Plasma Transglutaminase in Hypertrophic Chondrocytes: Expression and Cell-specific Intracellular Activation Produce Cell Death and Externalization

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Abstract. We previously used subtractive hybridization to isolate cDNAs for genes upregulated in chick hypertrophic chondrocytes (Nurminskaya, M., and T.F. Linsenmayer. 1996. *Dev. Dyn.* 206:260–271). Certain of these showed homology with the "A" subunit of human plasma transglutaminase (factor XIIIA), a member of a family of enzymes that cross-link a variety of intracellular and matrix molecules. We now have isolated a full-length cDNA for this molecule, and confirmed that it is avian factor XIIIA. Northern and enzymatic analyses confirm that the molecule is upregulated in hypertrophic chondrocytes (as much as eightfold). The enzymatic analyses also show that appreciable transglutaminase activity in the hypertrophic zone becomes externalized into the extracellular matrix. This externalization most likely is effected by cell death and subsequent lysis—effected by the transglutaminase itself. When hypertrophic chondrocytes are transfected with a cDNA construct encoding the zymogen of factor XIIIA, the cells convert the translated protein to a lower molecular weight form, and they initiate cell death, become permeable to macromolecules and eventually undergo lysis. Non-hypertrophic cells transfected with the same construct do not show these degenerative changes. These results suggest that hypertrophic chondrocytes have a novel, tissue-specific cascade of mechanisms that upregulate the synthesis of plasma transglutaminase and activate its zymogen. This produces autocatalytic cell death, externalization of the enzyme, and presumably cross-linking of components within the hypertrophic matrix. These changes may in turn regulate the removal and/or calcification of this hypertrophic matrix, which are its ultimate fates.

Key words: transglutaminase • factor XIIIA • hypertrophic chondrocyte • cell death • cartilage

During the development of long bones, chondrocytes within the epiphyseal growth region undergo proliferation, maturation, and hypertrophy. During this progression, many cellular and matrix components undergo developmentally regulated changes. For the chondrocytes themselves these changes result in hypertrophy, and eventually apoptosis (Gibson et al., 1995, 1997) or their transformation into osteogenic cells (Galotto et al., 1994). The matrix undergoes calcification and/or removal, and is ultimately replaced by bone or a marrow cavity.

As chondrocytes enter the "hypertrophic program" they undergo changes in their biosynthetic repertoire that range from alterations in the collagens and proteoglycans (Shinomura et al., 1984; Shinomura and Kimata, 1992) and growth factors (Ballock et al., 1993), to the stimulation of enzymes that degrade the matrix (Dean et al., 1985) or induce calcification within it (Osdoby and Caplan, 1981; Schmid et al., 1990; Kirsch and von der Mark, 1991). Recently, another type of enzyme has been shown to be upregulated during hypertrophy—the molecular cross-linker, transglutaminase (Aeschlimann et al., 1993, 1995; Nurminskaya and Linsenmayer, 1996), which presumably alters preexisting cellular and matrix components.

The transglutaminases comprise a family of Ca^{2+}-dependent enzymes that catalyze the formation of γ-glutamyl cross-links. Their substrates include a variety of macromolecular components, both intracellular and extracellular, so they can alter multiple properties of tissues in which they are active. The family (Greenberg et al., 1991; Fesus et al., 1996) consists of at least five members, including the plasma form (also called coagulation factor XIII) and the tissue/cytosolic form. Both of these forms have been shown to be upregulated in hypertrophic cartilage—the plasma form by subtractive hybridization as an upregu-

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lated gene in chick hypertrophic chondrocytes (Nurminskaya and Linsenmayer, 1996), and the tissue form by immunohistochemical identification in hypertrophic cartilage in rats (Aeschlimann et al., 1993) and in articular cartilage in rabbits (Demignot et al., 1995).

The plasma transglutaminase molecule, as isolated from blood, is a tetrameric zymogen (factor XIII) consisting of two catalytic "A" subunits (termed factor XIIIA) and two non-catalytic "B" subunits. Upon thrombin cleavage within the A subunit, the complex dissociates, resulting in the active enzyme composed only of A subunits. In certain cell types, such as platelets and macrophages, factor XIII can exist intracellularly as a dimer of two zymogenic A subunits. The activation mechanism for this intracellular form of factor XIII is unknown, but has been suggested to involve conformational changes induced by the ionic environment rather than proteolysis (Fesus et al., 1996). We will show that hypertrophic chondrocytes most likely have an intracellular mechanism for activation that involves proteolytic cleavage of the zymogen.

Tissue transglutaminase differs, because it is monomeric and cytosolic. It is expressed in certain cells, such as hepatocytes and erythrocytes, and is upregulated in many cell types undergoing apoptosis. For activation, its only known requirement is a critical concentration of calcium (Fesus et al., 1996).

Functions have been proposed for some transglutaminases. These include the plasma transglutaminase-mediated stabilization of the fibrin matrix during blood clotting and the keratinocyte transglutaminase cross-linking of the keratin envelopes formed during epidermal cell differentiation (Thacher, 1989). Tissue transglutaminase has been implicated in many roles, including: the determination of cell shape by cross-linking the actin cytoskeleton and its associated proteins (Nemes et al., 1997), the promotion of cell adhesion (Borge et al., 1996; Ueki et al., 1996), and the stabilization of extracellular matrices (Aeschlimann et al., 1995). Its activity has also been reported to correlate with the regulation of cell growth and differentiation (Borge et al., 1996; Katoh et al., 1996) and apoptosis (Borge et al., 1996; Cummings, 1996; Fesus et al., 1996). As we will show, the plasma transglutaminase that consists only of A subunits (factor XIIIA) can also produce cell death—at least in hypertrophic chondrocytes.

All the transglutaminases seem to be capable of cross-linking a broad, similar range of substrates. Intracellular substrates include proteins of the actin cytoskeleton (Zhu et al., 1994) and histones (Ballestar et al., 1996); extracellular substrates include certain collagens, osteopontin, vitronectin and osteonectin (Mosher and Proctor, 1980; Prince et al., 1991; Beninati et al., 1994; Aeschlimann et al., 1996). Many of these are found in the hypertrophic cartilage matrix, and possibly, the extent to which these and other components are cross-linked alters the susceptibility of the matrix to proteolysis, and thus affects the rate of its removal during development. Fibronectin, for example, is sensitive to cleavage by MMPs only after it has been cross-linked by transglutaminase (Bini et al., 1996).

The expression of two transglutaminases in hypertrophic chondrocytes—the tissue form that is constitutively active, and the plasma form that requires proteolytic activation—suggests that each may serve different roles. In the present study, we have begun to elucidate these functions in the avian growth cartilage, with emphasis on the enzymatically activated form of factor XIIIA. We have observed by sequencing and enzymatic assays, that factor XIIIA is the major form of the molecule in avian growth region cartilage, and that it is upregulated as much as eightfold as the chondrocytes undergo hypertrophy. Since neither factor XIIIA nor the tissue form of transglutaminase has a signal peptide, we examined the mechanism of externalization. By transfection analyses, it seems likely that externalization is effected through cell death and subsequent lysis. When hypertrophic chondrocytes are transfected with a cDNA construct encoding the zymogen of factor XIIIA, the cells effect conversion of the translated protein to a lower molecular weight form, and this occurs concomitant with cell death. Subsequently the cells undergo permeabilization/lysis, allowing for externalization of the enzyme, and presumably of other cytoplasmic components. Non-hypertrophic cells transfected with the same construct show none of these changes. These results suggest that hypertrophic chondrocytes have tissue-specific mechanisms that upregulate the synthesis of plasma transglutaminase and activate its zymogen, resulting in autocatalytic cell death and externalization of the enzyme.

