development of hair follicles, there is an inverse correlation between the expression of CD44 by the cells of the dermal condensation and the presence of hyaluronan in this region (Ueberhill, 1993). Thus, the removal of hyaluronan by the osteoprogenitor cells may be part of a more generalized system for regulating the distribution of hyaluronan during development.

The presence and removal of hyaluronan in the zone of erosion may have important biological consequences. For example, hyaluronan may act as a substratum to direct the migration of osteoprogenitor cells into this region. Indeed, hyaluronan has been shown to be a haptotactic agent that stimulates the migration of cultured melanocytes that express CD44 (Thomas et al., 1992, 1993). Another possibility is that the fragments of hyaluronan produced in the process of degradation stimulate the ingrowth of blood vessels. During enchondral ossification of the long bone growth plate, the transverse septa of cartilage matrix are quickly invaded by newly formed blood vessels. West et al. (1985) have previously shown that fragments of hyaluronan (4 to 25 disaccharide units) have a marked stimulatory effect on angiogenesis. Thus, it is possible that the fragments produced as by-products of hyaluronan degradation could act as inductive agents for endothelial cells, resulting in the continuous extension of the blood vessels.

In conclusion, we have shown that large amounts of hyaluronan are present in the lacunae of chondrocytes in the zone of hypertrophy, and are subsequently removed by osteoprogenitor cells in the zone of erosion. We speculate that this dynamic change in the hyaluronan may play a critical role in the elongation of the growth plate. Clearly this possibility deserves further investigation.

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