Transglutaminase-catalyzed Matrix Cross-linking in Differentiating Cartilage: Identification of Osteonectin as a Major Glutaminyl Substrate

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Abstract. The expression of tissue transglutaminase in skeletal tissues is strictly regulated and correlates with chondrocyte differentiation and cartilage calcification in endochondral bone formation and in maturation of tracheal cartilage (Aeschlimann, D., A. Wetterwald, H. Fleisch, and M. Paulsson. 1993. *J. Cell Biol.* 120:1461–1470). We now demonstrate the transglutaminase reaction product, the γ-glutamyl-ε-lysine cross-link, in the matrix of hypertrophic cartilage using a novel cross-link specific antibody. Incorporation of the synthetic transglutaminase substrate monodansylcadaverine (amine donor) in cultured tracheal explants reveals enzyme activity in the pericellular matrix of hypertrophic chondrocytes in the central, calcifying areas of the horseshoe-shaped cartilages. One predominant glutaminyl substrate (amine acceptor) in the chondrocyte matrix is osteonectin as revealed by incorporation of the dansyl label in culture. Indeed, nonreducible osteonectin-containing complexes of ~65, 90, and 175 kD can be extracted from mature tracheal cartilage. In vitro cross-linking of osteonectin by tissue transglutaminase gives similar products of ~90 and 175 kD, indicating that the complexes in cartilage represent osteonectin oligomers. The demonstration of extracellular transglutaminase activity in differentiating cartilage, i.e., cross-linking of osteonectin in situ, shows that tissue transglutaminase-catalyzed cross-linking is a physiological mechanism for cartilage matrix stabilization.

Long bone development and growth occurs by endochondral ossification (Hunziker and Schenk, 1989; Solursh, 1989). In this process chondrocytes pass through a series of differentiation stages in which they rapidly proliferate, synthesize matrix constituents at high rate and become hypertrophic, before the cartilage matrix calcifies. The calcified matrix is subsequently replaced by bone. The differentiating chondrocytes also change their repertoire of biosynthetic products and express e.g., collagen X, osteopontin, and osteonectin (for references see Aeschlimann et al., 1993). Rat tracheal cartilage undergoes maturation and calcification in a manner resembling chondrocyte differentiation during endochondral bone formation (Aeschlimann et al., 1993), with the exception that matrix calcification is not followed by cartilage resorption, vascularization, and bone formation.

We previously reported that tissue transglutaminase is synthesized and externalized by terminally differentiating chondrocytes both in endochondral bone formation and in maturation of tracheal cartilage (Aeschlimann et al., 1993). The transglutaminase catalyzes the posttranslational modification of proteins referred to as R-glutamyl-peptide, amine-γ-glutamyl transfer reaction (EC 2.3.2.13). The Ca^{2+}-dependent reaction results in the formation of new γ-amide bonds between γ-carboxamide groups of peptide-bound glutamine residues and various primary amines (for review see Folk and Finlayson, 1977; Lorand and Conrad, 1984). Most commonly, γ-glutamyl-ε-lysine cross-links are formed in or between proteins by reaction with ε-amino groups of peptide-bound lysine residues. Osteonectin is a potent in vitro glutaminyl substrate for tissue transglutaminase and is coexpressed with the enzyme in differentiating cartilage (Aeschlimann et al., 1993). It appears that tissue transglutaminase may function in cartilage by cross-linking matrix proteins like osteonectin before mineralization of the tissue.

Osteonectin was initially identified as one of the most abundant noncollagenous proteins in bone (Termine et al., 1981*a*; Bolander et al., 1988), but is also widely distributed in other tissues, although in lower amounts (Dziadek et al., 1986; Mason et al., 1986; Stenner et al., 1986; Lankat-Buttgereit et al., 1988). It has been implicated in the miner-

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alization of cartilage and bone because it contains multiple calcium-binding domains, one of them in the form of an EF-hand (Engel et al., 1987; Maurer et al., 1992), and appears to have affinity for hydroxy-apatite (Termine et al., 1981*b*). However, as osteonectin is not bone specific, this line of reasoning is insufficient to explain all biological functions of this protein, and tissue-specific posttranslational modifications may modulate some of its functions (Kelm and Mann, 1991).

Transglutaminases form a large protein family, with members specialized for protein cross-linking in different biological systems (Aeschlimann and Paulsson, 1994). Examples are the stabilization of the fibrin clot in hemostasis by the activated plasma transglutaminase, factor XIIIa, and the role of transglutaminases in the process of physiological cell death, e.g., in the formation of the cornified envelope by epidermal transglutaminases in keratinocyte terminal differentiation, and in the cross-linking of membrane and cytoskeletal components by tissue transglutaminase in aging of erythrocytes (Lorand and Conrad, 1984; Aeschlimann and Paulsson, 1994). Tissue transglutaminase may also play a role in programmed death of other cells since it accumulates in the cytoplasm of e.g., hepatocytes undergoing terminal differentiation both in vivo and in vitro (for review see Fésus et al., 1991). There is also increasing evidence for the occurrence of tissue transglutaminase-catalyzed reactions outside the cell, although the mechanism of enzyme externalization remains unclear (Aeschlimann and Paulsson, 1991; Aeschlimann et al., 1993). This evidence includes the identification of a number of different extracellular matrix proteins as specific glutaminyl substrates (for references see Aeschlimann et al., 1992; Aeschlimann and Paulsson, 1994), the demonstration of tissue transglutaminase activity in the pericellular matrix of cultured hepatocytes and endothelial cells (Barsigian et al., 1991, and references therein), and of tissue transglutaminase protein in various extracellular matrices in vivo (Aeschlimann and Paulsson, 1991; Aeschlimann et al., 1993). Extracellular tissue transglutaminase may play a role in the assembly of the matrix, but also in events related to wound healing and excessive tissue repair since active enzyme binds to the extracellular matrix with high affinity after wounding of cells (Upchurch et al., 1991) and may modulate matrix production by regulating the conversion of latent into active TGF-β (Kojima et al., 1993).

The present study concerns the expression of transglutaminase activity during cartilage maturation and the identification of substrate proteins in cartilage cross-linked by transglutaminases in situ. The preparation of antibodies specific for the γ-glutamyl-ϵ-lysine cross-link allowed the immunohistochemical analysis of tissues for cross-linking by transglutaminases in vivo. Transglutaminase cross-linking in cartilage was predominant in the hypertrophic zone of long bone growth plate and of maturing trachea. Explant culture of rat trachea in the presence of the transglutaminase amine donor substrate monodansylcadaverine revealed enzyme activity in the pericellular matrix of the chondrocytes in the calcifying areas. Osteonectin was identified as one predominant target protein for cross-linking in this tissue. These findings further support the physiological importance of (tissue) transglutaminase-catalyzed cross-linking in extracellular matrices.

Materials and Methods

Organ Culture

Tracheae were prepared under aseptic conditions from rats of defined age, cut longitudinally into halves, rinsed extensively with Earle's balanced salt solution, and cultured in medium M-199, containing 10% fetal calf serum and penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (2.5 μg/ml). (All cell culture reagents were from GIBCO BRL, Gaithersburg, MD.) The explants were maintained in 24-well plates with ~0.5 ml medium, a volume sufficient to just cover the tissue pieces. The tracheae were allowed to adapt to the culture conditions for 4 d with two medium changes before pulse-labeling with 0.1 or 0.5 mM of the transglutaminase substrate monodansylcadaverine (*N*-[5'-aminopentyl]-5-dimethylamino-l-naphthalene sulfonamide; Sigma Immunochemicals, St. Louis, MO) for 24 h, followed by a 24-h chase period. Control cultures were treated with the substrate analogue dansylamidopentanol (0.1 or 0.5 mM, see below). The dansyl compounds were dissolved in 0.1 M HCl and diluted 1:250 in the medium. The pH of the medium was readjusted and the concentration of either label verified by absorbance measurement at 335 nm. Cell viability was verified by pulse-labeling with 40 μCi/ml L-[^{35}S]methionine (1,000 Ci/mmol, Amersham Corp., Amersham, UK) after treatment with either substance. The cultured tracheae were rinsed with Earle's balanced salt solution before further processing as described below.