Materials and Methods

Chondrocyte Culture

Cartilages were dissected from 14- or 20-d-old chicken embryos (Spafas Inc., Norwich, CT). Hypertrophic chondrocytes were from the hypertrophic zone three (Kim and Conrad, 1977; Stocum et al., 1979; Schmid and Conrad, 1982*a*) of the tibia. Non-hypertrophic chondrocytes were from the caudal one-third of the sternum. Cells were dissociated by 0.125% trypsin (GIBCO BRL, Gaithersburg, MD), 0.3% collagenase type I (Sigma Chemical Co., St. Louis, MO), and 0.6% bovine testes hyaluronidase type I (Sigma Chemical Co.), and then cultured in DME (GIBCO BRL) containing 10% BCS (Hyclone Labs, Logan, UT) and 50 U/ml penicillin and streptomycin (GIBCO BRL). The hypertrophic chondrocytes were cultured for 2–4 wk and passaged three to four times before use. We (Schmid and Conrad, 1982*b*; Schmid and Linsenmayer, 1983) previously observed that when chondrocytes from the hypertrophic zone are put in cell culture, over a several-week period the relative synthesis of type X collagen, a marker for mature hypertrophic chondrocytes, progressively increases until it constitutes >90% of the collagens being synthesized. Thus, using such passaged cells ensures that the cells are mature, hypertrophic chondrocytes. On the other hand, non-hypertrophic chondrocytes were cultured for only 3–4 d before use, since with extended time in culture, some of these can progress to the hypertrophic state (Schmid and Linsenmayer, 1983).

In some experiments, ascorbic acid (50 μg/ml) was added to the culture medium, which was changed every other day.

Protein Extraction from Cartilage and Cell Cultures

Isolated pieces of sternum or hypertrophic cartilage were extracted in ice-cold 50 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl and protease inhibitors (2 mM DTT, 0.4 mM PMSF, 5 μg/ml leupeptin) for 2–4 h at 4°C. After removal of this supernatant, termed "matrix extract," the tissue was homogenized in a lysis buffer to produce a "tissue extract." Lysis buffer consisted of 5 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 0.2 mM Mg_2SO_4, 0.4% Triton X-100, and the same protease inhibitors as in the extraction buffer. The total protein content was determined by the BCA Protein Assay Reagent (Pierce Chemical Co., Rockford, IL).

For extracts of cell cultures, plates were rinsed twice with PBS, scraped in PBS, and then centrifuged at 400 *g* for 5 min. The cell pellets were stored frozen at −20°C until use. The cell pellets were suspended in the same lysis buffer used for tissue homogenization, sonicated four times for

15 s at 10-s intervals, and then centrifuged at 13,000 *g* for 5 min at 4°C. The supernatant was collected and assayed for transglutaminase activity and total protein.

Transglutaminase Assay

Transglutaminase activity was determined using a [^{14}C]putrescine assay described by Demignot et al. (1995), except that the reaction was stopped by adding an equal volume of 10% TCA. TCA precipitates were collected on Glass Microfibre filters (Whatman Inc., Clifton, NJ), washed three times with 1 ml of ice-cold 5% TCA followed by 1 ml of 96% ethanol, dried, and then counted in 10 ml of scintillation fluid (Beckman Ready Safe). Enzyme activity was expressed as fmol of putrescine incorporated per mg of protein in 1 min.

Preparation of RNA and Northern Analysis

PolyA$^+$ RNA was isolated using a Fast Track Kit (Invitrogen Corp., Carlsbad, CA) and cDNA was synthesized using the cDNA Synthesis System (Amersham Corp., Arlington Heights, IL). Total RNA was isolated with Trizol reagent (GIBCO BRL). RNA samples (2 μg polyA$^+$ or 20 μg total RNA) were subjected to electrophoresis through 1% agarose/2.2 M formaldehyde gels and transferred to a HybondN filter (Amersham Corp.). The filters were baked under vacuum and hybridized to labeled probes as described in Sambrook et al. (1989). The probe for chicken plasma transglutaminase was prepared by RT-PCR of hypertrophic chondrocyte cDNA using the following oligonucleotide primers: ATGTCTGAACCAGCGAGCAC (positions 94–114 in the partial cDNA sequence [Nurminskaya and Linsenmayer, 1996]) and TAGGGTCGGCTGAAGTCTATCTG (reverse sequence for positions 327–350). Amplification was performed for 30 cycles, with each cycle being at 94°C for 1 min, 56°C for 1 min, and 72°C for 1.5 min.

5′- and 3′-RACE

To extend the lengths of the previously published (Nurminskaya and Linsenmayer, 1996) partial cDNA sequence for plasma transglutaminase, 5′- and 3′-RACE was used, using a Marathon cDNA Amplification kit (CLONTECH Laboratories, Inc., Palo Alto, CA). The primer used for the 5′-RACE was TAGGGTCGGCTGAAGTCTATCTG (reverse sequence for positions 327–350) and for the 3′-RACE was GGCTCTGGAGACTGCTGTAATGT (positions 1,590–1,613 in the published sequence). The extended, amplified products were cloned into the plasmid pCRII (TA cloning kit; Invitrogen). For each RACE product, 24–48 colonies were picked and amplified by PCR using the same insert-specific primers. The positive clones were then analyzed for insert size by PCR amplification with vector primers.

DNA Sequencing

A set of gamma-delta transposon insertions (Strathmann et al., 1991) was obtained for each clone to be sequenced that was longer than 0.6 kb. Each clone was sequenced from both strands with at least threefold redundancy. Sequencing was performed using a DyePrimer Cycle Sequencing kit and the 373A automated DNA sequencer (PE Applied Biosystems, Foster City, CA). The results were analyzed with the Sequencher software program (GeneCodes Corp., Ann Arbor, MI).

Vector Construction, Transfection, and Immunochemical Analyses

A plasma factor XIIIA (FXIIIA)-green fluorescent protein (GFP)[1] expression vector (pFXIIIA-GFP) was constructed using the pGreenLantern-1 (GIBCO BRL) and pCR3.1 unidirectional expression vector (Invitrogen). A cDNA fragment encoding amino acid residues 1–555 of factor XIIIA was obtained by RT-PCR of the hypertrophic chondrocyte cDNA and cloned into pCR 3.1 following the manufacturer's instructions. pGreenLantern-1 vector was digested with NotI to obtain the DNA fragment coding for GFP. This fragment was ligated into the NotI site of the FXIIIA-pCR 3.1 plasmid. Transfections were performed in two-chamber slides (for immunohistochemistry) or in 60-mm dishes (for Western blot analysis). 7×10^4 hypertrophic chondrocytes or 7×10^3 non-hypertrophic chondrocytes were transfected with 1 μg of DNA using 7 μl of lipofectin (GIBCO BRL) according to the manufacturer's instructions. At intervals after transfection, the transfected cultures were analyzed by immunofluorescence and immunoblotting. For immunofluorescence, the cells were fixed in 4% PFA for 25 min at 4°C, permeabilized by methanol, and then reacted with an anti–type X collagen mAb (Schmid and Linsenmayer, 1985). The nuclei were visualized by Hoechst staining. For immunoblotting, the transfected cell cultures were washed with PBS and scraped into Laemmli lysis buffer (150 μl/60-mm dish; with DTT). 20-μl aliquots were analyzed on 4–20% gradient SDS-polyacrylamide gels (BIO-RAD Laboratories, Hercules, CA). The proteins were transferred to nitrocellulose membranes (BIO-RAD Laboratories), and the fusion proteins were detected by reaction with an anti-GFP polyclonal antibody (CLONTECH Laboratories, Inc.) followed by fluorescence visualization using an ECL kit (Amersham Corp.).

In Situ Hybridization

Tibiotarsi were dissected from 14- and 20-d-old chicken embryos (Spafas Inc., Norwich, CT) and fixed for 3 h in 4% PFA. Tissues were then washed in PBS, dehydrated in a series of ethanols and xylene, and then embedded in paraffin wax. Tissues were cut into 6-μm sections and mounted on Vectabond-coated slides (Vector Laboratories, Burlingame, CA).

