Expression of Tissue Transglutaminase in Skeletal Tissues Correlates with Events of Terminal Differentiation of Chondrocytes

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Abstract. Calcifying cartilages show a restricted expression of tissue transglutaminase. Immunostaining of newborn rat paw bones reveals expression only in the epiphyseal growth plate. Tissue transglutaminase appears first intracellularly in the proliferation/maturation zone and remains until calcification of the tissue in the lower hypertrophic zone. Externalization occurs before mineralization. Subsequently, the enzyme is present in the interterritorial matrix during provisional calcification and in the calcified cartilage cores of bone trabeculae. In trachea, mineralization occurring with maturation in the center of the cartilage is accompanied by expression of tissue transglutaminase at the border of the hydroxyapatite deposits.

Transglutaminase activity also shows a restricted distribution in cartilage, similar to the one observed for tissue transglutaminase protein. Analysis of tissue homogenates showed that the enzyme is present in growth plate cartilage, but not in articular cartilage, and recognizes a limited set of substrate proteins. Osteonectin is coexpressed with tissue transglutaminase both in the growth plate and in calcifying tracheal cartilage and is a specific substrate for tissue transglutaminase in vitro.

Tissue transglutaminase expression in skeletal tissues is strictly regulated, correlates with chondrocyte differentiation, precedes cartilage calcification, and could lead to cross-linking of the mineralizing matrix.

TRANSGLUTAMINASE (EC 2.3.2.13) is an enzyme that catalyzes a Ca^{2+}-dependent acyl-transfer reaction in which new γ-amide bonds are formed between γ-carboxamide groups of peptide-bound glutamine residues and various primary amines (for review see Lorand and Conrad, 1984; Greenberg et al., 1991). A glutamine residue serves as acyl-donor and the most common acyl-acceptors are primary amino groups of some naturally occurring polyamines, like putrescine or spermidine, or ϵ-amino groups of peptide-bound lysine residues. In the latter case, the reaction results in the formation of γ-glutamyl-ϵ-lysine cross-links in or between proteins. The number of glutaminyl substrates is highly restricted, while the tolerance to structural differences in acyl-acceptors is considerable.

Transglutaminases form a large protein family (Ichinose et al., 1990) and have a wide distribution amongst tissues and body fluids. Thus, proteins modified by transglutaminases are found throughout the organism, e.g., in fibrin clots in hemostasis and wound healing, in cell membranes of terminally differentiated erythrocytes, in extracellular matrices, and in the cornified envelope of the epidermis (for review see Greenberg et al., 1991). In some of these tissues, a role for transglutaminases in the apoptotic program has been postulated as enzymes of this class are accumulated in the cytoplasm of cells undergoing terminal differentiation both in vivo and in vitro (for review see Fésus et al., 1991).

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The abundant tissue transglutaminase is a monomeric globular protein with an M_r of ~77,000 (Ichinose et al., 1990; Greenberg et al., 1991). It is often highly expressed at sites of pathologic injury where it acts together with factor XIIIa, the plasma transglutaminase (Weinberg et al., 1991; Wiebe et al., 1991). Several extracellular proteins like fibrin(ogen) (Achyuthan et al., 1988; Shainoff et al., 1991), fibronectin (Fésus et al., 1986), vitronectin (Sane et al., 1988; Skorstengaard et al., 1990), nidogen/entactin (Aeschlimann and Paulsson, 1991; Aeschlimann et al., 1992), collagen type III N-propeptide (Bowness et al., 1987) and osteopontin (Prince et al., 1991) have been identified as specific glutaminyl substrates for tissue transglutaminase. Together with the observed association of tissue transglutaminase expression with programmed cell death and pathologic injury, the cross-linking of extracellular matrix components indicates a physiological function of the enzyme in maintaining the integrity of the tissue by fixation of the matrix at the site of the lesion (Upchurch et al., 1991).

During endochondral bone formation, the chondrocytes pass through a series of differentiation stages in which they undergo proliferation, a high rate of matrix synthesis, hypertrophy and calcification of the hypertrophied chondrocyte matrix (Hunziker and Schenk, 1989, and references therein). They are eventually removed from cartilage through tissue resorption, vascularization and bone remodeling. The calcified cartilage matrix serves as a substrate for initial osteogenesis.

Cartilage contains collagen type II, a fiber forming colla-

gen specific for cartilage and vitreous humor. The C-propeptide of type II collagen, termed chondrocalcin, is enriched in the epiphyseal growth plate and may play a role in cartilage calcification (Hinek et al., 1987). Several quantitatively minor collagens have been detected in cartilage. One of these, type X collagen, was shown to be exclusively expressed by hypertrophic chondrocytes in tissues undergoing endochondral bone formation (Schmid and Linsenmayer, 1985). Osteonectin is one of the most abundant noncollagenous proteins in bone (Termine et al., 1981a; Bolander et al., 1988), but may also be expressed in cartilage (Nomura et al., 1988; Metsäranta et al., 1989). It contains multiple calcium binding domains, one in the form of an EF-hand near the carboxyl terminus (Engel et al., 1987; Maurer et al., 1992), and might thus be involved in the mineralization process. The protein is also widely distributed in other tissues, although in lower amounts (Mason et al., 1986; Dziadek et al., 1986).

The present study addresses the distribution of tissue transglutaminase in skeletal tissues and the role of the enzyme in physiological cross-linking of the extracellular matrix. We show a correlation between the expression of tissue transglutaminase protein and transglutaminase activity with subsequent calcification of cartilage, and identify abundant proteins of cartilage and bone matrix, i.e., collagen type II and osteonectin as glutaminyl substrates for the enzyme.

Materials and Methods

Protein Reagents

Tissue transglutaminase was purified from guinea pig liver (Connellan et al., 1971) and osteonectin prepared from rat long bone by a modification of the method of Fisher et al. (1987). Native recombinant human BM-40/osteonectin (Nischt et al., 1991) was kindly provided by Dr. R. Timpl, Max-Planck-Institute for Biochemistry (Germany). Reduction and alkylation of guanidine-HCl/EDTA denatured recombinant BM-40/osteonectin was done as described (Aeschlimann et al., 1992). Human plasma fibronectin was a kind gift of Dr. K. Ingham, American Red Cross. BSA and N,N-dimethylcasein was from Serva (Heidelberg, Germany).