Dansylamidopentanol (*N*-[5'-hydroxypentyl]-5-dimethylamino-naphthalene-l-sulfonamide) was synthesized from l-amino-5-pentanol (0.9 g) dissolved in 10 ml ethanol by dropwise addition of dansylchloride (1.0 g; Fluka, Buchs, Switzerland) dissolved in 8.9 ml ether and 1.1 ml triethylamine with stirring for 2 h in the dark. The yellow oil obtained after evaporation of the solvent was dissolved in ether and extracted repeatedly with 50 mM HCl. The aqueous phases were combined and reextracted with a small volume of $CHCl_3$. After evaporation of the solvent, the yellow gum was dissolved in a small volume of 50 mM HCl and the product precipitated by addition of ~100 ml 0.2 M ammonium hydrogen carbonate. Dansylamidopentanol was allowed to crystallize from this solution at pH 9.0 and 4°C for 3–10 d. The pH was readjusted repeatedly. The product was recrystallized from 0.2 M ammonium hydrogen carbonate giving a final yield of 500–600 mg. The purity of the product was verified by TLC (Kieselgel 60; Merck, Darmstadt, Germany) in acetone and examination under UV light or after iodine staining. The identity of the product was confirmed by infrared spectroscopy (R-SO_2-N: 1150 and 1310 cm^{-1}, –OH: 3520 cm^{-1}), the absorbance spectrum (abs_{max} of fluorophore: 335 nm), and mass spectroscopy (experimentally determined: M_r = 336, M^{+1}, 20.88%; M^{+2}, 6.54%; calculated: M_r = 336; M^{+1}, 20.90%; M^{+2}, 5.03%).

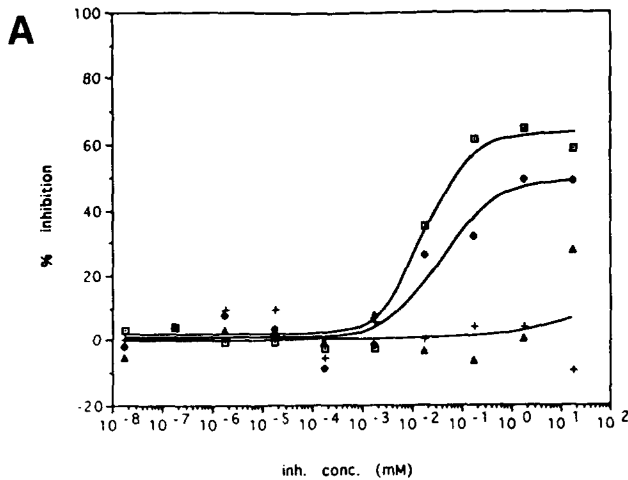
Protein Reagents

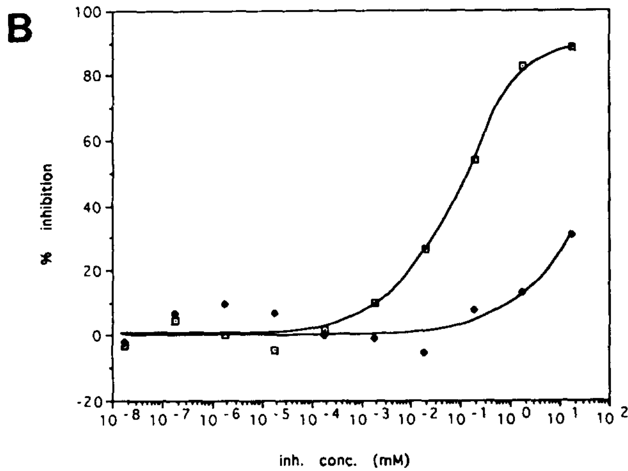
Tissue transglutaminase was purified from guinea pig liver (Connellan et al., 1971). Osteonectin was prepared from young adult rat long bone according to a modification of the procedure established for the human protein by Fisher et al. (1987). A purity of >90% was achieved after two additional chromatographic steps, i.e., on hydroxyapatite (Bio-Gel HTP; Bio-Rad Laboratories, Hercules, CA), eluted with a linear gradient of 10–500 mM sodium phosphate, pH 6.8, in 7 M urea, and on Mono S (fast protein liquid chromatography [FPLC][1]; Pharmacia LKB Biotechnology, Inc., Uppsala, Sweden), eluted with a linear gradient of 0–0.5 M NaCl in 20 mM sodium acetate, pH 4.8, 7 M urea. The rat osteonectin was finally characterized by NH_2-terminal sequencing after separation by SDS-PAGE and blotting to polyvinylidene difluoride membrane (Immobilon P; Millipore, Bedford, MA) as described (Aeschlimann et al., 1992). Native recombinant human BM-40/osteonectin (Nischt et al., 1991) was kindly provided by Dr. R. Timpl (Max-Planck-Institute for Biochemistry, Germany).

Antisera to guinea pig tissue transglutaminase (Aeschlimann and Paulsson, 1991), rat osteonectin, and dansylated (hapten) hemocyanin (Aeschlimann et al., 1993) were raised in rabbits and affinity purified when needed (Aeschlimann and Paulsson, 1991). A monoclonal antibody against collagen type II (CIICl; Holmdahl et al., 1986) was from the Developmental Studies Hybridoma Bank (University of Iowa) and antisera to mouse and human BM-40/osteonectin were generously supplied by Dr. R. Timpl.

An antiserum to the transglutaminase cross-link was raised against a

1. *Abbreviation used in this paper*: FPLC, fast protein liquid chromatography.





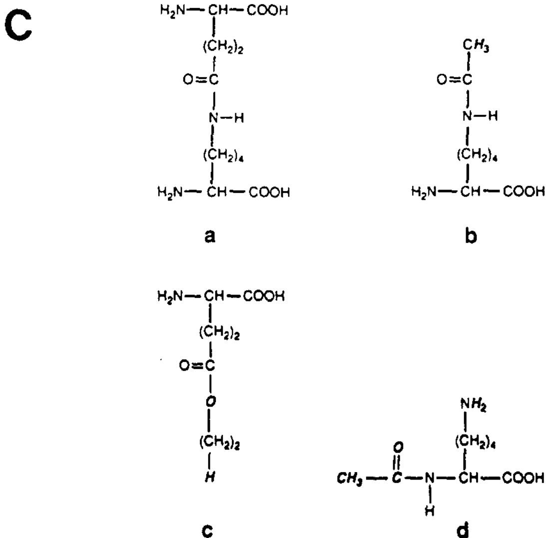


Figure 1. Characterization of antibodies raised against the transglutaminase cross-link. (*A*) The antigen, [*N*,*N*-didansyl(γ-Glu-ε-Lys)]$_n$hemocyanin, was coated to 96-well plates (at 5 μg/ml) and detected with hemocyanin-adsorbed antiserum to the cross-link conjugate that had been preincubated with serial dilutions of γ-Glu-ε-Lys (□; *C*, structural formula *a*), N_ϵ-acetyl-Lys (♦; *C*, *b*), Glu-γ-ethylester (▲; *C*, *c*) or N_α-acetyl-Lys (+; *C*, *d*), and peroxidase-conjugated secondary antibodies. (Other compounds tested and found to be noninhibitory include N_α-acetyl-Gln, N_α-carbobenzoxy-Gln-Gly, Lys, and N_α-acetyl-Lys-methylester). The residual reactivity of the adsorbed antiserum is directed mainly to the dansyl group, a synthetic compound that has no structural analogue in biological systems. The results are shown as a mean of two determinations. (*B*) The antiserum was adsorbed with a Sepharose affinity matrix made from a N_ϵ-acetyl-Lys-hemocyanin conjugate to remove antibodies cross-reactive with N_ϵ-acetyl-Lys and was subsequently tested as above (□, γ-Glu-ε-Lys; ♦, N_ϵ-acetyl-Lys). (*C*) Structural formulas of the various inhibitors are represented, with parts differing from the transglutaminase cross-link structure printed in italics.

hemocyanin conjugate. The γ-Glu-ε-Lys dipeptide (50 mg; Sigma) was NH_2-terminally blocked by dansylation in 9.6 mM sodium phosphate, pH 7.4, 140 mM NaCl (PBS), using cyclohepta-amylose·dansylchloride (10-fold molar excess) as described (Aeschlimann et al., 1993). The product was purified on a Bio-Gel P-2 column (Bio-Rad) in the same buffer (recording of absorbance at 335 nm) and coupled to *Limulus polyphemus* hemocyanin (10 mg; Sigma) via its free carboxyl groups by a modified carbodiimide protocol (Staros et al., 1986). The protein conjugate was finally separated from low molecular mass compounds on a PD-10 column (Pharmacia LKB Nuclear, Gaithersburg, MD), equilibrated in PBS, and used for antibody production in rabbits (250 μg/injection) as described (Aeschlimann and Paulsson, 1991). The antiserum was adsorbed either with a hemocyanin–Sepharose matrix (Aeschlimann and Paulsson, 1991) or with a Sepharose affinity matrix obtained by coupling a N_ϵ-acetyl-Lys-hemocyanin conjugate (obtained as described above) to remove contaminating hemocyanin antibodies and antibodies cross-reactive with N_ϵ-acetyl-Lys, respectively, and the specificity verified in an inhibition-style ELISA (Fig. 1).