For in situ hybridization sections were dewaxed in xylene and rehydrated in a series of ethanol washes. After a 20-min wash in 0.2 N HCl in PBS and two 5-min washes in PBS, the tissues were treated with 1 μg/ml proteinase K for 30 min at 37°C, quenched with 0.1% glycine, refixed in 4% PFA for 10 min at RT, washed in PBS for 5 min and 0.1 M triethanolamine, pH 8.0, for 10 min, acetylated with 0.25% acetic anhydride for 10 min, and then dehydrated in a series of ethanol washes. Sections were then air-dried for at least 1 h. The DIG-labeled riboprobes were diluted in hybridization buffer (50% formamide, 10% dextran sulfate, 4× SSC, 1× Denhardt's solution, 1 mg/ml yeast tRNA, 10 mM DTT) to a final concentration of 5 ng/μl for plasma transglutaminase. The probes were denatured at 65°C for 10 min and hybridization was performed overnight in a humid chamber at 52°C. To reduce non-specific background, the slides were washed in 4× SSC, incubated with 8.25 μg/ml RNase A for 30 min at 37°C, and finally washed in a series of SSC washes with decreasing salt concentrations and increasing temperature. Slides were then incubated overnight at RT with an alkaline phosphatase conjugated anti-digoxigenin antibody (Boehringer Mannheim Corp., Indianapolis, IN), and the hybridization signal was visualized by addition of NBT/BCIP.

Preparation of Riboprobes

Probes were amplified from cDNA, and the PCR fragments were ligated into the pCR2.1 vector (Invitrogen). Plasmids containing the inserts in the forward or reverse orientation were linearized with BamHI, and labeled sense (negative control) or antisense RNA was generated by in vitro transcription in the presence of digoxigenin-UTP and unlabeled nucleotides using the T7 promoter. The 548-bp PCR fragment for the plasma transglutaminase was generated with the forward primer CACCGACAGAGAGAAGAAACGACCTC and reverse primer TCAGTTGCCGAGTTT.

Results

Isolation and Characterization of a Full-Length cDNA for Plasma Transglutaminase (Factor XIIIA)

We previously used subtractive hybridization to isolate cDNA clones for genes upregulated in chicken hypertrophic chondrocytes. Two of these clones, when extended by RACE, generated products totaling 1.6 kb. The sequences of these showed high homology with the A subunit of human plasma transglutaminase (factor XIIIA).

These clones were used as probes for Northern analyses of total RNA from cell cultures of hypertrophic and non-hypertrophic chondrocytes. The data (Fig. 1) give the size of the mRNA as 4.3 kb.

To obtain a full-length cDNA we further used 5′- and 3′-RACE. This sequence shows an open reading frame of 2.5 kb, encoding a 749–amino acid protein with a predicted

1. *Abbreviation used in this paper*: GFP, green fluorescent protein.

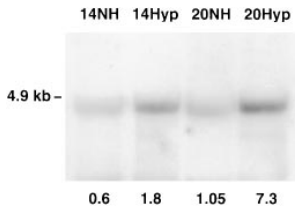

Figure 1. Northern analysis of factor XIIIA expression in hypertrophic (*Hyp*) and non-hypertrophic (*NH*) chondrocytes. Total RNA (10 μg) was isolated from cell cultures from 14- and 20-d-old embryos. Relative abundance is normalized to the housekeeping gene GAPDH.

molecular mass of ~75 kD. The actual protein could, however, be somewhat larger, as two potential sites for *N*-glycosylation (N-Y-S sequence) are present (positions 345 and 549). The conceptual protein lacks a signal peptide, as based on the hydrophobicity profile and the criteria of the "-3,-1" rule (von Heijne, 1986). The deduced amino acid sequence has 75% identity to human factor XIIIA (67% identity at the nucleotide level). The identity is even higher (up to 90%) within the functional domains responsible for enzymatic activity (residues 319–347) and for Ca^{2+} binding (residues 468–488).

Upregulation of Plasma Transglutaminase mRNA in Hypertrophic Chondrocytes

To determine the course of the upregulation of plasma transglutaminase during chondrocyte development we first performed Northern analysis on hypertrophic and non-hypertrophic chondrocytes cultured from two different stages of embryos—14 d, which is a time when rapid embryonic growth is still occurring, and 20 d, when growth has reached a plateau in preparation for hatching. The mRNA for the plasma transglutaminase (Fig. 1), when normalized to that for the "housekeeping" enzyme GAPDH, showed a relative increase in the hypertrophic chondrocytes versus non-hypertrophic chondrocytes of three- to fourfold in the cultures from 14-d embryos, and six- to eightfold in those from 20-d embryos.

The upregulation was confirmed for growth cartilages in vivo by in situ hybridization (Fig. 2). In the growth region of tibiotarsi from 14-d embryos, the signal for plasma transglutaminase shows a large increase in cells of the hypertrophic zone (Fig. 2 *A*, and panels demarcated by *arrows*). In comparison to collagen type X protein, as visualized by immunofluorescence (Fig. 2 *A′*), the increased signal for the transglutaminase seems to occur slightly earlier than the appearance of this hypertrophic-specific collagen.[2] Both remain present throughout the remainder of the hypertrophic zone. In tissues from 20-d embryos (Fig. 2 *B*), the mRNA for plasma transglutaminase becomes restricted to a narrower zone. By comparison with the immunolocalization of the type X collagen (Fig. 2 *B′*) this is the zone of early hypertrophy.

2. We used this combination of in situ hybridization and immunofluorescence to compare precisely the localizations of transglutaminase and type X collagen in the same section. Obviously the immunofluorescence for the type X collagen is visualizing the protein and not the mRNA. However, we feel that this comparison is valid, since our previous studies on type X collagen show concomitant appearance of the mRNA and protein.

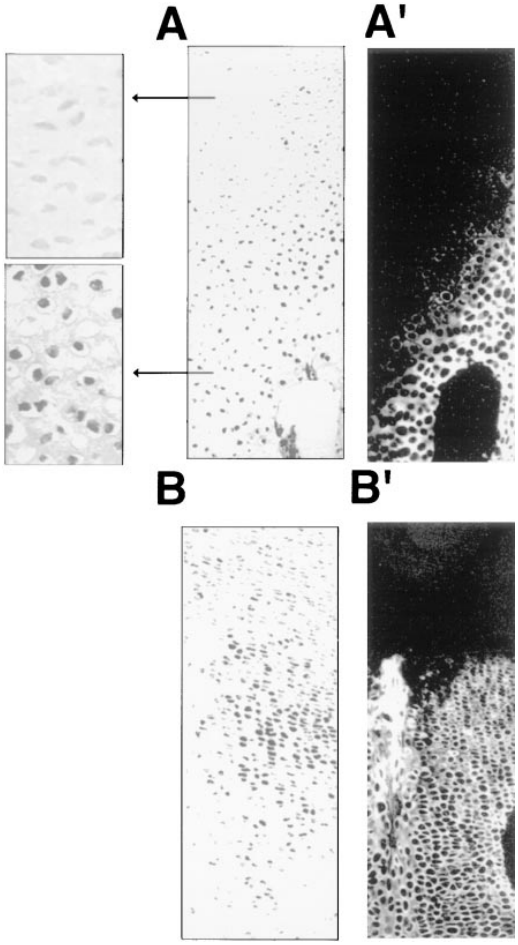

Figure 2. Upregulation of plasma transglutaminase in hypertrophic cartilage in vivo. In situ hybridization for factor XIIIA in the tibiotarsal growth region of 14-d (*A*) or 20-d (*B*) embryos. The arrows in *A* demarcate enlarged images from the pre-hypertrophic (*top*) and hypertrophic (*bottom*) zones. *A′* and *B′* are fluorescent images of the same field as in *A* and *B* reacted with a mAb against collagen type X.