Histochemical Methods

Antisera to guinea pig tissue transglutaminase (Aeschlimann and Paulsson, 1991), rat osteonectin, and dansylated (hapten) *Limulus polyphemus* hemocyanin (carrier; Sigma Chemical Co., St. Louis, MO) were raised in rabbits and affinity purified when needed (Aeschlimann and Paulsson, 1991). Cycloheptaamylose·dansyl-chloride complex was prepared (Kinoshita et al., 1974) and proteins dansylated by addition of 20 mg complex per 10 mg protein in 1 ml of 0.1 M sodium phosphate buffer, pH 7.7. The mixture was stirred for 90 min in the dark and low molecular weight compounds removed by passage over a PD 10 column (Pharmacia, Uppsala, Sweden). The specificity of the antibodies was demonstrated in immunoblots of crude extracts of the tissue of origin.

Tissues were prepared for paraffin embedding by fixation in 4% (wt/vol) paraformaldehyde in PBS (8 mM sodium phosphate, pH 7.4, 0.15 M sodium chloride) at 4°C overnight and complete demineralization in 0.42 M EDTA, 0.5% (wt/vol) paraformaldehyde. For cryopreservation, tissues were embedded in Tissue-Tek® (Miles, Inc., Naperville, IL) and frozen on dry ice.

For immunohistochemistry, sections (5 μm) were cut, mounted on gelatine-coated slides and dried. Paraffin-embedded sections were prepared for immunolabeling by deparaffinization and rehydration in 50 mM Tris/HCl, pH 7.4, 0.15 M NaCl (TBS). Cryosections were used unfixed or after post-fixation (the expression, "post-fixation" is used to describe fixation of tissue sections that had been adsorbed onto slides and dried) in 4% (wt/vol) paraformaldehyde, acetone or methanol. To increase antibody penetration, sections were digested for 1 h with 40 mU/ml chondroitinase ABC (Sigma Chemical Co.) in TBS containing 0.01% (wt/vol) BSA where indicated. Endogenous peroxidase was blocked by incubation in methanol containing 1% (vol/vol) H_2O_2 and nonspecific antibody binding by treatment with 1% (wt/vol) BSA in TBS. Sections were treated with specific antibodies, nonimmune ChromPure rabbit IgG (Jackson ImmunoResearch Labs, West Grove, PA) or preimmune rabbit serum for 1 h, followed by peroxidase-conjugated swine anti-rabbit IgG (Dakopatts, Glostrup, Denmark) for 45 min. Antibodies were diluted in 1% BSA/TBS. The slides were developed with 3-amino-9-ethylcarbazole (Sigma Chemical Co.) /H_2O_2 and counterstained with Mayers Hämalaun (Merck, Darmstadt, Germany).

For detection of transglutaminase activity, unfixed BSA-treated cryosections were incubated for 1 h in 0.1 M Tris/HCl, pH 8.3, 5 mM $CaCl_2$, 12 μM monodansylcadaverine, with or without addition of 10.8 μg/ml of exogenous tissue transglutaminase (250 μl/section). Inhibition was done by addition of EDTA to 25 mM. Protein-bound label was detected with anti-dansyl antiserum as described above. Hydroxyapatite was shown by von Kossa's stain for calcium phosphate.

Radioactive Transglutaminase Assay

Transglutaminase-catalyzed incorporation of [1,4-^{3}H]putrescine (24.8 Ci/mmol, Amersham Corp., Amersham, UK) into putative substrate proteins was done for 30 min at 37°C as described previously (Aeschlimann and Paulsson, 1991).

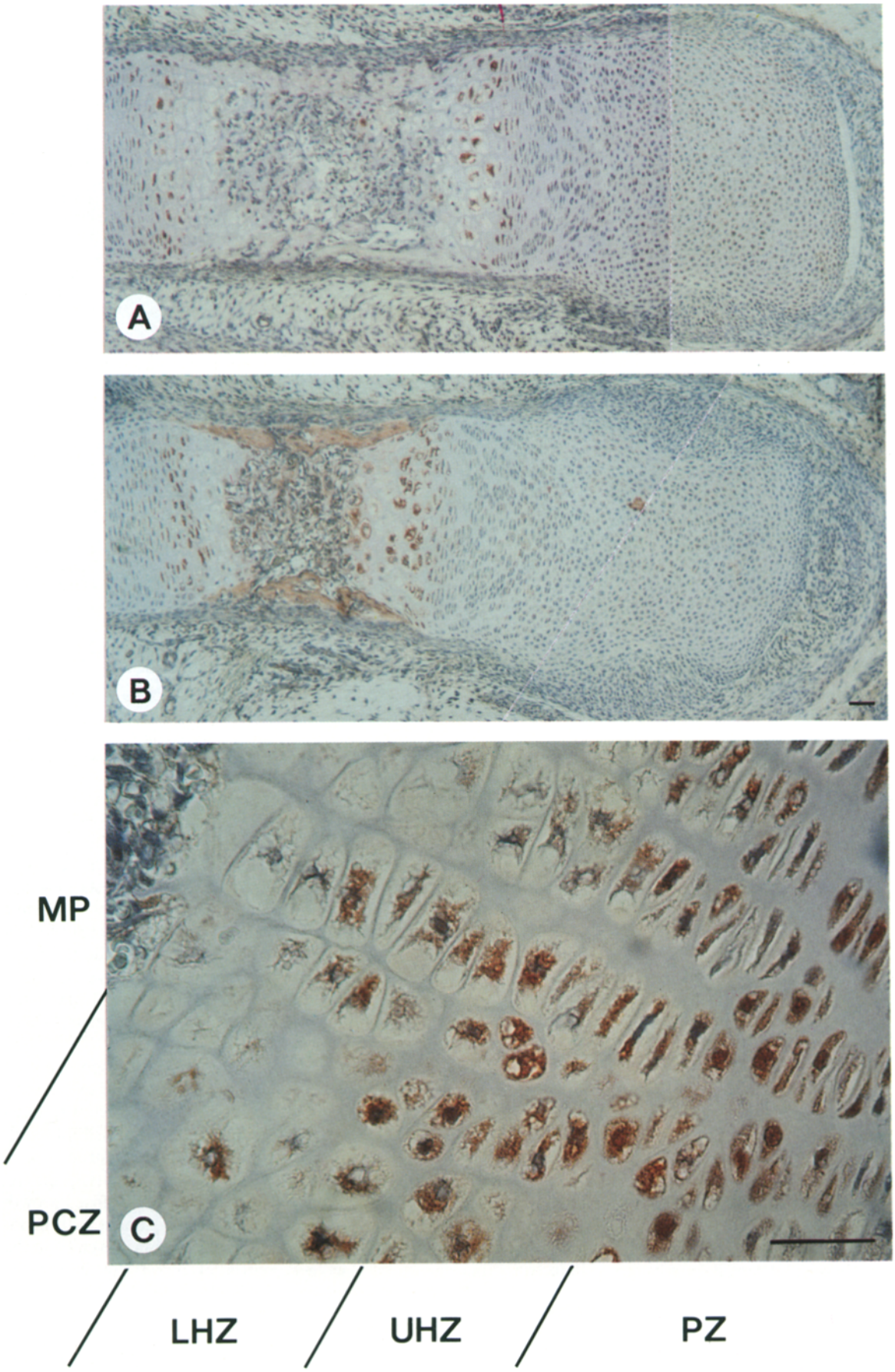
Detection of Transglutaminase Activity in Cartilage Homogenates