Histochemical Methods

Tissues were frozen on dry ice in Tissue-Tek® (Miles, Inc., Naperville, IL). For immunohistochemistry, 5-μm sections were cut, adsorbed to gelatin-coated slides, air dried, and immunolabeled with the indicated antibodies using the peroxidase protocol as previously described (Aeschlimann et al., 1993). To increase antibody penetration, sections were demineralized in TBS (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl) containing 0.42 M EDTA and digested for 1 h with 40 mU/ml chondroitinase ABC (Seikagaku Corp., Tokyo, Japan) in 0.1 M Tris-HCl, 0.1 M sodium acetate, pH 7.4, containing 0.01% (wt/vol) bovine serum albumin, where indicated. Calcification of tissues was visualized by von Kossa's stain for calcium phosphate (Schenk et al., 1984). For autoradiography, sections were exposed to Kodak NTB-2 liquid emulsion.

Protein Extraction from Tracheal Cartilage

Tracheal cartilage was dissected from adhering soft tissue and, when obtained from explant cultures, briefly homogenized in 10 ml/g wet tissue of 4 M guanidine HCl, 50 mM Tris-HCl, pH 6.0, containing 0.1 M EDTA and the protease inhibitors *N*-ethylmaleimide (1 mM NEM), PMSF (1 mM), benzamidine (10 mM), and 6-aminohexanoic acid (0.1 M), and extracted by stirring for 2 h at room temperature. Directly isolated cartilage was sequentially extracted by homogenization in 13 ml/g wet tissue of (*a*) ice-cold 50 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and the protease inhibitors, and by stirring for 2 h at 4°C in *b* the same buffer containing in addition 0.42 M EDTA and in *c* 4 M guanidine HCl, 50 mM Tris-HCl, pH 7.4, containing 0.42 M EDTA and the protease inhibitors. The homogenates were centrifuged at 35,000 *g* for 20 min, and extracted proteins precipitated from the supernatants with ice-cold ethanol (1:9 vol/vol). The precipitates were collected by centrifugation and resuspended by boiling in SDS-PAGE sample mixture containing 4 M urea.

Digestion of cartilage from cultured tracheal explants was performed with 20 mg α-chymotrypsin (bovine pancreas, 45 U/mg; Serva Biochemicals, Paramus, NJ)/g wet tissue in 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.1 M EDTA, and 10 mM NEM for 48 h at room temperature while shaking. The digestion was stopped by addition of a concentrated stock solution to yield the same 4 M guanidine HCl-extraction buffer as above, in this instance containing 10 mM PMSF, and the tissue extracted as above.

Transglutaminase Assay

In vitro cross-linking of recombinant BM-40/osteonectin and incorporation of monodansylcadaverine into *N,N*-dimethylcasein (Serva) with tissue transglutaminase was done as described previously (Aeschlimann and Paulsson, 1991).

SDS-PAGE and Immunoblotting

SDS-PAGE (Laemmli, 1970) was done in 4–20% gradient gels. Proteins were reduced with 2-mercaptoethanol (1% vol/vol) when desired, and detected by staining with Coomassie brilliant blue R or immunochemically after transfer onto nitrocellulose (Towbin et al., 1979). Binding of primary antibody was visualized using peroxidase-conjugated swine anti–rabbit IgG (Dakopatts, Glostrup, Denmark) and the ECL kit (Amersham) according to the manufacturer's protocol. Stripping and reprobing of membranes was done as described (ECL kit). When using the antibodies to the transglutaminase cross-link, nonspecific binding was blocked with bovine serum albumin, which had been shown not to be a transglutaminase substrate (Aeschlimann and Paulsson, 1991) and does therefore not contain cross-link epitopes, and binding of primary antibodies was detected with ^{35}S-labeled donkey anti–rabbit IgG (Amersham).

Results

Characterization of the Antibodies to the Transglutaminase Cross-link

To demonstrate transglutaminase cross-linking in vivo we raised polyclonal antibodies against the unique reaction product of these enzymes, the γ-Glu-ϵ-Lys dipeptide. For this purpose the dipeptide was coupled to hemocyanin after blockage of amino groups by dansylation ([*N,N*-didansyl(γ-Glu-ϵ-Lys)]$_n$hemocyanin). The antiserum was purified by adsorption to a hemocyanin–Sepharose matrix and the specificity of the remaining antibodies was tested in an inhibition-style ELISA (Fig. 1 *A*). Among the structural analogues to the antigen tested, only γ-Glu-ϵ-Lys and N_ϵ-acetyl-Lys were able to inhibit antibody binding at concentrations below 20 mM (γ-Glu-ϵ-Lys gave half-maximal inhibition at ~10 μM, N_ϵ-acetyl-Lys at three- to fourfold higher concentration). The reactivity of the antibodies with N_ϵ-acetyl-Lys was expected as the compound is structurally identical to γ-Glu-ϵ-Lys but smaller (Fig. 1 *C*). The maximum inhibition with N_ϵ-acetyl-Lys is lower, indicating that a subpopulation (~22%) of the antibodies recognizing the γ-Glu-ϵ-Lys dipeptide is completely specific for the cross-link. This antibody population was further purified by adsorption of the antiserum to a N_ϵ-acetyl-Lys-hemocyanin–Sepharose matrix, giving a fraction of antibodies that is inhibited by N_ϵ-acetyl-Lys only at >1,000-fold higher concentrations than of γ-Glu-ϵ-Lys (Fig. 1 *B*). N_ϵ-acetylation of lysines occurs physiologically in nuclear proteins (Turner, 1991) while transglutaminase cross-linking has been reported in the cytosol, at the plasma membrane, and in the extracellular space (Aeschlimann and Paulsson, 1994). The compartmentalization of the respective reactions as well as the removal of cross-reactive antibodies allows discrimination between these structures.

To further demonstrate the specificity of the antibodies, we performed immunostaining on skin and immunoblotting on in vitro cross-linked proteins (Fig. 2). The massive intracellular cross-linking by epidermal transglutaminases in terminal differentiation of keratinocytes leads to the formation of the cornified envelopes (Thacher and Rice, 1985; Hohl et al., 1991; Kim et al., 1993; Aeschlimann and Paulsson, 1994). Immunostaining with the novel antibodies revealed increasing levels of cross-linking in the upper spinous and granular layers of epidermis and in the stratum corneum (Fig. 2 *A*). This staining agrees with the expression pattern of keratinocyte transglutaminase (Thacher and Rice, 1985). Intense staining for the cross-link is also observed in the in-