Transglutaminase Activity

To determine whether transglutaminase enzymatic activity also becomes elevated during hypertrophy, we used the standard assay measuring [^{14}C]putrescine incorporation into casein (Demignot et al., 1995). This was examined both in cell cultures (Fig. 3) and in isolated tissues (Fig. 4; Table I). Each sample was analyzed before and after thrombin activation. The activity requiring thrombin should reflect only the factor XIIIA, as this is the member of the transglutaminase family that is synthesized as a zymogen. The activity already present before thrombin treatment (constitutive activity), however, is probably more complex. In the non-hypertrophic cells, the constitutive activity should reflect largely the tissue form, as these cells do not seem able to activate the factor XIIIA zymogen (see below). In the hypertrophic cells, however, the constitutive activity most likely includes both the tissue form and that portion of the factor XIIIA that has already undergone activation—since hypertrophic chondrocytes have the ability to activate the zymogen intracellularly (shown later).

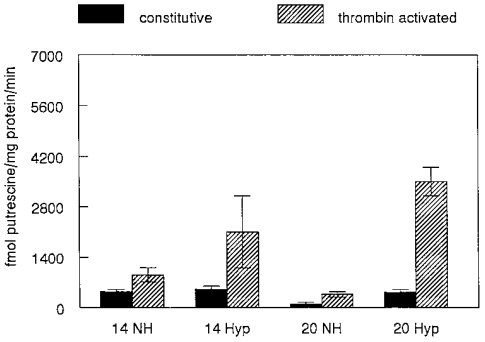

Figure 3. Enzymatic assays for plasma transglutaminase in cultures of hypertrophic (*Hyp*) and non-hypertrophic (*NH*) chondrocytes. Activities were measured without thrombin activation (*solid bar*) and with activation (*cross-hatched bar*). Cell cultures were initiated with chondrocytes from 14- and 20-d-old embryos.

In lysates of hypertrophic and non-hypertrophic chondrocytes cultured from 14- or 20-d-old embryos (Fig. 3), the constitutive activity (*solid bars*) was relatively low and constant in both cell types. The major activity required thrombin activation (Fig. 3, *cross-hatched bars*), with this being four- to sixfold greater in hypertrophic cells. These observed differences in thrombin-activation between the hypertrophic and non-hypertrophic cells are consistent with those obtained for the mRNA of the plasma form,

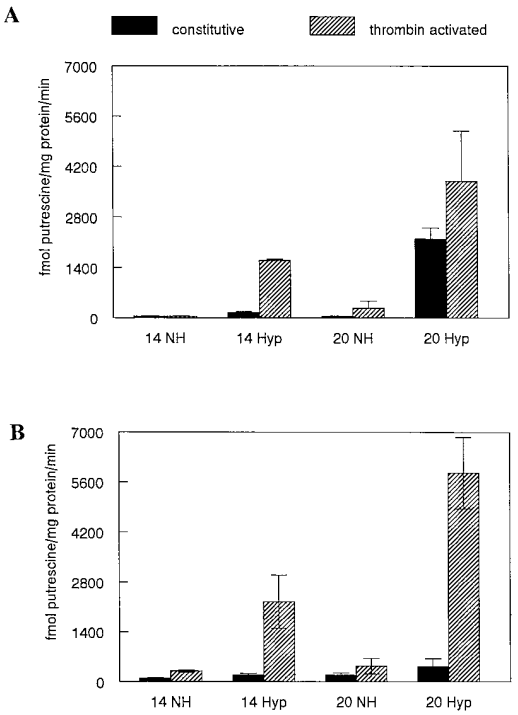

Figure 4. Transglutaminase enzymatic activity in non-hypertrophic (*NH*) and hypertrophic (*Hyp*) cartilage tissues dissected from 14- and 20-d embryonic tibiotarsal growth regions. Both matrix extract (neutral salt extractable) (*A*) and tissue lysate (detergent extracted) (*B*) pools were examined, and activities were measured both without thrombin activation (*solid bar*) and with activation (*cross-hatched bar*).

Table I. Distribution of Transglutaminase Activity in the Growth Plate of the 14-d-old Chicken Embryo

	Matrix extracts		Tissue lysate	
	constitutive	thrombin activated	constitutive	thrombin activated
sterna	ND	ND	110	330
non-hypertrophic zone	32	280	160	730
hypertrophic zone	170	1600	190	2250
bone marrow	ND	ND	20	150

Activity is given in fmol of [^{14}C]putrescine incorporated into casein by 1 mg of total protein in 1 min.

which showed a similar degree of upregulation (above). The culture media showed little of either type of activity, if any at all (data not shown). Identical results were obtained in cultures supplemented with exogenous ascorbic acid for 4–5 d preceding the assay.

To determine whether the enzymatic analyses of cells in culture reflected the conditions in vivo, we examined transglutaminase activities in freshly dissected cartilages from the growth region of limbs. As shown in the diagram in Table I, non-hypertrophic cartilage was from the upper one-third of the growth region, which included the proliferative zone and part of the zone of maturation, and hypertrophic cartilage was from the lower one-third of the region. For comparisons and controls, we also examined the caudal sternum and bone marrow. The caudal sternum is essentially a permanent cartilage, and thus provides a pure source of non-hypertrophic material. Bone marrow is virtually impossible to remove from the vascular channels in hypertrophic cartilage, and thus is a potential source of contaminating cells. If the enzymatic activity in marrow were either low or nonexistent, it would ensure that our results were not influenced by contaminant cells. For each tissue, two fractions were examined. An extracellular/matrix–associated pool (termed matrix extract) was obtained by extraction of the intact tissue with 0.15 M NaCl (Fig. 4 *A*), and an intracellular pool (termed tissue lysate) was obtained by subsequent homogenization of the remaining material in the presence of detergent (Fig. 4 *B*).

The data for 14-d-old tissues is shown in Table I. The matrix extracts of the non-hypertrophic, caudal sternal control showed no detectable constitutive enzyme, and after thrombin activation only a small amount of additional activity was detected (data not shown). In the matrix of the growth region of the limb, the non-hypertrophic zone showed a small amount of constitutive activity, and after thrombin activation, somewhat more. In the hypertrophic zone both activities were increased five- to sixfold. Bone marrow had essentially no activity. The tissue lysates in all cartilages and zones contained most of the activity. The constitutive activity again was minor and constant throughout all of the zones, with most of the activity requiring thrombin activation. In the hypertrophic zone the thrombin-activated form was threefold greater than in the non-hypertrophic zone of the limb and sevenfold higher

than in the caudal sternum. The bone marrow, again, had little activity.

When these data on the 14-d limb tissues were compared with those from 20-d embryos (Fig. 4), the most obvious difference was the heightened activity in the extracellular matrix of the hypertrophic zone at the later stage. In the tissue lysates from this zone, by far the major activity required thrombin activation (Fig. 4, *cross-hatched bars*), and in the matrix extracts this thrombin-activated (plasma) form also predominated. In the matrix extracts constitutive activity was also elevated. However, since hypertrophic chondrocytes can activate the plasma form, which is subsequently released into the matrix (described next), a large portion of this activity is also likely to be the plasma transglutaminase.

Thus, these data on enzymatic activities are consistent with the plasma form being the major transglutaminase in developing avian growth cartilage, with this activity becoming greatly upregulated during hypertrophy and with increasing developmental age. They also suggest that in the hypertrophic region a large amount of plasma transglutaminase (and presumably also the tissue form) is released into the matrix.