Articular and epiphyseal growth zone (Hinek et al., 1987) cartilage was isolated from calf tibial and femoral bones. Cartilage (0.25 g wet tissue/ml) was briefly homogenized in 0.1 M Tris/HCl, pH 8.3, containing 0.3 M NaCl, 50 mM $CaCl_2$, 5 mM dithiothreitol, the protease inhibitors phenylmethanesulfonyl fluoride (1 mM), benzamidine HCl (10 mM) and 6-aminohexanoic acid (0.1 M) and the transglutaminase substrate (amine donor) monodansylcadaverine (1 mM, Serva). To one half of each homogenate 1 mg exogenous tissue transglutaminase/g wet tissue was added and incubation was done at 37°C for 3 h. Homogenates incubated either in the presence or absence of exogenous tissue transglutaminase were centrifuged at 5,000 *g* for 15 min, the pellets washed with reaction buffer by repeated suspending and centrifugation, and the supernatants pooled to yield a total of 5 ml/g tissue of buffered salt extract. Pellets were extracted in a total of 5 ml/g tissue each of 4 M guanidine HCl/50 mM Tris/HCl, pH 6.0, containing protease inhibitors (see above), N-ethylmaleinimide (1 mM) and EDTA (0.1 M), first while shaking at room temperature for 75 min and then by washing as above. Insoluble material was washed in 0.5 M acetic acid and digested with porcine pepsin (Serva, 17 mAnson U/mg; 2 mg/g tissue wet weight) in a total of 3 ml/g tissue of 0.5 M acetic acid at 4°C for 14 h to solubilize type II collagen (Miller, 1971).

SDS-PAGE and Immunoblotting

SDS-PAGE (Laemmli, 1970) was done in 4–20% gradient gels. Proteins

Figure 1. Expression of tissue transglutaminase and osteonectin in developing long bones of 4-d old rat. Serial sections (5 μm) of fixed, decalcified and paraffin-embedded tissue, showing a tarsal bone (*A* and *B*) or the distal growth plate of tibia (*C*), were incubated with antibodies to tissue transglutaminase (*A* and *C*) or osteonectin (*B*), developed with peroxidase-conjugated secondary antibodies (3-amino-9-ethylcarbazole/H_2O_2 used as peroxidase substrate solution: *brown stain*), and counterstained with Mayers Hämalaun (*blue stain*). (Negative controls for immunochemical results are not shown but were submitted to the journal and available to the referees.) In *B*, the section was digested with chondroitinase ABC before incubation with the antibody. The differentiation stages of the chondrocytes were assigned according to the cellular morphology and are indicated on the left and below: proliferation zone (*PZ*), upper hypertrophic zone (including the maturation zone) (*UHZ*), lower hypertrophic zone (*LHZ*) with the zone of provisional calcification (*PCZ*; cartilage calcification was assessed by von Kossa staining on cryosections of the same tissue), and the metaphysis (*MP*). Bars, 50 μm.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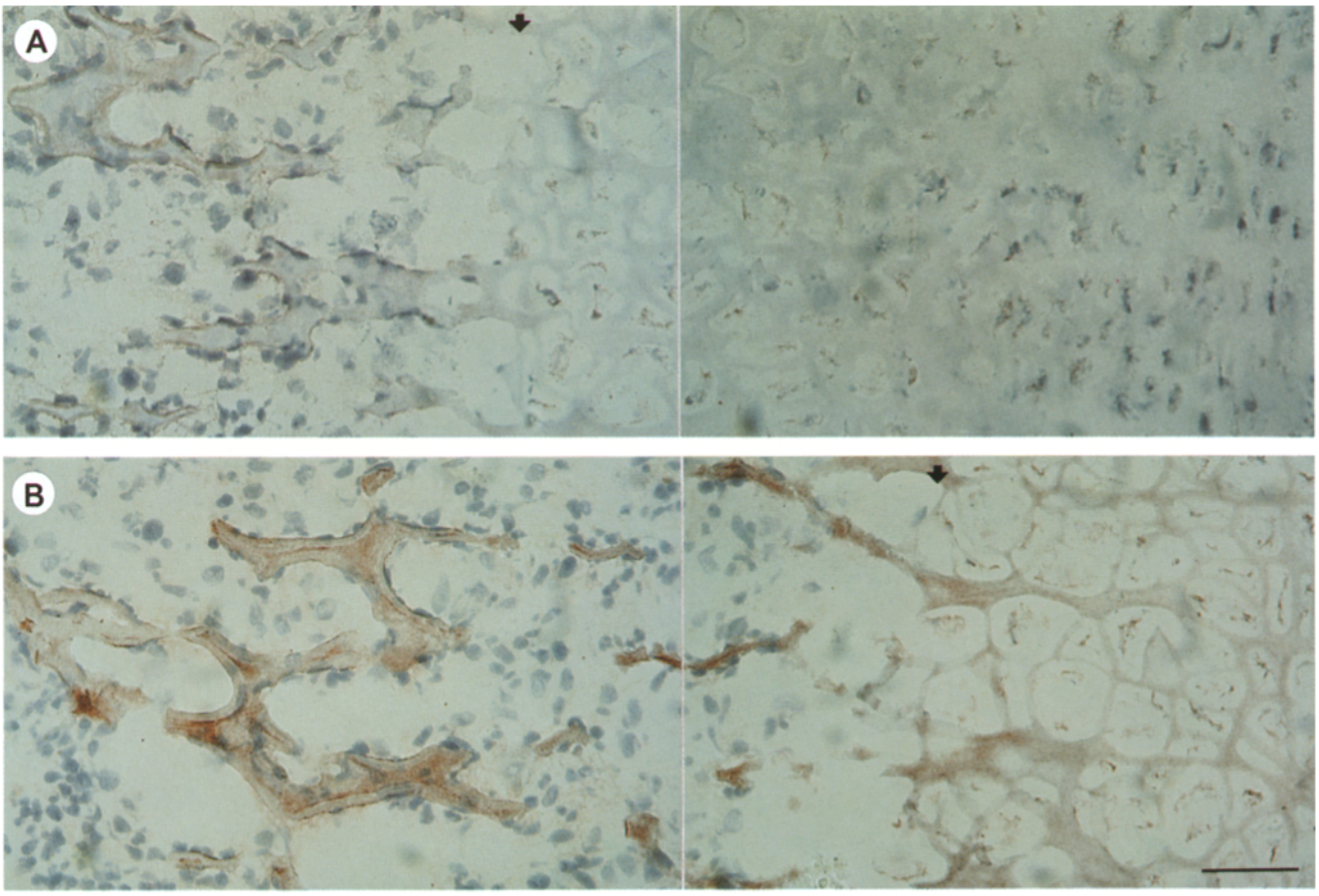