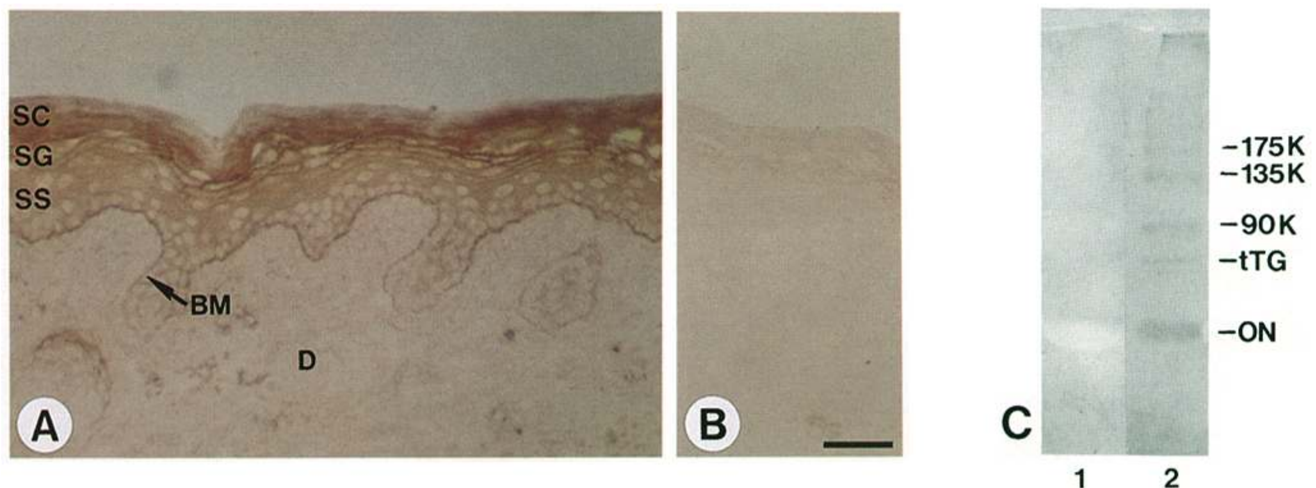


Figure 2. Detection of the transglutaminase cross-link on sections of skin and of in vitro cross-linked proteins by immunoblotting. 5-μm cryosections of rat paw skin (*A* and *B*) were incubated with antibodies specific to the transglutaminase cross-link (*A*; compare Fig. 1 *B*) or nonimmune serum (*B*), and developed using peroxidase-conjugated secondary antibodies. Tissue structures in skin are indicated as follows: basement membrane (*BM*), separating dermis (*D*), and epidermis (spinous layer [*SS*], granular layer [*SG*] and stratum corneum [*SC*]). Bar, 50 μm. (*C*) Human recombinant osteonectin was incubated with guinea pig liver tissue transglutaminase in the presence of lysine (1 mM) and either 25 mM EDTA (lane *1*) or 5 mM Ca^{2+} (lane *2*) for 30 min at 37°C. The reaction mixture was subjected to SDS-PAGE under reducing conditions, then proteins were transferred to a nitrocellulose membrane and epitopes generated by the action of the Ca^{2+}-dependent transglutaminase were detected with the antibodies to the cross-link and ^{35}S-labeled secondary antibodies. The protein bands assigned to osteonectin (*ON*), osteonectin oligomers (*90K, 135K, 175K*; compare Fig. 9), and tissue transglutaminase (*tTG*) are indicated on the right.

ner root sheath of hair follicles (results not shown), consistent with the function of epidermal transglutaminase in cornification of this tissue (Kim et al., 1993; Lee et al., 1993). In addition, transglutaminase cross-linking is observed in the basement membrane underlying epidermis (Fig. 2 *A*) and surrounding hair follicles (results not shown), presumably due to tissue transglutaminase (Lichti et al., 1985) catalyzed cross-linking of e.g., nidogen and BM-40/osteonectin (Aeschlimann and Paulsson, 1991; Aeschlimann et al., 1992). Incorporation of lysine into recombinant BM-40/osteonectin by tissue transglutaminase generated epitopes that are reactive with the antibodies to the cross-link (Fig. 2 *C*). The generation of such epitopes also in tissue transglutaminase is due to the autocatalytic activity of the enzyme (Aeschlimann and Paulsson, 1991).

Detection of the Transglutaminase Cross-link in Differentiating Cartilages

Transglutaminase cross-links were revealed in the mineralized matrix of the hypertrophic zone of growth plate cartilage and in bone matrix of developing rat long bones by antibody labeling after decalcification of the tissue and digestion of glycosaminoglycans with chondroitinase ABC (Fig. 3 *B*). Preincubation of sections with Ca^{2+}-containing buffer gave labeling of also not digested and demineralized tissue, reflecting the activation of the intracellular pool of transglutaminase in the earlier chondrocyte maturation steps (Fig. 3 *D*; *large arrow*) and in osteoblasts (Fig. 3 *D*; e.g., along the bone trabeculae). Control sections preincubated with EDTA-containing buffer were negative (similar to Fig. 3 *A*). This pattern of cross-linking in epiphyseal growth plate cartilage correlates with the distribution of tissue transglutaminase protein (Aeschlimann et al., 1993). The enzyme responsible for cross-linking of bone matrix is likely to be a different member of the transglutaminase protein family (Aeschlimann et al., 1993). Transglutaminase cross-linking in the matrix of growth plate cartilage (Fig. 3 *B*, *small arrow*) occurs in parallel with the deposition of osteonectin (Fig. 3 *E*) and matrix mineralization (Fig. 3 *C*).

In maturing tracheal cartilage, the transglutaminase cross-link was detected around the calcium phosphate mineral deposits in the center of the horseshoe-shaped cartilages (Fig. 3 *F*; compare with Fig. 6 *C*, *inset*), which is in agreement with the expression of tissue transglutaminase (Aeschlimann et al., 1993). Foci of intense staining (*arrowhead*) appear to be single hypertrophic chondrocytes which are cross-linked intracellularly and/or in the pericellular matrix,

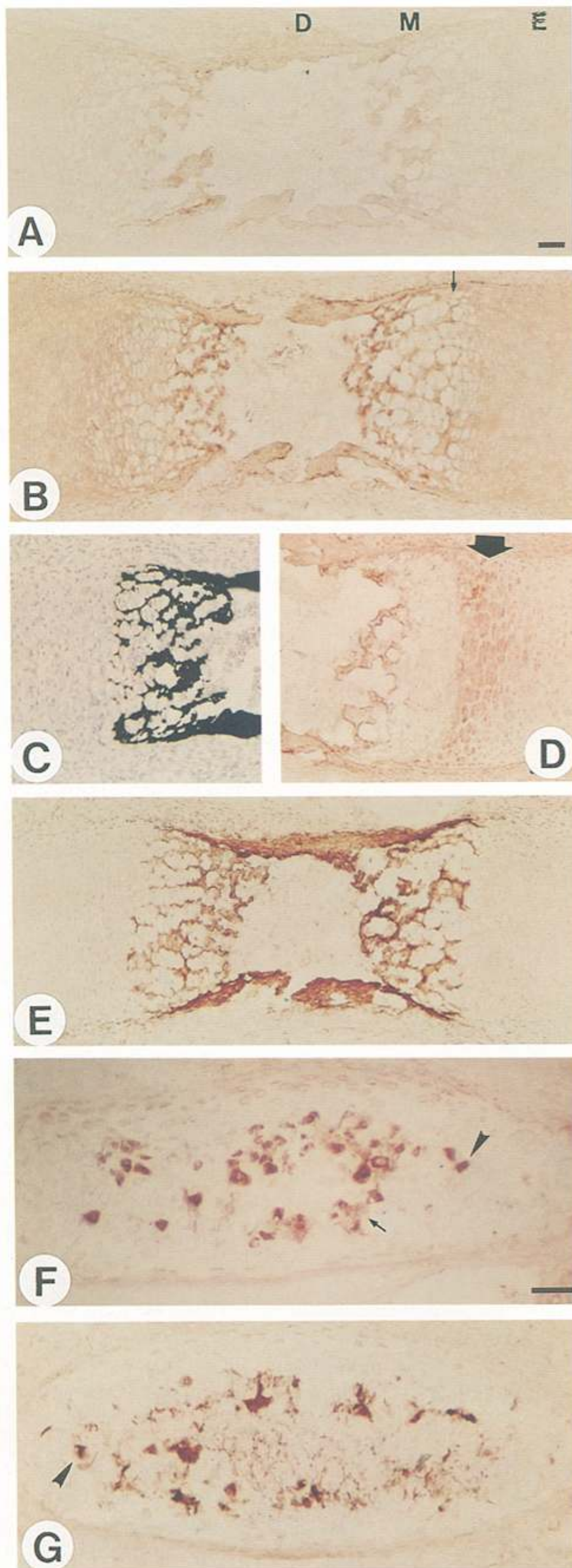


Figure 3. Detection of the transglutaminase cross-link on sections of developing long bone and maturing tracheal cartilage. 5-μm cryosections of a newborn rat tarsal bone (*A–E*) and of a 9-wk-old rat tracheal cartilage (*F* and *G*) were incubated with antibodies specific to the transglutaminase cross-link (*B*, *D*, and *F*; compare Fig. 1 *B*), to osteonectin (*E* and *G*) or with nonspecific serum (*A*). Binding of primary antibodies was visualized using peroxidase-conjugated secondary antibodies. Sections *A*, *B*, *E*, *F*, and *G* were postfixed with methanol, decalcified and digested with chondroitinase ABC before immunolabeling, while section D was preincubated with Ca^{2+}-containing buffer (Aeschlimann et al., 1993). Mineralization was revealed by von Kossa's stain for calcium phosphate (*C*). Histologically discernible zones in long bones are marked (*A*). *E*, epiphysis; *M*, metaphysis; and *D*, diaphysis. Bars, 50 μm.

while patchy staining areas (*small arrow*) indicate matrix cross-linking. Also the staining for immunoreactive osteonectin in the periphery of the mineralized area occurs frequently in foci (Fig. 3 *G*, *arrowhead*). The absence of staining for the cross-link in the fully mineralized matrix could be due to limited penetration of the antibodies even after decalcification and chondroitinase digestion.