Transglutaminase Externalization

As neither the plasma nor tissue form of transglutaminase possesses a consensus signal peptide, the question arises how they become externalized, and, in growth cartilage, why this preferentially occurs in the hypertrophic region. Two possibilities were examined. One is that these enzymes are components of the matrix vesicles that are preferentially shed from hypertrophic chondrocytes; the other is that the enzymes are released after cell death. We failed to detect transglutaminase in matrix vesicles isolated from hypertrophic cell cultures (data not shown), so we examined the possible role of cell death. Cell death is known to occur in 8–10% of the cells throughout the growth plate, and this rises to 30% of the cells that have entered the hypertrophic program (Gibson et al., 1995; Aizawa et al., 1997). Also, the tissue form of transglutaminase has been reported to be involved in apoptosis in other cell types.

To address this we transfected hypertrophic chondrocytes with an expression plasmid (pFXIIIA-GFP) containing a construct for the zymogen form of factor XIIIA fused with GFP as a tag to identify the transfected cells. The construct consisted of the coding sequence for the first 555 amino acids of the transglutaminase linked to that encoding 238 residues of GFP, all driven by a CMV promoter. The construct was designed such that the size of the fusion protein produced would be similar to that of the native protein. This was achieved by replacing the sequence encoding 194 amino acids from the COOH-terminal end of transglutaminase with 238 amino acid residues of GFP. The only potential alteration this might produce in enzyme function would be a lowered catalytic efficiency. Studies on human factor XIIIA have shown that deletion of 218 amino acid residues from the COOH terminus results in a fourfold reduction in activity (Lai et al., 1996).

We transfected hypertrophic chondrocytes with this construct and then examined the effect on the cells by fluorescence microscopy. After several days, the transfected

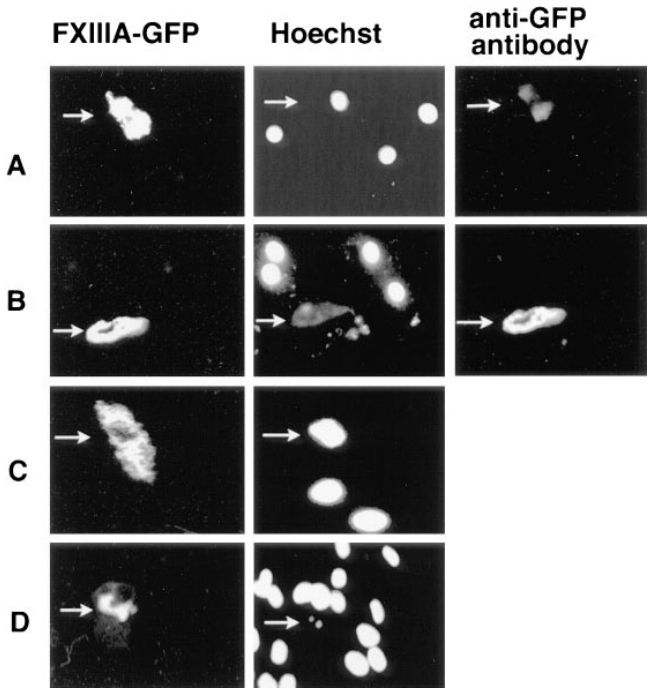

Figure 5. Fluorescence micrographs of hypertrophic (*A* and *B*) and non-hypertrophic chondrocytes (*C* and *D*) transfected with the full-length (*A–C*) and truncated (constitutively active) (*D*) pFXIIIA-GFP constructs. Transfected cells (*arrows*) were identified by the intrinsic fluorescence of the GFP fusion protein (*left column*); nuclear morphology was visualized by Hoechst staining (*middle column*), and the integrity of cell membranes was determined by intracellular reactivity of non-permeabilized cells with rhodamine-conjugated anti-GFP antibody (*right column*). 2 d after transfection of hypertrophic cells with the full-length FXIIIA-GFP construct (*A*) no changes are seen in the nuclear morphology compared with non-transfected cells. Also, the fusion FXIIIA-GFP protein in these non-permeabilized cells is not reactive with the anti-GFP antibody. By 4 d (*B*), the Hoechst staining shows shrunken and fragmented nuclei in those cells transfected with pFXIIIA-GFP, and now the anti-GFP antibody can penetrate the non-permeabilized cells and shows reactivity. Non-hypertrophic chondrocytes, transfected with the pFXIIIA-GFP construct, still maintain a normal nuclear morphology 6 d after transfection (*C*). However, the nuclei of non-hypertrophic chondrocytes transfected with the truncated (constitutively active) pFXIIIA-GFP (*D*) become shrunken and fragmented by day 4.

cells showed a spectrum of changes indicative of cellular death, including shrunken and fragmented nuclei (Fig. 5 *B*, *middle column*). Eventually, all that remained of the transfected cells were patches of GFP-containing cytoplasm (Fig. 5 *B*, *right column*). Control hypertrophic cells transfected with a plasmid expressing GFP only showed no cellular changes, and several days after transfection were found frequently in nests of two or four cells, suggesting that they had continued to undergo division (data not shown).

Further evidence for transglutaminase-mediated cell lysis and the release of cytoplasmic components was obtained by immunohistochemistry with an antibody against the GFP tag (Fig. 5). Shortly after transfection (2 d) with the pFXIIIA-GFP plasmid (when cell nuclei still appeared normal), the cells required permeabilization for reactivity

with the anti-GFP antibody, suggesting that their membranes were intact (Fig. 5 *A*). Later (4–5 d) after transfection, however, when nuclei were undergoing degenerative changes, reactivity with the antibody could be achieved without permeabilization (Fig. 5 *B*). Thus, the cell membranes had become leaky to large macromolecules, or possibly had been destroyed.

Non-hypertrophic cells, when transfected with the full-length pFXIIIA-GFP construct, encoding the zymogenic form of FXIIIA exhibited no such degenerative changes (Fig. 5 *C*). The nuclei did not undergo morphologically degenerative changes, and the GFP fusion protein remained in discrete cytoplasmic granules/vacuoles, the identity of which is unknown. Thus, non-hypertrophic chondrocytes either differ in their susceptibility to transglutaminase activity, or they lack the ability to convert the zymogen to an active enzyme.

To determine whether the non-hypertrophic chondrocytes are susceptible to the action of transglutaminase, we transfected these cells with a truncated ptFXIIIA-GFP fusion construct from which the inhibitory domain of the zymogen had been removed (by deleting 144 amino acid residues from the NH_2-terminal end of the molecule). Thus, the enzyme produced by this construct should be constitutively active. Control hypertrophic cells transfected with this truncated construct showed that the enzyme produced was active. Such transfected cells underwent nuclear changes that were indistinguishable from those of the cells transfected with the full-length construct. Likewise, when non-hypertrophic cells were transfected with this truncated construct, they also underwent similar degradative changes (Fig. 5 *D*). Thus, the non-hypertrophic cells are not refractory to the effects of the enzyme. Instead, the most likely reason they were unaffected by transfection with the full-length construct was an inability to activate the zymogen.

Hypertrophic Chondrocyte-mediated Conversion of the Zymogen

Extracellular activation of the zymogen of human plasma transglutaminase—as occurs during blood clotting—involves thrombin-mediated cleavage of the A subunit at an Arg-Gly site at position 38 (Fig. 6 *A*, *circle* in human sequence). The transfections of the avian hypertrophic chondrocytes described above, however, raise the possibility that this cell type can activate the zymogen intracellularly. In the chicken plasma transglutaminase, the deduced amino acid sequence contains two Arg-Gly residues (at positions 46 and 87; see Fig. 6 *A*, *boxes*) that, by comparison to the human and other species, are potential sites for activation.