Figure 2. Tissue transglutaminase antigen in the growth plate matrix is revealed by chondroitinase ABC digestion. Unfixed cryosections (5 μm) showing the distal growth plate of tibia of a 4-d old rat were stained with antibodies to tissue transglutaminase without (*A*) or with (*B*) prior treatment with chondroitinase ABC. (Negative controls for immunochemical results are not shown but were submitted to the journal and available to the referees.) The arrows indicate the border between the lower hypertrophic zone of the growth plate and the metaphysis. Bar, 50 μm.

reduced with 2-mercaptoethanol (1%, vol/vol) were detected by staining with Coomassie brilliant blue R or after transfer onto nitrocellulose (Towbin et al., 1979). Binding of primary antibody was visualized using peroxidase-conjugated swine anti-rabbit IgG (Dakopatts) and 4-chloro-1-naphthol as the chromogenic agent.

Results

Expression of Tissue Transglutaminase and Osteonectin in Long Bones

The distribution of tissue transglutaminase was studied in the tarsal and metatarsal bones as well as in the distal portion of the tibia from 1–4-d old rats. Affinity purified antibodies were used in combination with immunoperoxidase staining. Tissue transglutaminase was found to be preferentially expressed in the growth plate. An acceptable preservation of the cellular morphology and retention of the intracellular material was obtained in fixed, paraffin-embedded sections. Tissue transglutaminase is detected mainly intracellularly, and appears first in the proliferation/maturation zone of the growth plate (Fig. 1 *A*). Subsequently, the enzyme is selectively accumulated in the cytoplasm of the cells during the maturation steps. Intracellular expression of the enzyme ceases in the lower layers of hypertrophic chondrocytes before calcification of the tissue (Fig. 1 *C*). When sections were digested with chondroitinase ABC to remove chondroitin/dermatan sulfate chains from the matrix, the staining pattern was similar to that without enzyme treatment. (Negative controls for immunohistochemical results are not shown but were submitted to the journal and available to the referees.) Some cells in the resting cartilage became positive, but much more weakly so than those in the growth plate. Thus, tissue transglutaminase might be expressed also by resting chondrocytes, but if so at low levels.

The disappearance of intracellular tissue transglutaminase before calcification, led us to try to demonstrate an extracellular pool by use of frozen sections. In such sections, much intracellular material is lost, but a better accessibility and/or antigenicity of the extracellular matrix structures is achieved. The remaining cellular immunoreactivity for tissue transglutaminase was restricted to the collapsed growth plate chondrocytes (Fig. 2 *A*). Upon treatment with chondroitinase ABC, tissue transglutaminase was revealed in the interterritorial cartilage matrix of growth plate and the calcified cartilage cores of trabeculae (Fig. 2 *B*). The expression appears as a gradient in the hypertrophic zone and reaches maximal intensity in the zone of provisional calcification. The enzyme is externalized and incorporated into the extracellular matrix. It persists in the calcified carti-

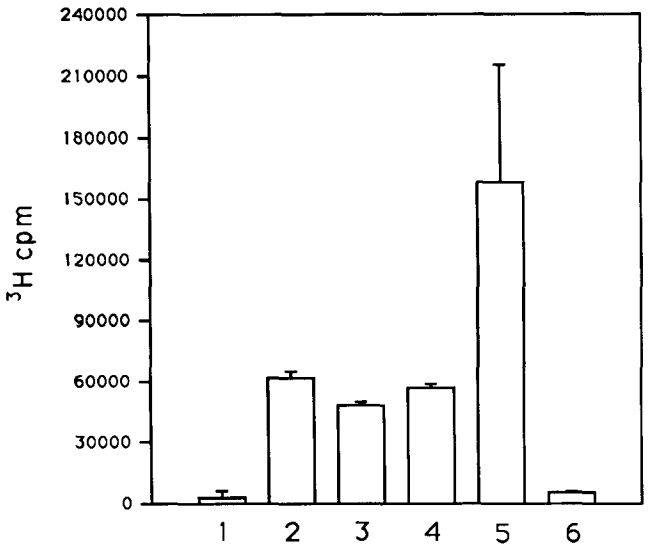

Figure 3. Tissue transglutaminase catalyzed incorporation of [^{3}H]putrescine into osteonectin and collagen type II. The incorporation of [^{3}H]putrescine into rat bone osteonectin/BM-40 (*1*), human recombinant osteonectin/BM-40 (*2*), bovine articular collagen type II (pepsin fragment) (*3*) was compared to the incorporation into the reference proteins bovine milk N,N-dimethylcasein (*4*; contains one amine incorporation site), human plasma fibronectin (*5*; contains several amine incorporation sites) and BSA (*6*; does not contain any amine incorporation sites) as previously described (Aeschlimann and Paulsson, 1991). All values are the average of three to six independent measurements. The bars indicate the standard deviation from mean values.

lage cores of trabeculae until it is removed by bone remodeling (Fig. 2 *B*). Tissue transglutaminase was detectable neither in mineralized matrix of cortical or trabecular bone nor in newly deposited osteoid along the trabecular surface (Figs. 1 *A*, 2, *A* and *B*). "Post-fixation" of the tissue before chondroitinase ABC digestion abolished the unmasking effect, explaining the absence of extracellular immunoreactivity in paraffin sections.

Osteonectin is predominantly present in cortical and trabecular bone matrix, but is similarly to tissue transglutaminase also expressed by growth plate chondrocytes (Fig. 1 *B*). Osteonectin is seen first in the maturation/upper hypertrophic zone. It appears slightly later in the chondrocyte differentiation program than tissue transglutaminase.