Characterization of the Cartilage Culture System Used to Detect Transglutaminase Activity In Situ

To determine the physiological role of cartilage matrix cross-linking by transglutaminases, we developed an organ culture system for tracheal cartilage which allowed us to detect transglutaminase activity and target proteins for cross-linking in situ. We used the synthetic amine substrate monodansylcadaverine, previously used in other culture systems (Cornwell et al., 1983; Kvedar et al., 1992) to detect transglutaminase activity and synthesized an inactive derivative, dansylamidopentanol (Fig. 4 *A*) for use as a control. (The toxic concentration reported for dansyl compounds, i.e., monodansylcadaverine, in cell culture is ~0.2 mM [Cornwell et al., 1983]. We observed detachment of cells from the substratum at those concentrations of monodansylcadaverine and dansylamidopentanol with cultured A204 human rhabdomyosarcoma cells [J.-P. Kleman, D. Aeschlimann, M. Paulsson, and M. van der Rest, manuscript submitted for publication], but we observed good viability at up to ~1 mM in tracheal explant culture. This higher tolerance is consistent with results obtained with skin explants [Kvedar et al., 1992] and might be explained by a diffusion gradient across the explants. We obtained identical results when using 0.1 and 0.5 mM monodansylcadaverine and 0.5 mM monobiotincadaverine [Lee et al., 1992] in our experiments.) We verified the validity of dansylamidopentanol as a control by incubation of tissue transglutaminase with the glutaminyl substrate protein *N,N*-dimethylcasein and either monodansylcadaverine or dansylamidopentanol at various pH values (from 5 to 9). The reactions gave incorporation of the amine, but not the alcohol (Fig. 4, *B* and *C*). Potential toxicity of the dansyl compounds at the concentrations used in culture (0.1 or 0.5 mM) could be excluded as metabolic labeling of the cultures with [^{35}S]methionine after treatment with either compound and quantitation of protein-bound radioactivity gave identical values as control cultures (results not shown; see also Fig. 7).

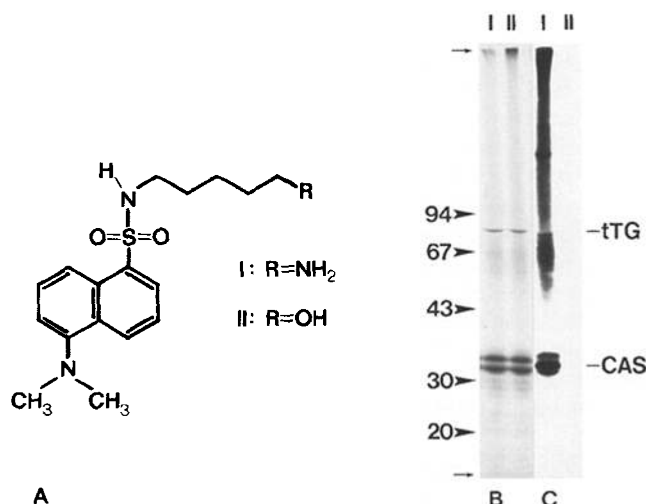


Figure 4. Tissue transglutaminase-catalyzed protein cross-linking in presence of monodansylcadaverine (dansylamidopentylamine) (*A*–C, *I*) or dansylamidopentanol (*A*–C, *II*). The structural formulas of the dansyl compounds are given in *A*. Guinea pig liver tissue transglutaminase was incubated with *N,N*-dimethylcasein at pH 7.0 in presence of either compound for 30 min at 37°C, the reaction mixture separated by SDS-PAGE and the proteins stained with Coomassie blue (*B*) or after transfer to nitrocellulose by incubation with anti-dansyl antiserum (*C*). M_r standards and the top and bottom of the separating gel (*arrows*) are indicated on the left, the protein bands assigned to *N,N*-dimethylcasein (*CAS*) and tissue transglutaminase (*tTG*) on the right.

Transglutaminase Activity Is Associated with Hypertrophic Chondrocytes in the Mineralizing Areas of Cultured Tracheae

Tracheae of young adult rats were incubated in culture for 24 h with the transglutaminase substrate monodansylcadaverine or the substrate analogue dansylamidopentanol and chased in medium without label. Immunohistochemical detection of the dansyl group revealed that label was incorporated in tissue incubated with the amine substrate, but not with the control derivative (Fig. 5, *A* and *B*). Staining was predominantly present in a ring around the chondrocytes, probably due to cross-linking of the dansyl label to proteins of the pericellular matrix and/or deposited at the intracellular face of the plasma membrane (Fig. 5 *C*). The labeling intensity increased with chondrocyte maturation (Fig. 5 *C*) which proceeds towards the center of the tracheal cartilages where the tissue undergoes mineralization (Fig. 6). Transglutaminase activity is highest in the layer of hypertrophic chondrocytes surrounding the mineral deposits (Fig. 5 *C*). This activity pattern is consistent with the previously observed expression of tissue transglutaminase on the protein level (Aeschlimann et al., 1993). Indeed, staining of serial sections for immunoreactive tissue transglutaminase and for incorporated monodansylcadaverine revealed coinciding patterns (results not shown).

To determine the onset of transglutaminase activity during tracheal cartilage development we studied explants taken at different time points in the rat postnatal growth phase. In explants from newborn rats, transglutaminase activity could not be detected (results not shown), consistent with the previously observed absence of tissue transglutaminase protein at this stage in vivo (Aeschlimann et al., 1993). In explants from 4–5-wk-old rats, the first cells expressing transglutaminase activity were detectable in the center of a few tracheal cartilages, whereas in those from 6–7-wk-old rats, enzyme activity was prominent in the hypertrophic zone of all horseshoe-shaped cartilages (Fig. 6, *A* and *B*). Mineralization started around week 7, corresponding to the age of puberty (Fig. 6 *B*). Expression of transglutaminase activity preceded the onset of mineralization, in agreement with the pattern seen in mature cartilage where enzyme activity is highest in the layer of hypertrophic chondrocytes adjacent to mineral deposits (Fig. 5 *C*). The occurrence of both processes in rapid succession in the postnatal development indicates that expression of active transglutaminase and mineralization may be coupled. Explants from animals of eight weeks or older resemble the situation in adult animals where

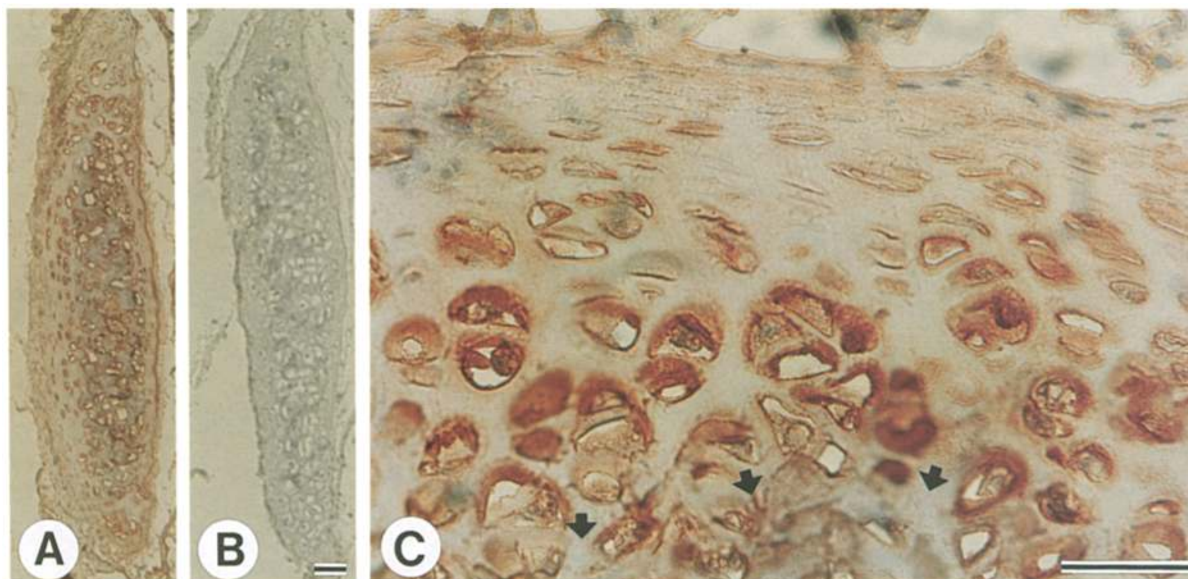


Figure 5. Detection of incorporated monodansylcadaverine on sections of tracheae after in situ labeling. Adult rat (∼3 mo) tracheae were cultured for 24 h in the presence of 0.5 mM of either the transglutaminase substrate monodansylcadaverine (*A* and *C*) or the control derivative dansylamidopentanol (*B*) and chased in medium without label. The tissue was cryosectioned (5 μm), incubated with anti-dansyl antiserum, 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*). The calcified area in *C* is indicated by arrows. Bars, 50 μm.

transglutaminase activity is observed in the hypertrophied and calcified areas of the horseshoe-shaped cartilages, but is absent from resting cartilage (Fig. 6 *C*).