To examine which, if either, of these sites can be cleaved by hypertrophic chondrocytes, cells were transfected with the pFXIIIA-GFP construct. Then, 24 and 150 h later, cell lysates were prepared and the molecular weight(s) of the fusion protein were determined by Western blotting with an antibody against the GFP moiety (Fig. 6 *B*). At 24 h after transfection only a single band was detected. At 150 h, however, an additional lower molecular weight band was also present. The difference in the molecular weights between these two is 8 kD, which is consistent with a proteolytic cleavage at residue 85 (Fig. 6 *A*, *circle* in chicken sequence). Thus, hypertrophic chondrocytes contain an intracellular thrombin-like activity that can cleave the zymogen form of plasma transglutaminase.

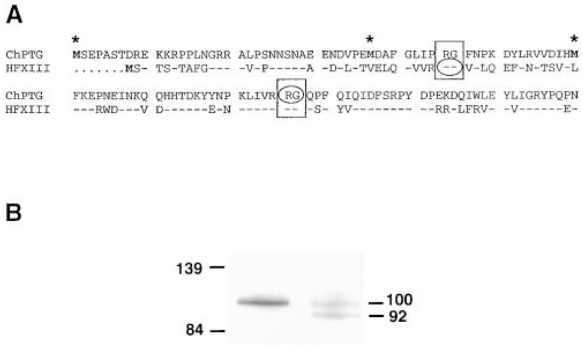

Figure 6. (*A*) Amino acid alignment of the chicken (*ChPTG*) and human (*HFXIII*) plasma transglutaminase. Asterisks indicate potential translation start sites in the chicken. Boxes mark the Arg-Gly residues of the potential activating sites at positions 46–47 and 87–88. Different sites are used by the chicken and human as designated by the circles. (*B*) Immunoblot of extracts of hypertrophic chondrocytes transfected with the pFXIIIA-GFP construct and probed with an antibody against the GFP moiety (*B*). At 24 h after transfection, only a single band was detected (of the size predicted for the zymogen), whereas by 150 h another band was also present, suggesting the conversion/activation of some of the zymogen.

Discussion

Previous immunohistochemical studies showed an increased level of the tissue form of transglutaminase in the hypertrophic zone of the growth plate of juvenile rats (Aeschlimann et al., 1993, 1995). In agreement with this, we (unpublished observations) also have observed the mRNA for this form of the enzyme to be upregulated in the hypertrophic zone of the avian growth region. Compared with the plasma form (see below), however, this represents a small portion of overall transglutaminase activity produced by hypertrophic chondrocytes.

The possibility that more than one transglutaminase might be present in cartilage was suggested by enzymatic analysis of cultured rabbit articular chondrocytes. These showed two activities that differed in their temporal patterns of expression and in their responses to retinoic acid (Demignot et al., 1995). The minor activity was later identified immunohistochemically as the tissue form, but the major one was characterized only as activated by trypsin. Trypsin is known to substitute for thrombin in activating the plasma form of the molecule, raising the possibility that the major activity was coming from factor XIII. Consistent with this identification, as an upregulated gene in hypertrophic chondrocytes we previously isolated a form of transglutaminase that we tentatively identified as the A subunit of factor XIII (Nurminskaya and Linsenmayer, 1996).

The additional data presented here solidify this identification. The full-length cDNA shows 75% identity with the A-chain of human factor XIII, as compared with only 47% with the tissue form (from chicken erythrocytes). It has

both enzymatic and Ca^{2+}-binding regulatory domains, and these show even higher (~90%) identity with human factor XIIIA. Also, like the factor XIIIA from other species, it is synthesized as a zymogen that can be enzymatically activated in in vitro assays by thrombin. The transfection studies show that this zymogen can also be activated within hypertrophic chondrocytes, and this, apparently, is also by an enzymatic activity. The identity of this putative enzyme within hypertrophic chondrocytes is still unknown. However, the sequence cleaved (Arg-Gly) may be the same as used by thrombin. The peptide removed by hypertrophic chondrocytes from the zymogen is consistent with cleavage of an Arg-Gly site found 85 amino acids from the NH_2-terminal end of the molecule. In the human zymogen during blood coagulation an identical sequence is cleaved at position 38.

Our data suggest that, in chicken growth cartilage, factor XIIIA is quantitatively the predominant transglutaminase, and that it undergoes appreciable upregulation during chondrocyte maturation/hypertrophy. This was observed at the level of mRNA, by both Northern analysis and in situ hybridization. It was also observed at the level of enzymatic activity. Throughout the growth region, the largest proportion of the activity requires thrombin activation, and in the hypertrophic region this is increased greatly. Also, at 20 d, as compared with 14 d, a much greater proportion of the enzymatic activity is found in the matrix fraction, suggesting a more active externalization—possibly resulting in increased cross-linking of matrix components, and through this either altering the rate of their removal or promoting calcification of the matrix.

The ultimate fate of the transglutaminases in hypertrophic cartilage is to be released into the matrix. There, most of the transglutaminase-induced cross-links are in the non-collagenous fraction of the extracellular matrix (Bowness and Tarr, 1997), with osteonectin and fibrillin being two known substrates (Aeschlimann et al., 1995; Bowness and Tarr, 1997). Additional substrates are also likely to exist, such as fibronectin (Beninati et al., 1994; Komarek et al., 1996), which is present in developing hypertrophic cartilage (Chen and Linsenmayer, 1993). Immunohistochemical analysis (Aeschlimann et al., 1995) using antibodies against the major cross-link produced by the enzyme (the γ-Glu-ϵ-Lys dipeptide), shows a low level of reactivity in the zone of maturation, and this activity is intracellular. In the zone of hypertrophy there is increased activity, and much of this is now within the extracellular matrix. Our enzymatic analyses are consistent with transglutaminase activity becoming externalized into the extracellular matrix of the hypertrophic zone. In this location, a portion of the enzyme is already active (especially in the older embryos) and a portion requires thrombin activation. The active enzyme could be comprised of both the tissue form and the factor XIIIA that had already been activated; the inactive enzyme most likely represents only the factor XIIIA zymogen that has been externalized.

Externalization of the transglutaminase(s) into the matrix is unlikely to occur through the conventional exocytotic pathway, as neither form has a consensus signal peptide. Externalization of other proteins that lack a signal peptide has been suggested to occur through a loosely defined "non-conventional pathway," or possibly through cell lysis. In hypertrophic chondrocytes, one such non-conventional pathway is through the shedding of membrane-bound matrix vesicles, which are thought to be involved in calcification (Wuthier et al., 1985). This pathway has been suggested for externalization of tissue transglutaminase by hypertrophic chondrocytes (Aeschlimann et al., 1995). We were unable, however, to detect transglutaminase activity in matrix vesicles isolated from cultures of hypertrophic chondrocytes, although our preparations did contain alkaline phosphatase, a marker for these structures (unpublished observations). Thus, if transglutaminase is a component of matrix vesicles, it must be in extremely low concentration.

Instead, our data from transfection studies are most consistent with a mechanism in which some portion of the plasma transglutaminase zymogen undergoes intracellular activation within hypertrophic chondrocytes, resulting in cell death, lysis, and subsequent externalization of the active enzyme, as well as the zymogen itself. The pFXIIIA-GFP zymogen construct employed in these studies contained the functional regions required for regulation (the activation peptide and Ca^{2+}-binding domains) and for cross-linking (the catalytic domain) (Lai et al., 1996).

Transfections of hypertrophic chondrocytes with this construct led to morphological signs of nuclear degeneration and cell death. The nuclear changes, as observed by staining with Hoechst dye or propidium iodide, include shrinkage, fragmentation, and eventual loss. We have not, however, observed typical apoptotic staining of the nuclei by end labeling (TUNEL). Thus, the transglutaminase-induced cell death may differ from apoptotic death, both in nuclear changes and in the fate of cytoplasmic components. In general, it is believed that during apoptosis cytoplasmic components are not externalized (Jacobson et al., 1997), but apparently they are during the transglutaminase-induced cell death.