Native Osteonectin is a Transglutaminase Substrate

The coexpression of tissue transglutaminase and osteonectin in the growth plate led us to investigate whether osteonectin is a transglutaminase substrate. This was done by incubation of purified tissue transglutaminase with either osteonectin (also known as BM-40 [Dziadek et al., 1986] or SPARC [Mason et al., 1986]) purified from rat bone (Fig. 3, bar *1*) or with the recombinant human protein (Fig. 3, bar 2) in the presence of [^{3}H]putrescine and Ca^{2+} (Aeschlimann and Paulsson, 1991). Isolation of osteonectin from rat bone had been done by decalcification with EDTA in the presence of the denaturing agent guanidine HCl, and this protein preparation was inactive in the assay. Human recombinant osteonectin/BM-40 had been purified avoiding denaturing steps, and incorporated the radioactive amine. Treatment of native recombinant osteonectin with guanidine HCl and EDTA led to 89–95% loss in [^{3}H]putrescine incorporation and reduction and alkylation of the denatured recombinant protein completely abolished incorporation. Thus, osteonectin/BM-40 has to be in the native conformation to act as a glutaminyl substrate for tissue transglutaminase. The fact that osteonectin is a substrate supports the notion that the enzyme may act in the growth plate by cross-linking matrix proteins.

Distribution of Transglutaminase Activity in Long Bones

Transglutaminase activity was detected on unfixed cryosections of the distal growth plate of the tibia by incubation with the substrate monodansylcadaverine and with Ca^{2+} either in presence or absence of exogenous enzyme (Aeschlimann and Paulsson, 1991). Monodansylcadaverine that had been incorporated into protein was subsequently detected by antibodies raised against the dansyl group. The distribution of enzyme activity in cartilage corroborated the previous localization of tissue transglutaminase protein. In the absence of exogenous enzyme, the activity was found only in chondrocytes and matrix of the growth plate (Fig. 4 *A*). The activity gradually increases with successive steps of chondrocyte differentiation, but could not be detected in the calcified cartilage cores of bone trabeculae, indicating that the immunoreactive enzyme present in this compartment (Fig. 2 *B*) is inactive due to entrapment in hydroxyapatite. Transglutaminase activity was also detected in newly deposited osteoid. This result, together with the demonstration that tissue transglutaminase antigen is absent from mineralized bone matrix and osteoid (Fig. 2 *B*), indicates that osteoblasts and/or bone marrow cells express a transglutaminase immunochemically distinct from the tissue type enzyme. Incubation of sections with exogenous tissue transglutaminase gave a homogenous strong staining, showing that glutaminyl substrates are present throughout the cartilage (Fig. 4 *B*). This demonstrates that the confined distribution of endogenous transglutaminase activity is indeed due to a restricted expression of the enzyme and not of the glutaminyl substrate proteins. Because transglutaminase activity is strongly Ca^{2+}-dependent, the specificity of the assay was demonstrated by inhibition with EDTA. (Negative controls for immunohistochemical results are not shown but were submitted to the journal and available to the referees.)

Transglutaminase Activity and Substrate Proteins in Homogenates of Epiphyseal Growth Plate and Articular Cartilage

Homogenates of bovine epiphyseal growth plate and of bovine articular cartilage were incubated in Ca^{2+}-containing buffer with the transglutaminase substrate monodansylcadaverine. Incubation was done either in the absence or presence of exogenous tissue transglutaminase to detect both intrinsic enzyme activity and substrate proteins that can be labeled by excess exogenous enzyme. After labeling, the homogenates were sequentially extracted with a buffered salt solution (extract I) and a guanidine HCl/EDTA solution (extract II). The residue was digested with pepsin to solubilize the triple helical domains of cartilage collagens, mainly type II collagen (extract III). Analysis by SDS-PAGE followed by