The apparent activation of the transglutaminase in the late stages of chondrocyte differentiation led us to examine the viability of the cells in the terminal differentiation program as externalization and/or intracellular activation of the transglutaminase might occur by cell death in combination with membrane disintegration and leakage of intracellular components or a rise in intracellular [Ca^{2+}], respectively. Metabolic labeling of the cultures with [^{35}S]methionine, followed by autoradiography of tissue sections demonstrated that all chondrocytes were metabolically active (Fig. 7). No signs of cell death were seen in any stage of chondrocyte differentiation, and even cells fully embedded in a calcified matrix were active in protein synthesis (Fig. 7 *C*, *arrowhead*). Thus, the abundance of transglutaminase activity associated with all maturing chondrocytes is clearly not due to cell death.

Substrate Proteins of the Endogenous Transglutaminase in Tracheal Cartilage

Tracheal explants were labeled in culture and dissected cartilages extracted with 4 M guanidine HCl in the presence of inhibitors of transglutaminases (EDTA, *N*-ethylmaleimide) and proteases. SDS-PAGE and immunoblotting with anti-dansyl antibodies revealed covalent attachment of the dansyl compound to proteins from explants incubated with monodansylcadaverine (Fig. 8, lanes *a* and *c*), but not from explants incubated with dansylamidopentanol (results not shown). Most of the dansyl label was detected in high molecular mass proteins or protein complexes, but a prominent glutaminyl substrate protein with an apparent molecular mass of ∼38 kD without and ∼43 kD with prior reduction can be clearly distinguished (Fig. 8, lanes *a* and *c*). The characteristic shift in mobility of this labeled protein following reduction resembles the behavior of osteonectin. Indeed, immunoblots of the tracheal extract probed with antibodies to the dansyl moiety (Fig. 8, lanes *a* and *c*) and to osteonectin (Fig. 8, lanes *b* and *d*) reveal comigration of these proteins, supporting the identity of the dansyl-labeled protein with osteonectin. Osteonectin with dansyl label attached migrated slightly slower upon reduction than osteonectin without the modification, resulting in a doublet-band (Fig. 8, lanes *c* and *d*; compare Fig. 9, lane *h*).

Extensive digestion of tracheae, labeled with monodansylcadaverine in culture, with α-chymotrypsin before extraction and immunoblotting with anti-dansyl antiserum, resulted in disappearance of the labeled high molecular mass proteins or protein complexes while fragments of an apparent molecular mass of ∼55, 60, and 100 kD were stable to further cleavage (Fig. 8, lane *e*). The ∼100-kD component was by immunoblotting shown to represent type II collagen (Fig. 8, lane *f*).

The demonstration of cross-linking of the dansyl label to extracellular matrix proteins, i.e., osteonectin and collagen II (Fig. 8), shows that at least part of the transglutaminase activity associated with hypertrophic chondrocytes (Fig. 5) is present in the pericellular matrix. We cannot exclude that intracellular cross-linking does also occur, although the present results are in full agreement with an extracellular cross-linking. This supports the previous immunohistochemical observation of an intracellular accumulation of tissue transglutaminase in the early steps of chondrocyte differentiation and its subsequent externalization before mineralization of the tissue (Aeschlimann et al., 1993). The unmasking of γ-glutamyl-ε-lysine cross-links in cartilage upon digestion with chondroitinase ABC (Fig. 3) is also consistent

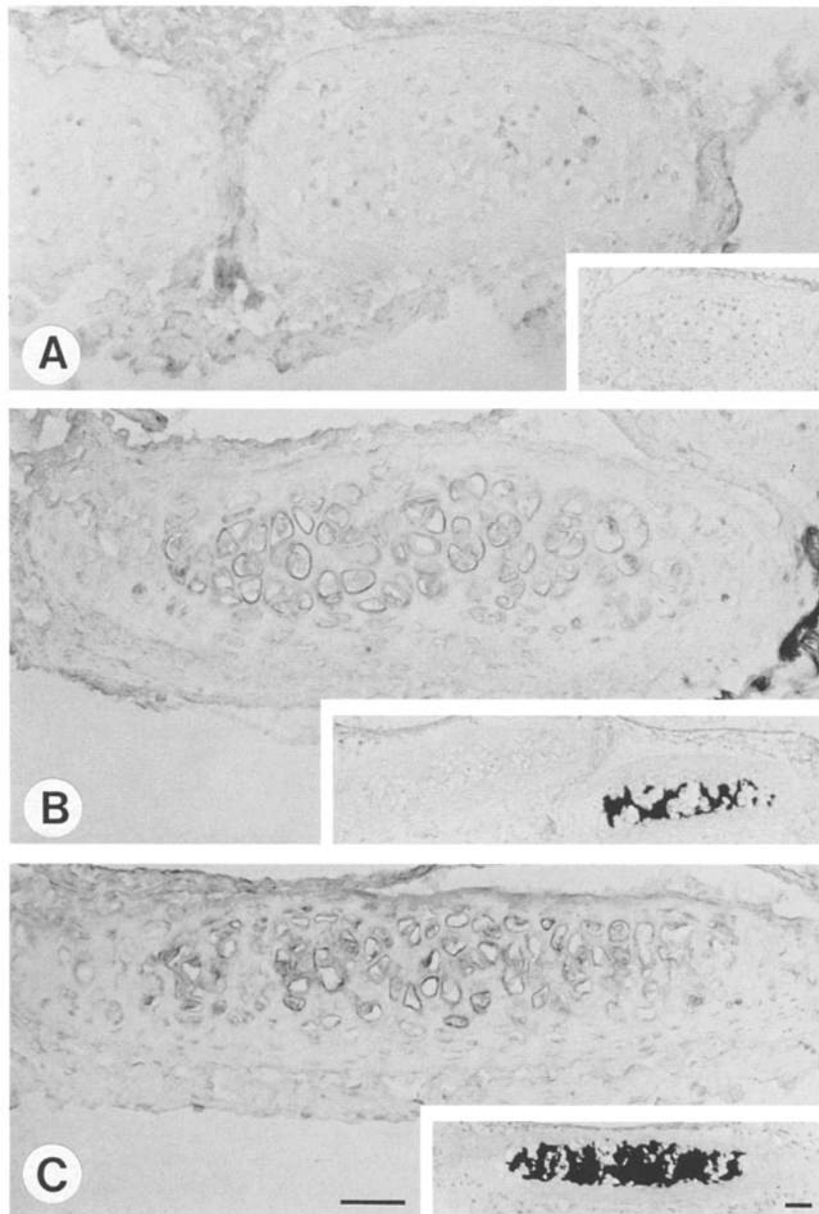


Figure 6. Transglutaminase activity in tracheal cartilage during the rat postnatal growth phase. Tracheal explants from 4- (*A*), 6- (*B*), and 8- (*C*) wk-old rats were incubated in culture with 0.5 mM monodansylcadaverine for 24 h and chased in medium without label. The tissue was cryosectioned (5 μm) and incorporated label detected with anti-dansyl antiserum and peroxidase-conjugated secondary antibodies. Calcium phosphate-containing mineral deposits were visualized by von Kossa's stain for calcium phosphate, shown in the insets. Not all cartilages are at exactly the same developmental stage at a given time point as exemplified by the differing extent of mineralization in two neighboring cartilages in panel *B*. Monodansylcadaverine incorporation is shown for a cartilage that has not yet become mineralized. Bar, 50 μm.

with extracellular transglutaminase activity. Indeed, immunohistochemical detection of extracellular matrix proteins in cartilage, e.g., cartilage proteoglycan aggregate (Poole et al., 1982) or fibronectin (Mackie et al., 1987), does often reveal predominant staining in the chondrocyte pericellular matrix, presumably due to masking of the proteins in the mature matrix. Further, incorporation of the transglutaminase into the matrix may lead to its inactivation as indicated by e.g., the presence of tissue transglutaminase antigen in the cartilage cores of bone trabeculae but lack of transglutaminase activity in this location (Aeschlimann et al., 1993). Pulse-labeling with monodansylcadaverine (Fig. 5) might reveal only the active enzyme in the newly deposited matrix. The demonstration of metabolic activity in chondrocytes expressing high levels of transglutaminase activity (Fig. 7) indicates that externalization of tissue transglutaminase must occur by a mechanism other than cellular disintegration.