Externalization of cytoplasmic components is an important feature of our model, as this is required for the subsequent transglutaminase-mediated modifications of matrix components. Observations suggest that transglutaminase-induced cell death can result in externalization. Shortly after transfection, when the nuclei still appear normal, immunohistochemical visualization of the intracellular transglutaminase fusion protein (via its GFP moiety) requires permeabilization of the cells. Later however, when signs of nuclear degeneration are apparent, permeabilization is no longer required for visualization of intracellular transglutaminase. Thus, the cell membranes have become "leaky" to macromolecules at least as large as the antibodies used for immunohistochemical detection. Eventually all that remains of the transfected cells are patches of GFP-positive cytoplasm/matrix on the surface of the culture dish.

The cellular death caused by the factor XIIIA-GFP fusion protein most likely requires activation of the zymogen through a mechanism that seems specific/selective for hypertrophic chondrocytes. However, the details of this mechanism are unknown. The zymogen of factor XIII has been detected in a variety of cells (Adany, 1996). However, only stimulated platelets and macrophages have been shown capable of its intracellular activation (Muszbek et al., 1995), and this is thought not to involve proteolytic ac-

tivation. In hypertrophic chondrocytes, however, our transfection studies suggest proteolytic activation is involved, as immunoblotting showed intracellular conversion of some factor XIIIA fusion protein zymogen to a lower molecular weight form. This conversion is consistent with cleavage at an Arg-Gly site that can also be cleaved by thrombin or trypsin. The identity of the intracellular protease involved remains to be determined. However, it too is most likely an upregulated/specific component of the hypertrophic program (see below), and through its ability to effect activation of the zymogen, it potentially adds another level of regulation to the transglutaminase-mediated effects.

Specificity of this activation for hypertrophic cells is evidenced by the observation that non-hypertrophic cells transfected with the same construct showed no indications of the nuclear changes seen in hypertrophic cells. Instead, these cells retained a healthy appearance, although they clearly contained large amounts of GFP-labeled protein localized in discrete cytoplasmic regions/structures that do not colocalize with markers for either Golgi or lysosomes (data not presented), and whose identity, therefore, is unknown. The non-hypertrophic chondrocytes were not simply refractory to the action of the enzyme. These cells, when transfected with a truncated construct from which the activation domain had been removed (which thus produced constitutively active enzyme) underwent the sequence of morphological changes indicative of cellular death. Therefore the non-hypertrophic cells most likely lack the activation mechanism for the zymogen, or it is at a much lower level (see below). Alternatively, they may have a mechanism for sequestering the zymogen in a subcellular compartment in which it is protected from activation.

Our determinations of enzymatic activity in vivo are also consistent with intracellular activation of the enzyme by hypertrophic chondrocytes. Appreciable enzymatic activity is present in the hypertrophic matrix, and this increases with developmental stage. As both the constitutively active enzyme and the zymogen are present in the extracellular matrix, not all the enzyme is required for the proposed cell lysis.

This raises the question whether a critical level of active enzyme (or time of exposure) results in cell death with the subsequent release of active enzyme plus the remaining store of zymogen. It also raises the question why massive death is not observed in cultures of hypertrophic cells, unless the quantity of enzyme is artificially increased by transfection with a transglutaminase-expressing construct. At present, no definitive answers to these questions are available. However, a major difference between hypertrophic chondrocytes in vivo (where a high proportion of them die) and in cell culture (where they do not die) is cellular proliferation. In vivo, hypertrophic chondrocytes are postmitotic, but in culture they rapidly proliferate. Possibly, cell division dilutes the enzyme or restricts the time available for it to alter cellular component(s).

Once the enzyme is externalized, the number of substrates potentially available for cross-linking is large, including: osteonectin and collagen type II (Aeschlimann et al., 1995), fibronectin (Mosher and Proctor, 1980; Komarek et al., 1996) and cartilage matrix protein and the proteoglycan aggrecan (Hauser et al., 1996). Transglutaminases may also stabilize heterotypic collagen fibrils, and may even be involved in the activation of latent growth factors (Kojima et al., 1997). The current observation that two different forms of transglutaminase are present in hypertrophic cartilage, at least in the chicken, adds an additional level of regulation for modifying these matrix molecules during endochondral bone development. As the substrate specificity of the tissue and plasma forms of the enzyme are thought to be similar (Komarek et al., 1996), their respective roles may be quantitative rather than qualitative. It remains to be tested whether the changes effected by either enzyme alter calcification and/or removal of the hypertrophic matrix, which are its ultimate fates.

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References

Adany, R. 1996. Intracellular factor XIII: cellular distribution of factor XIII subunit a in humans. *Semin. Thromb. Hemost.* 22:399–408.

Aeschlimann, D., A. Wetterwald, H. Fleisch, and M. Paulsson. 1993. Expression of the tissue transglutaminase in skeletal tissues correlated with events of terminal differentiation of chondrocytes. *J. Cell Biol.* 120:1461–1470.

Aeschlimann, D., O. Kaupp, and M. Paulsson. 1995. Transglutaminase-catalyzed matrix cross-linking in differentiating cartilage: Identification of osteonectin as a major glutaminyl substrate. *J. Cell Biol.* 129:881–892.

Aeschlimann, D., D. Mosher, and M. Paulsson. 1996. Tissue transglutaminase and factor XIII in cartilage and bone remodeling. *Semin. Thromb. Hemost.* 22:437–443.

Aizawa, T., S. Kokubun, and Y. Tanaka. 1997. Apoptosis and proliferation of growth plate chondrocytes in rabbits. *J. Bone Jt. Surg. Br. Vol.* 79:483–486.

Ballestar, E., C. Abad, and L. Franco. 1996. Core histones are glutaminyl substrates for tissue transglutaminase. *J. Biol. Chem.* 271:18817–18824.

Ballock, R.T., A. Heydemann, L.M. Wakefield, K.C. Flanders, A.B. Roberts, and M.B. Sporn. 1993. TGF-b1 prevents hypertrophy of epiphyseal chondrocytes: regulation of gene expression for cartilage matrix proteins and metalloproteases. *Dev. Biol.* 158:414–429.

Beninati, S., D.R. Senger, E. Cordella-Miele, A.B. Mukherjee, I. Chackalaparampil, V. Shanmugam, K. Singh, and B.B. Mukherjee. 1994. Osteopontin: its transglutaminase-catalyzed posttranslational modifications and cross-linking to fibronectin. *J. Biochemistry (Tokyo).* 115:675–682.

Bini, A., Y. Itoh, B.J. Kudryk, and H. Nagase. 1996. Degradation of cross-linked fibrin by matrix metalloproteinase 3 (stromelysin 1): hydrolysis of the gamma Gly 404-Ala 405 peptide bond. *Biochemistry.* 35:13056–13063.

Borge, L., S. Demignot, and M. Adolphe. 1996. Type II transglutaminase expression in rabbit articular chondrocytes in culture: relation with cell differentiation, cell growth, cell adhesion and cell apoptosis. *Biochim. Biophys. Acta.* 1312:117–124.

Bowness, J.M., and A.H. Tarr. 1997. (gamma-Glutamyl)lysine crosslinks are concentrated in a non-collagenous microfibrillar fraction of cartilage. *Biochem. Cell Biol.* 75:89–91.

Chen, Q., and T.F. Linsenmayer. 1993. Distributions of fibronectin in the developing avian cartilagious growth plate. *In* Fourth International Conference on Limb Development and Regeneration (Part B). J.F. Fallon, P.F. Goetinck, R.O. Kelley, and D.L. Stocum, editors. John Wiley & Sons, Inc., New York. 495–504.

Cummings, M. 1996. Apoptosis of epithelial cells *in vivo* involves tissue transglutaminase upregulation. *J. Pathol.* 179:288–293.

Dean, D.D., O.E. Muniz, I. Berman, J.C. Pita, M.R. Carreno, J.F. Woessner, Jr., and D.S. Howell. 1985. Localization of collagenase in the growth plate of rachitic rats. *J. Clin. Invest.* 76:716–722.