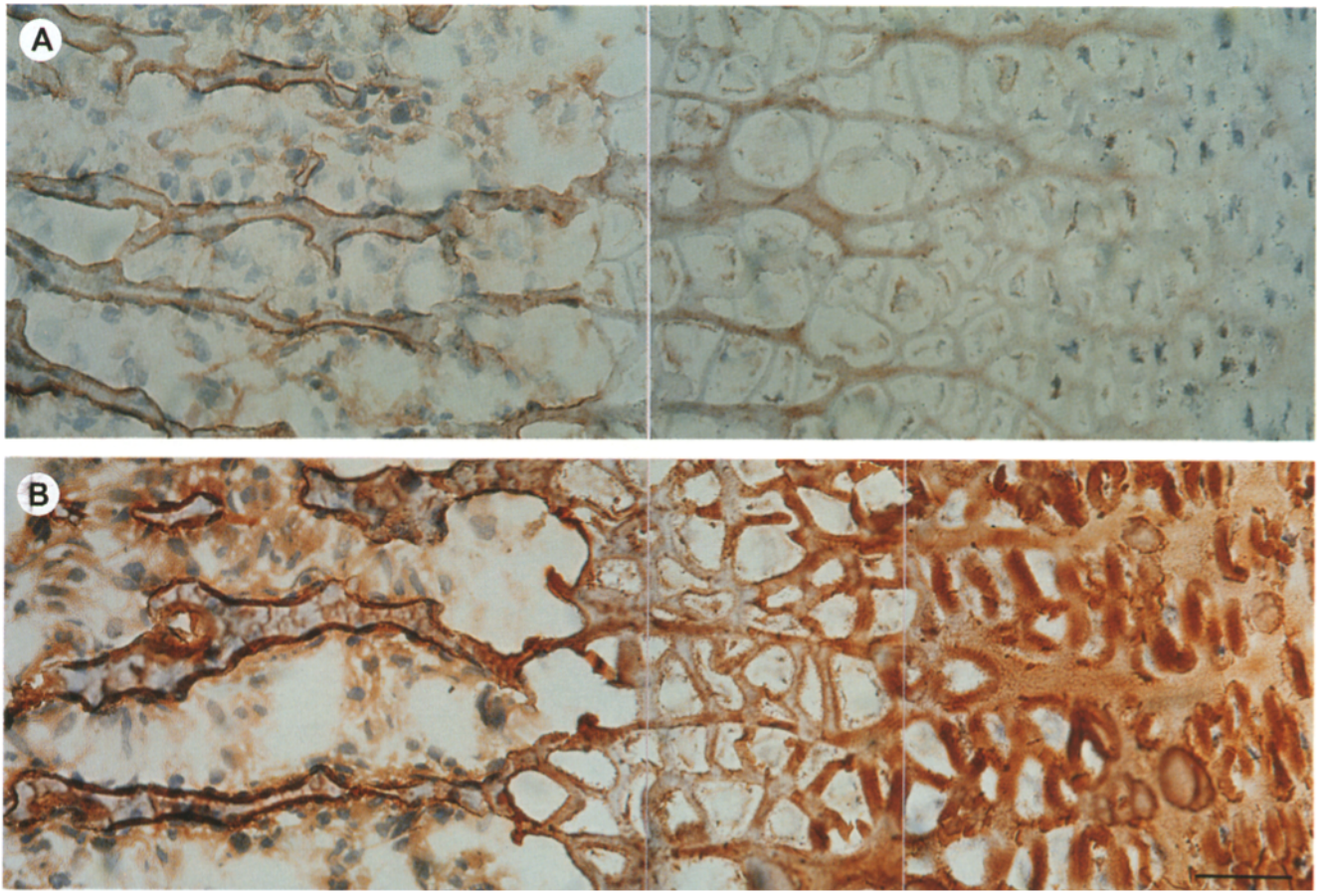

Figure 4. Detection of transglutaminase activity present in sections of the distal growth plate of the tibia of 4-d old rat. Unfixed cryosections (5 μm) were incubated with monodansylcadaverine alone (*A*) or monodansylcadaverine together with purified tissue transglutaminase (*B*) in Ca^{2+}-containing buffer. Protein-bound monodansylcadaverine was subsequently detected by use of anti-dansyl antiserum and peroxidase-conjugated secondary antibodies. (Negative controls for immunochemical results are not shown but were submitted to the journal and available to the referees.) Bar, 50 μm.

Coomassie-staining (Fig. 5 *A*) or immunoblotting with anti-dansyl antiserum (Fig. 5, *B–D*) revealed that many proteins of different M_r are able to incorporate the labeled amine when treated with exogenous tissue transglutaminase, both in the epiphyseal growth zone and articular cartilage (Fig. 5, *C* and *D*). The band patterns obtained from the two tissues were comparable, indicating that a similar set of proteins act as glutaminyl substrates for tissue transglutaminase in both tissues. Collagen type II was heavily labeled and appears to be a glutaminyl substrate in the cartilage matrix (Fig. 5, *C* and *D*; extract III). Similarly, in vitro incubation of collagen type II and tissue transglutaminase in the presence of [^{3}H]putrescine and Ca^{2+} led to an incorporation of the radioactive amine into collagen type II (Fig. 3, bar *3*). Tissue transglutaminase is itself able to act as a glutaminyl substrate (Birckbichler et al., 1977; Aeschlimann and Paulsson, 1991) and is therefore also labeled in the cartilage extracts where exogenous enzyme had been added (Fig. 5, *C* and *D*; extract I, II).

Labeled proteins were detected in the extract from growth plate cartilage also without the addition of exogenous tissue transglutaminase (Fig. 5 *B*), but not in the extract from articular cartilage (results not shown). Endogenous enzyme is either absent from articular cartilage or present in quantities not detectable with this assay. It cannot be excluded that some of the endogenous transglutaminase activity observed in the growth plate homogenate may be due to a contamination of blood plasma derived factor XIIIa. However, the use of protease inhibitors directed at thrombin makes an activation of factor XIII unlikely. The antibodies used in immunolocalization of tissue transglutaminase in the rat growth plate do not cross-react with factor XIII (Aeschlimann and Paulsson, 1991). Tissue transglutaminase, which does not require cleavage by thrombin for activation, must therefore be present. The endogenous transglutaminase showed a more restricted substrate pattern (Fig. 5 *B*) and only a subset of those proteins detected by the exogenous enzyme (Fig. 5 *C*) were labeled. A predominantly labeled protein of apparent M_r ~30,000 was present in the growth plate but not in articular cartilage (Fig. 5 *B*; extract I, II; *asterisk*). Collagen type II was not a major glutaminyl substrate for the endogenous enzyme under the conditions used (Fig. 5 *B*; extract III). Possibly, the affinity of the enzyme for collagen type II might be low compared to other substrate proteins, and the level of endogenous enzyme in the growth plate insufficient to give saturation labeling. There might also be differences in the substrate specificity of the endogenous enzyme in growth plate cartilage and of liver tissue transglutaminase,