Osteonectin Is Present in Cross-linked Complexes in Skeletal Tissues In Vivo

Adult rat tracheal cartilage was sequentially extracted with buffered saline, saline supplemented with EDTA, and a 4 M guanidine HCl/EDTA solution in presence of inhibitors of transglutaminases and proteases. SDS-PAGE and immunoblotting with antibodies to osteonectin revealed several immunoreactive bands of higher apparent molecular mass than the monomeric protein (Fig. 9). Bands of ~65, 90, and 175 kD were resistant to reduction, even after boiling in urea and SDS (Fig. 9, lanes *a–c*). To exclude a contaminating reactivity in our antiserum to rat bone osteonectin we verified this result with an antiserum raised against the mouse Engelbreth-Holm-Swarm tumor-derived protein (results not shown). Presumably, these bands represent covalently stabilized protein complexes with osteonectin as a constituent. In vitro cross-linking of recombinant BM-40/osteonectin by tis-

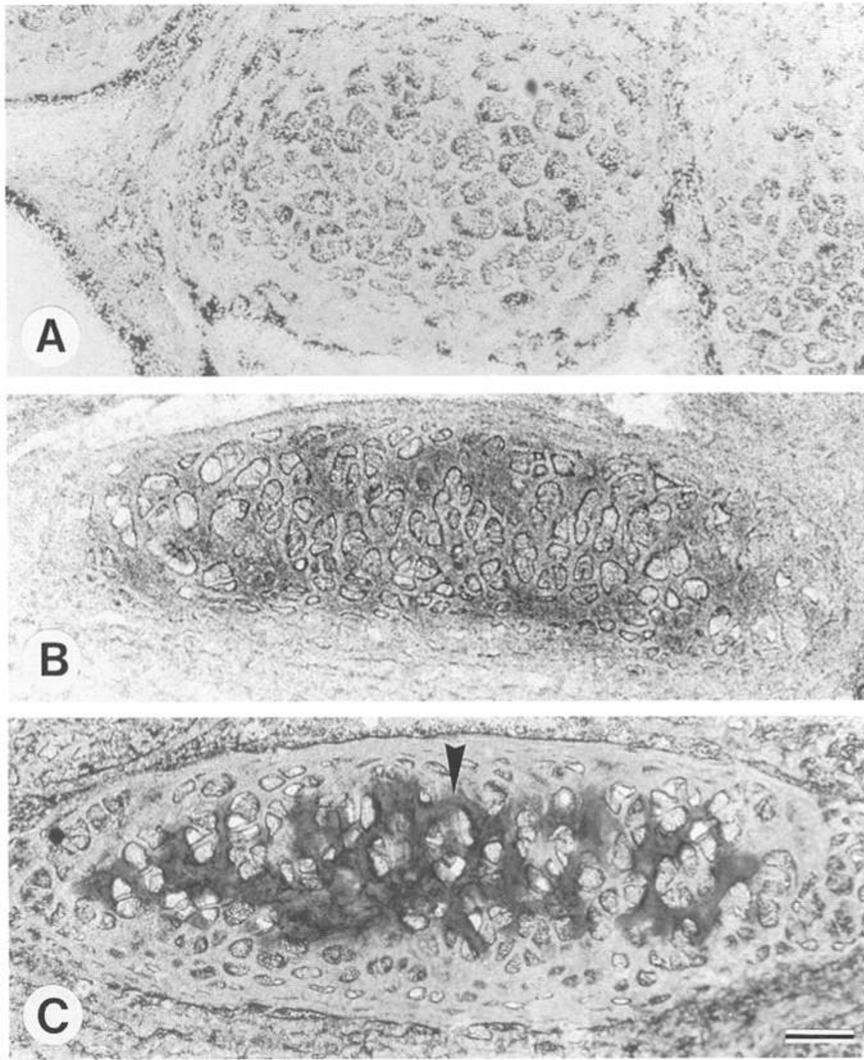


Figure 7. Autoradiography of sections from cultured tracheae following metabolic labeling with [^{35}S]methionine. Tracheae of 4- (*A*), 6- (*B*), and 8- (*C*) wk-old rats were cultured in the presence of 0.5 mM monodansylcadaverine, subsequently pulse-labeled with [^{35}S]methionine for 24 h and chased in medium without label. 5-μm cryosections of the tissue were covered with photographic emulsion and exposed for 2 d. The photographic reaction was developed (*black grains*) and the tissue weakly counterstained with Mayers Hämalaun. The darker staining (Hämalaun) area in the center of the horseshoe-shaped cartilage in *C* indicates cartilage calcification. Bar, 50 μm.

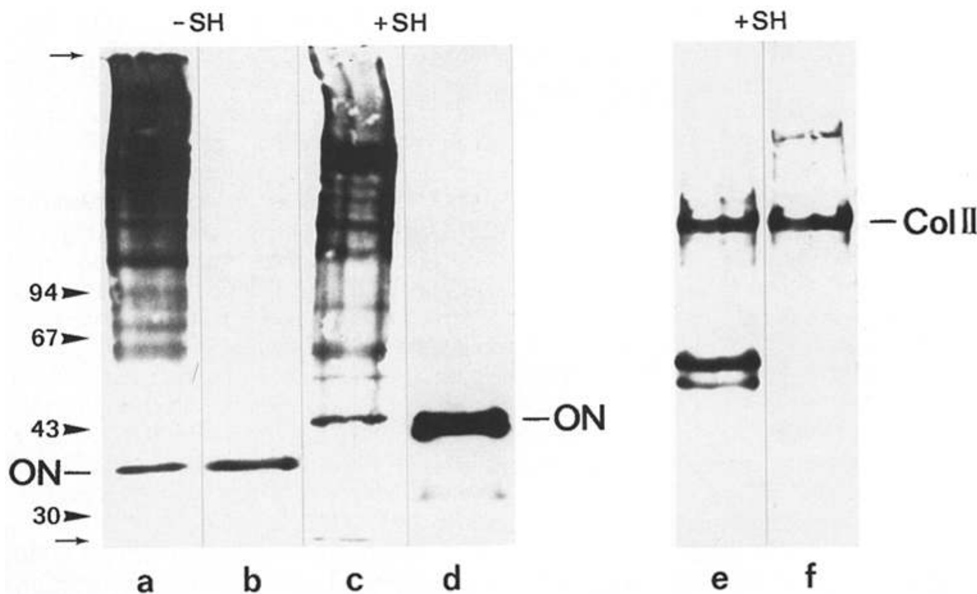


Figure 8. Analysis of transglutaminase substrate proteins in tracheal cartilage after in situ labeling with monodansylcadaverine. Tracheae were cultured for 24 h in the presence of 0.5 mM monodansylcadaverine and chased in medium without label. The proteins were extracted with 4 M guanidine HCl containing buffer (in the presence of transglutaminase inhibitors), either directly (lanes *a–d*) or after extensive digestion of the tissue with α-chymotrypsin (lanes *e* and *f*), and separated in 4–20% SDS-polyacrylamide gels without (*−SH*) or with (*+SH*) prior reduction. Proteins were detected after transfer to nitrocellulose by incubation with antibodies to the dansyl moiety (lanes *a*, *c*, and *e*), to osteonectin (lanes *b* and *d*) or to collagen type II (lane *f*) by stripping and reprobing the membranes. M_r standards as well as top and bottom of the separating gels (*arrows*) are indicated on the left. The protein bands assigned to osteonectin and collagen type II are marked with the label *ON* and *Col II*, respectively.