Demignot, S., L. Borge, and M. Adolphe. 1995. Transglutaminase activity in rabbit articular chondrocytes in culture. *Biochim. Biophys. Acta.* 1266:163–170.

Fesus, L., A. Madi, Z. Balajthy, Z. Nemes, and Z. Szondy. 1996. Transglutaminase induction by various cell death and apoptosis pathways. *Experientia (Basel).* 52:942–949.

Galotto, M., G. Campanile, G. Robino, F. Descalzi-Cancedda, P. Bianco, and R. Cancedda. 1994. Hypertrophic chondrocytes undergo further differentiation to osteoblast-like cells and participate in the initial bone formation in developing chick embryo. *J. Bone Miner. Res.* 9:1239–1249.

Gibson, G.J., W.J. Kohler, and M.B. Schaffler. 1995. Chondrocyte apoptosis in endochondral ossification of chick sterna. *Dev. Dyn.* 203:468–476.

Gibson, G.J., D.L. Lin, and M. Roque. 1997. Apoptosis of terminally differenti-

ated chondrocytes in culture. *Exp. Cell Res.* 233:372–382.
Greenberg, C.S., P.J. Birckbichler, and R.H. Rice. 1991. Transglutaminases: multifunctional cross-linking enzymes that stabilize tissues. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 5:3071–3077.
Hauser, N., M. Paulsson, D. Heinegård, and M. Mörgelin. 1996. Interaction of cartilage matrix protein with aggrecan—Increased covalent cross-linking with tissue maturation. *J. Biol. Chem.* 271:32247–32252.
Jacobson, M.D., M. Weil, and M.C. Raff. 1997. Programmed cell death in animal development. *Cell.* 88:347–354.
Katoh, S., N. Nakagawa, Y. Yano, K. Satoh, H. Kohno, Y. Ohkubo, T. Suzuki, and K. Kitani. 1996. Hepatocyte growth factor induces transglutaminase activity that negatively regulates the growth signal in primary cultured hepatocytes. *Exp. Cell Res.* 222:255–261.
Kim, J.J., and H.E. Conrad. 1977. Properties of cultured chondrocytes obtained from histologically distinct zones of the chick embryo tibiotarsus. *J. Biol. Chem.* 252:8292–8299.
Kirsch, T., and K. Von der Mark. 1991. Ca^{2+} binding properties of type X collagen. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 294:149–152.
Kojima, S., T. Inui, H. Muramatsu, Y. Suzuki, K. Kadomatsu, M. Yoshizawa, S. Hirose, T. Kimura, S. Sakakibara, and T. Muramatsu. 1997. Dimerization of midkine by tissue transglutaminase and its functional implication. *J. Biol. Chem.* 272:9410–9416.
Komarek, D., T.C. Achyuthan, T.C. Rowland, P.J. Birckbichler, K.N. Lee, P. Bishop, and A.M. Achyuthan. 1996. Hierarchies in the binding of human factor XIII, factor XIIIa, and endothelial cell transglutaminase to human plasma fibrinogen, fibrin, and fibronectin. *Mol. Cell. Biochem.* 162:43–49.
Lai, T.S., T.F. Slaughter, C.M. Koropchak, Z.A. Haroon, and C.S. Greenberg. 1996. C-terminal deletion of human tissue transglutaminase enhances magnesium-dependent GTP/ATPase activity. *J. Biol. Chem.* 271:31191–31195.
Mosher, D.F., and R.A. Proctor. 1980. Binding and factor XIIIa-mediated cross-linking of a 27-kilodalton fragment of fibronectin to Staphylococcus aureas. *Science.* 209:927–929.
Muszbek, L., G. Haramura, and J. Polgar. 1995. Transformation of cellular factor XIII into an active zymogen transglutaminase in thrombin-stimulated platelets. *Thromb. Haemostasis.* 73:702–705.
Nemes, Z., Jr., R. Adány, M. Balázs, P. Boross, and L. Fésüs. 1997. Identification of cytoplasmic actin as an abundant glutaminyl substrate for tissue transglutaminase in HL-60 and U937 cells undergoing apoptosis. *J. Biol. Chem.* 272:20577–20583.
Nurminskaya, M., and T.F. Linsenmayer. 1996. Identification and characterization of up-regulated genes during chondrocyte hypertrophy. *Dev. Dynam.* 206:260–271.
Osdoby, P., and A.I. Caplan. 1981. First bone formation in the developing chick limb. *Dev. Biol.* 86:147–156.
Prince, C.W., D. Dickie, and C.L. Krumdieck. 1991. Osteopontin, a substrate for transglutaminase and factor XIII activity. *Biochem. Biophys. Res. Commun.* 177:1205–1210.
Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
Schmid, T.M., and H.E. Conrad. 1982*a*. A unique low molecular weight collagen secreted by cultured chick embryo chondrocytes. *J. Biol. Chem.* 257: 12444–12450.
Schmid, T.M., and H.E. Conrad. 1982*b*. Metabolism of low molecular weight collagen by chondrocytes obtained from histologically distinct zones of the chick embryo tibiotarsus. *J. Biol. Chem.* 257:12451–12457.
Schmid, T.M., and T.F. Linsenmayer. 1983. A short chain (pro)collagen from aged endochondral chondrocytes. *J. Biol. Chem.* 258:9504–9509.
Schmid, T.M., and T.F. Linsenmayer. 1985. Immunohistochemical localization of short chain cartilage collagen (type X) in avian tissues. *J. Cell Biol.* 100: 598–605.
Schmid, T.M., R.G. Popp, and T.F. Linsenmayer. 1990. Hypertrophic cartilage matrix: Type X collagen, supramolecular assembly, and calcification. *Ann. NY Acad. Sci.* 580:64–73.
Shinomura, T., and K. Kimata. 1992. Proteoglycan-Lb, a small dermatan sulfate proteoglycan expressed in embryonic chick epiphyseal cartilage, is structurally related to osteoinductive factor. *J. Biol. Chem.* 267:1265–1270
Shinomura, T., K. Kimata, Y. Oike, N. Maeda, S. Yano, and S. Suzuki. 1984. Appearance of distinct types of proteoglycan in a well-defined temporal and spatial pattern during early cartilage formation in the chick limb. *Dev. Biol.* 103:211–220.
Stocum, D.L., R.M. Davis, M. Leger, and H.E. Conrad. 1979. Development of the tibiotarsus in the chick embryo: biosynthetic activities of histologically distinct regions. *J. Embryol. Exp. Morphol.* 54:155–170.
Strathmann, M., B.A. Hamilton, C.A. Mayeda, M.I. Simon, E.M. Meyerowitz, and M.J. Palazzolo. 1991. Transposon-facilitated DNA sequencing. *Proc. Natl. Acad. Sci. USA.* 88:1247–1250.
Thacher, S.M. 1989. Purification of keratinocyte transglutaminase and its expression during squamous differentiation. *J. Invest. Dermatol.* 92:578–584.
Ueki, S., J. Takagi, and Y. Saito. 1996. Dual functions of transglutaminase in novel cell adhesion. *J. Cell Sci.* 109:2727–2735
von Heijne, G. 1986. A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* 14:4683–4690.
Wuthier, R.E., J.E. Chin, J.E. Hale, T.C. Register, L.V. Hale, and Y. Ishikawa. 1985. Isolation and characterization of calcium-accumulating matrix vesicles from chondrocytes of chicken epiphyseal growth plate cartilage in primary culture. *J. Biol. Chem.* 260:15972–15979.
Zhu, Y., L. Tassi, W. Lane, and M.E. Mendelsohn. 1994. Specific binding of the transglutaminase, platelet factor XIII, to HSP72. *J. Biol. Chem.* 269:22379–22384.