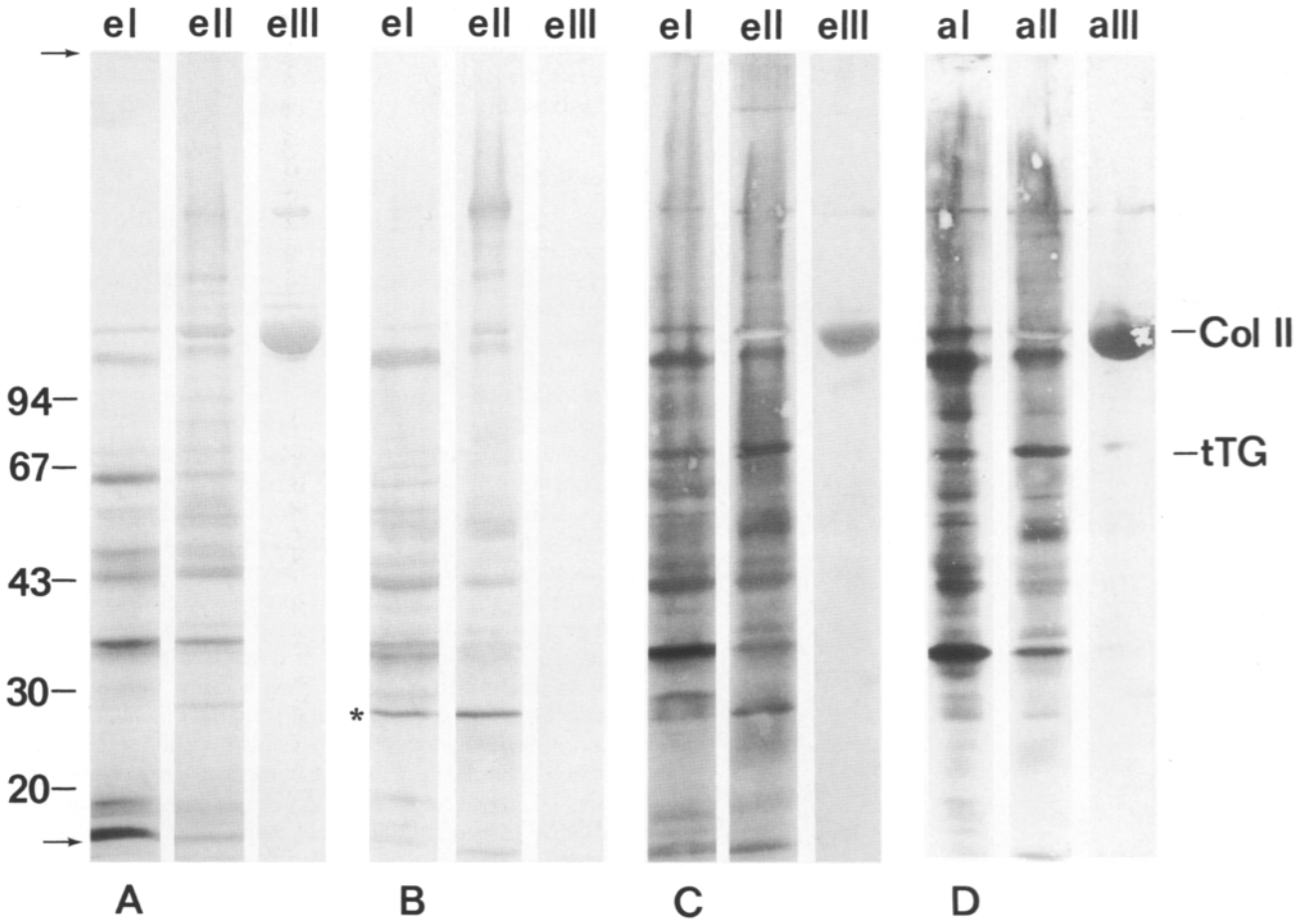

Figure 5. Transglutaminase activity and protein substrates present in homogenates of growth plate cartilage (*e*) and articular cartilage (*a*). Homogenates derived from either growth plate (*A–C*) or articular cartilage (*D*) were incubated with monodansylcadaverine either in the absence (*A*, *B*) or presence (*C*, *D*) of purified tissue transglutaminase, sequentially extracted with buffered salt (*I*), guanidine/EDTA (*II*), and finally digested with pepsin (*III*), and subsequently analyzed by SDS-PAGE on 4–20% gradient gels under reducing conditions. The gels were stained either with Coomassie brilliant blue R (*A*) or after electrophoretic transfer to nitrocellulose by incubation with antidansyl antiserum (*B–D*). M_r standards as well as the top and bottom of the separating gel (*arrows*) are indicated on the left. Bands assigned to collagen type II (*Col II*) and tissue transglutaminase (*tTG*) are indicated on the right. The prominent glutaminyl substrate protein of the endogenous transglutaminase ($M_r \sim 30{,}000$) is marked with an asterisk (*B*).

even though the enzymes share epitopes and antibodies cross-react strongly.

Tissue Transglutaminase Expression in Cartilages that Do Not Undergo Bone Formation and in Bones Formed by Intramembraneous Ossification

Unfixed cryo-sections of 4-d old or adult rat trachea were assayed for tissue transglutaminase antigen or enzyme activity. The staining patterns were compared to the ones obtained with antibodies against osteonectin. In 4-d old rats, neither tissue transglutaminase nor osteonectin is expressed in the tracheal cartilage rings. (Negative controls for immunohistochemical results are not shown but were submitted to the journal and available to the referees.) This is in agreement with the observation that resting chondrocytes in long bones do not express osteonectin nor tissue transglutaminase. Surprisingly, in adult tracheal cartilage substantial amounts of osteonectin were found in the center of the rings (Fig. 6 *A*). This could be correlated to calcification of the cartilage as seen with von Kossa's stain for calcium phosphate (Fig. 6 *C*). Mineralization starts in the center of the tracheal rings and proceeds outwards with increasing age of the animal. Moreover, tissue transglutaminase expression was also observed in adult trachea, either as intense spots corresponding to single cells or as a faint staining of the calcified cartilage matrix (Fig. 6 *B*). Careful comparison of the staining patterns for tissue transglutaminase and calcium phosphate deposits revealed that cells expressing the enzyme in large quantities are located at the calcification border (Fig. 6, *B* and *C*). These cells probably follow a differentiation program similar to that of growth plate chondrocytes, with the exception that no subsequent osteogenesis takes place. Similar results were obtained by staining tracheal sections for enzyme activity. Only adult rats were positive. Endogenous activity was present in the central, but not in the peripheral region of the tracheal rings. The activity was cell associated in regions not yet calcified and extracellular in the calcified regions of the cartilage (results not shown).

Similar to cortical bone and the bone matrix of trabeculae, the calvaria showed no expression of tissue transglutaminase protein (results not shown). In contrast, endogenous enzyme activity was present at high levels in newly deposited osteoid

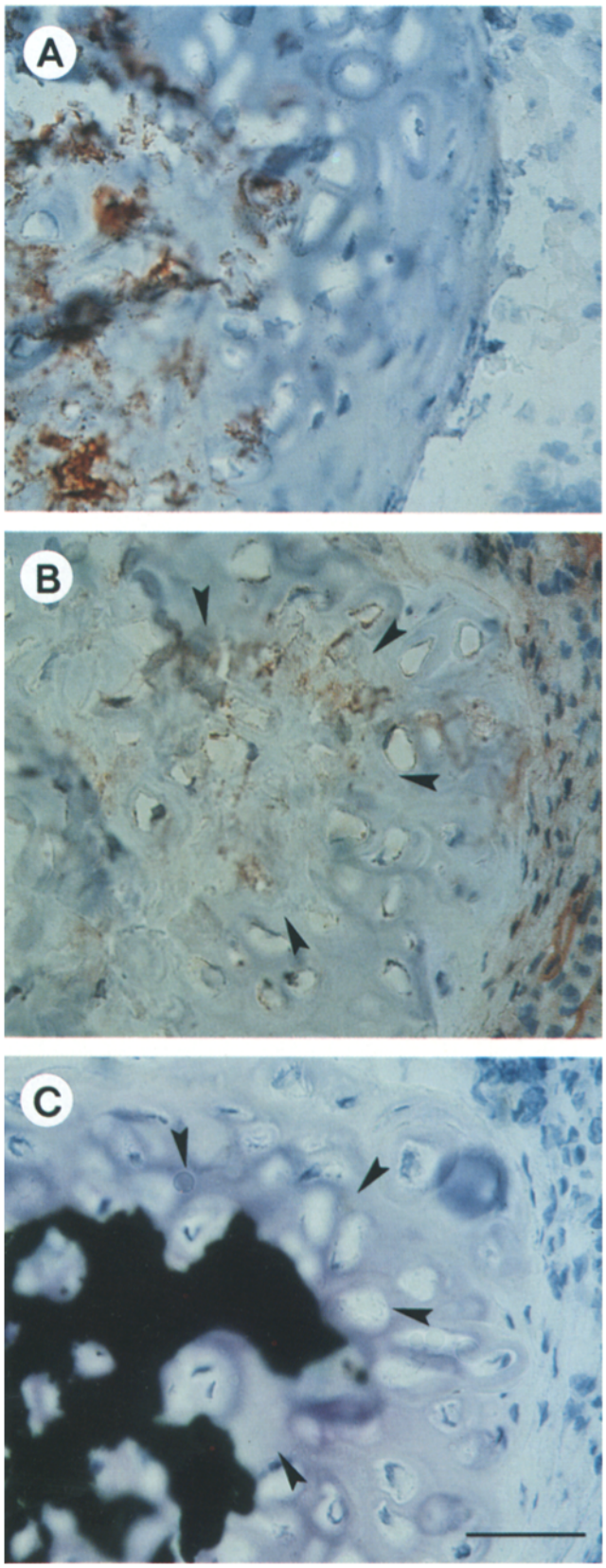

Figure 6. Expression of tissue transglutaminase and osteonectin in calcified tracheal cartilage. Unfixed cryosections (5 μm) of adult rat trachea were stained with antibodies directed to osteonectin (*A*) or tissue transglutaminase (*B*) (negative controls for immunochemical results are not shown but were submitted to the journal and available to the referees), or were stained for hydroxyapatite with von Kossa's stain for calcium phosphate (*C*). In *B* and *C* is the same field on serial sections shown, with arrowheads marking identical positions. Bar, 50 μm.

(results not shown), in agreement with the distribution in long bone trabeculae (Fig. 4 *A*). This further supports the hypothesis that osteoblasts express a different member of the transglutaminase family.

Discussion

In the present study we investigated the expression of tissue transglutaminase in skeletal tissues. We found a correlation between the expression of the enzyme and the terminal differentiation of chondrocytes, both in endochondral bone formation and in the calcification of tracheal cartilage occurring with maturation.

In long bones, tissue transglutaminase expression is induced in chondrocytes in the proliferation or maturation zone and the protein is subsequently accumulated in the cytoplasm of the cells during further differentiation steps. Thus, tissue transglutaminase protein is highly enriched in hypertrophic chondrocytes, and is subsequently released in the zone of provisional calcification. Externalization of the enzyme might possibly be due to physiologically occurring cell death or occur by an alternative way of secretion. Conventional secretion is unlikely as tissue transglutaminase, as well as factor XIII, does not have a signal peptide, is not glycosylated and does not contain disulfide bonds (Ichinose et al., 1990). One possible mechanism would be release of membrane-bound vesicles with cytoplasm-derived contents. So called matrix vesicles have been described in cartilage and other tissues and have been implicated in calcification (Wu et al., 1991). It is likely that the enzyme becomes activated at the elevated extracellular Ca^{2+} concentrations encountered upon externalization. Specific glutaminyl substrates for tissue transglutaminase like fibronectin (Fésus et al., 1986), osteopontin (Prince et al., 1991) and the N-propeptide of collagen type III (Bowness et al., 1987) are present in the cartilage and/or bone matrix (Heinegård and Oldberg, 1989; Keene et al., 1991). Native osteonectin/BM40 and collagen type II, which are present in the matrix of the growth plate, are additional glutaminyl substrates for tissue transglutaminase, at least under in vitro conditions. The presence of specific substrates in the matrix of the growth plate, the observed redistribution of tissue transglutaminase from an intracellular pool to the cartilage matrix during calcification, and the demonstration that the externalized enzyme is active, indicates a role for the enzyme in cross-linking of the matrix during terminal differentiation of chondrocytes. The process of accumulation and consecutive activation of tissue transglutaminase during differentiation of chondrocytes resembles the well-characterized events occurring in ageing of erythrocytes and terminal differentiation of epidermal keratinocytes. In leaking erythrocytes, tissue transglutaminase cross-links integral plasma membrane proteins and cytoskeletal proteins underlying the plasma membrane to a rigid coat (Lorand and Conrad, 1984), and in epidermis the keratinocyte transglutaminase cross-links a subset of cytoplasmic proteins, e.g., involucrin and loricrin, to form the cornified envelope of the epidermis (Thacher and

Rice, 1985; Simon and Green, 1988; Hohl et al., 1991). The function of this sealing process might differ between tissues and physiological situations. It might play a role in preventing leakage of substances influencing cell growth, proliferation and differentiation. The sealing might also function in maintaining the mechanical stability of the tissue and in scar formation.

In tracheal cartilage, a tissue that does not undergo proper osteogenesis, we observed that calcification occurs with maturation, as previously described by other investigators (Bonucci et al., 1974). Calcification starts in the center of the rings and proceeds towards the periphery. It was accompanied by prominent expression of osteonectin as well as expression of tissue transglutaminase, analogous to the pattern observed in the growth plate. Tissue transglutaminase antigen and activity was observed most prominently in the cartilage surrounding the hydroxyapatite deposits, which shows some similarity to the hypertrophic cartilage of growth plate. Thus, tissue transglutaminase and osteonectin expression are intimately associated with hypertrophy of chondrocytes and calcification of cartilage tissue, but not necessarily with bone formation. There might be a second, immunochemically distinct transglutaminase acting in bone, as indicated by the fact that in newly deposited osteoid in long bones and in calvaria enzyme activity was observed but tissue transglutaminase protein could not be detected.

We observed osteonectin in hypertrophic chondrocytes of growth plate cartilage, in osteoblasts, in newly deposited osteoid and mineralized bone matrix of long bones and calvaria. This agrees with the high level of expression of the osteonectin gene found in growth plate cartilage, in bone tissue of long bones and calvaria, and in the perichondrium and periosteum (Nomura et al., 1988; Metsäranta et al., 1989). In situ hybridization of developing long bones revealed that osteoblasts and hypertrophic chondrocytes contain a high level of mRNA whereas resting chondrocytes do not. In agreement, we did not observe any staining with the anti-osteonectin antiserum in the resting cartilage of developing long bones. Osteonectin has been proposed to have affinity for collagen and hydroxyapatite, and a potential role in the mineralization process was suggested (Termine et al., 1981*b*). As the major cartilage collagen as well as osteonectin and fibronectin (Fésus et al., 1986) are specific substrates for tissue transglutaminase, it is likely that these proteins are cross-linked upon release and activation of tissue transglutaminase in growth plate. It is quite possible that the cross-linking of the organic matrix influences the structure and mechanical properties of the bone formed.

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