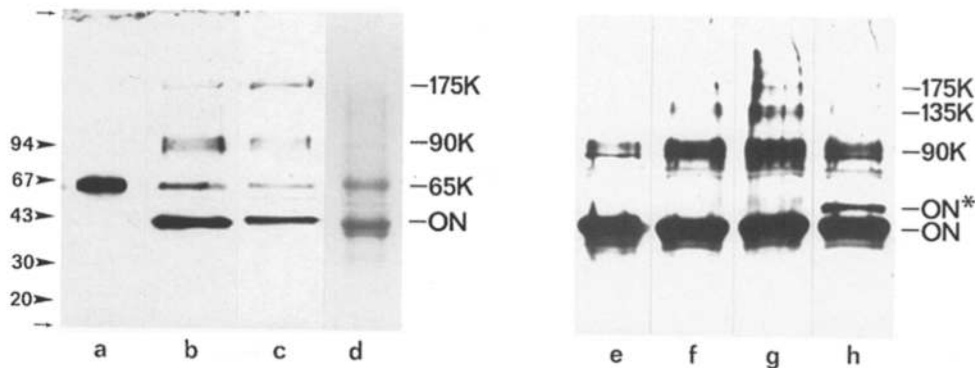


Figure 9. Characterization of osteonectin and osteonectin complexes present in cartilage and bone. Proteins sequentially extracted from adult rat tracheal cartilage with buffered saline (lane *a*), containing EDTA (lane *b*) and guanidine HCl/EDTA (lane *c*), were separated by SDS-PAGE performed under reducing conditions and after transfer to nitrocellulose stained by incubation with antibodies to osteonectin (lanes *a–c*). Osteonectin prepared from rat long bone was revealed by Coomassie blue staining after SDS-PAGE separation and transfer to polyvinylidene difluoride membrane (lane *d*). Human recombinant osteonectin was incubated with guinea pig liver tissue transglutaminase in presence of 25 mM EDTA (lane *e*), 5 mM Ca^{2+} (lanes *f* and *g*), or 5 mM Ca^{2+} and 20 μM monodansylcadaverine (lane *h*) for 30 min (lanes *e*, *f*, and *h*) or 2 h (lane *g*) at 37°C and the proteins were subsequently separated by SDS-PAGE and detected after transfer to nitrocellulose with antibodies to osteonectin. M_r standards as well as top and bottom of the separating gels (*arrows*) are indicated on the left. The protein bands assigned to osteonectin (*ON*), osteonectin-monodansylcadaverine complex (*ON**), osteonectin oligomers (*90K*, *135K*, and *175K*) and a 65-kD osteonectin complex (*65K*) are indicated on the right.

sue transglutaminase yields products which resemble the 90- and 175-kD osteonectin complexes isolated from cartilage, indicating that these complexes represent osteonectin dimers and tetramers, respectively (Fig. 9, lanes *e–g*). Furthermore, incorporation of monodansylcadaverine into recombinant BM-40/osteonectin results in a slightly retarded migration of the protein in SDS-PAGE (Fig. 9, lane *h*), resembling the pattern observed in the cartilage explant cultures with a doublet-band in the monomer position (compare Fig. 8, lanes *c* and *d*). A pattern of osteonectin-immunoreactive bands similar to that in tracheal cartilage extracts was obtained in extracts from long bones and purification of osteonectin from this source revealed, in addition to the monomeric protein, a copurifying complex of ~65 kD that was reactive with the antibodies to osteonectin (Fig. 9, lane *d*). NH_2-terminal sequencing of the two bands with apparent molecular masses of 40 and 43 kD in the monomer position (Fig. 9, lane *d*) gave both the sequence APQTEAAEEM that is closely similar to APQQTEVAEEI previously determined for mouse osteonectin (Mason et al., 1986), confirming them to be the rat osteonectin homologue. The ~65-kD complex gave APQT, representing the NH_2 terminus of osteonectin, and GA(hydroxyP)GP, originating from a collagen. The collagen sequence was too short to be unambiguously assigned to the subtype. It remains however unclear if the complex represents osteonectin covalently bound to another component, e.g., a collagen fragment, or only osteonectin, i.e., a degradation product of an oligomer.

Discussion

Amine incorporation in cultured tracheal explants revealed that in cartilage of adult rats, transglutaminase activity is predominantly associated with the chondrocytes surrounding the calcium phosphate deposits, which phenotypically resemble the hypertrophic chondrocytes of long bone growth plate. During the postnatal development of trachea, transglutaminase activity could first be detected around chondrocytes shortly before the onset of mineralization. This finding is consistent with the previously observed intracellular accumulation and subsequent externalization of tissue transglutaminase protein by chondrocytes in the hypertrophic zone of long bone growth plate and trachea in vivo (Aeschlimann et al., 1993). Indeed, detection of the unique transglutaminase reaction product, the γ-glutamyl-ϵ-lysine cross-link, with a specific antibody on tissue sections revealed the cross-link in the hypertrophic cartilage of the epiphyseal growth plate and the mineralizing area of tracheal cartilages, but not in resting cartilage. The demonstration of amine incorporation into matrix proteins in cultured tracheal explants (Fig. 8) together with the detection of incorporated amine around differentiating chondrocytes (Fig. 5) suggests that the cross-linking occurs predominantly in the pericellular matrix. The detection of γ-glutamine-ϵ-lysine cross-links (Fig. 3) and tissue transglutaminase antigen (Aeschlimann et al., 1993) in cartilage matrix and the requirement for depolymerization of glycosaminoglycan chains to unmask these epitopes are consistent with extracellular cross-linking. These results indicate that tissue transglutaminase expression, externalization and activation, and mineral formation are consecutive steps in the chondrocyte maturation program both during postnatal development of tracheal cartilage and in endochondral ossification. There is not necessarily a direct causal connection between these processes, even though transglutaminase-catalyzed cross-linking of the matrix could indeed alter its structure in a way that predisposes the tissue to calcification. Clustering of fixed charged groups by cross-linking of anionic matrix proteins, e.g., to the collagen fibril, might provide a surface which promotes nucleation and/or growth of hydroxyapatite crystals (Boskey, 1992). Osteonectin and osteopontin are present in mineralizing areas of cartilage and bone (Metsäranta et al., 1989; Aeschlimann et al., 1993; Ikeda et al., 1992; Denhardt and Guo, 1993), are anionic and contain calcium-binding domains (Engel et al., 1987; Maurer et al., 1992; Denhardt and Guo, 1993), and are transglutaminase substrates (Prince et al., 1991; Aeschlimann et al., 1993; Sorensen et al., 1994).

A prerequisite for the function of tissue transglutaminase

in matrix remodeling is its externalization to the extracellular space. It is unlikely that tissue transglutaminase is secreted by the conventional pathway as the enzyme has a cytoplasmic localization and lacks the characteristic features of secretory proteins, such as a signal peptide, glycosylation, and disulfide bonding, even though potential sites for such modifications are present in the sequence (for review see Aeschlimann and Paulsson, 1994). The present demonstration of protein synthesis by chondrocytes also in the mineralized areas of tracheal cartilage is consistent with metabolic and proliferative activity of chondrocytes in the calcified matrix of long bones detected by incorporation of [^{35}S]sulfate and [^{3}H]thymidine, respectively (Hunziker, 1992 and personal communication), and shows that externalization of the transglutaminase in the mineralizing matrix is not due to cell death. This is further supported by the fact that DNA-fragmentation, which accompanies apoptotic cell death in most cases (Fésus et al., 1991), has not been observed in the growth plate cartilage of the developing chicken limb (V. Thomazy, personal communication). In contrast, tissue transglutaminase expression in the interdigital segment of the limb correlates with DNA fragmentation and apoptosis. The fate of terminally differentiated chondrocytes remains uncertain (Gentili et al., 1993), but tissue transglutaminase externalization and activation should be considered as part of the differentiation program of chondrocytes, independent from potential cell death.

Since we and others failed to demonstrate widespread cell death and lysis in terminally differentiated cartilage, we conclude that tissue transglutaminase is externalized by another, not yet characterized mechanism. It has recently been shown that another transglutaminase family member, prostate transglutaminase, though absent from endoplasmic reticulum and Golgi complex, is released in apocrine secretory vesicles from dorsal prostate and coagulating gland during copulation (Seitz et al., 1990). Prostate transglutaminase is therefore likely to enter the vesicles directly from the cytoplasm. Membrane-bound vesicles with cytoplasm-derived contents shedded from the cell surface could similarly be responsible for the externalization of tissue transglutaminase. So called matrix vesicles that are selectively enriched in certain cytoplasmic proteins have been observed in many tissues, including cartilage (Hale and Wuthier, 1987).

Tissue transglutaminase binds tightly to components of the pericellular matrix upon release (Upchurch et al., 1991) and probably acts by cross-linking the surrounding matrix upon activation at the elevated Ca^{2+} concentration met in the extracellular space. Fibrinogen and fibronectin bind to the surface of hepatocytes and endothelial cells in suspension culture and become cross-linked into the pericellular matrix by tissue transglutaminase (Barsigian et al., 1991). Extraction of the tracheal cartilage after labeling with monodansylcadaverine in culture revealed a distinct set of labeled proteins. Among these, osteonectin and type II collagen were identified as predominantly labeled components. This finding is consistent with the previously observed amine incorporation into osteonectin and type II collagen in the transglutaminase-catalyzed reaction in vitro (Aeschlimann et al., 1993) but, in addition, demonstrates that these proteins are physiological substrates for the transglutaminase. Moreover, a previously described ~43-kD protein that acts as glutaminyl substrate for endogenous transglutaminase in homogenates of bovine epiphyseal growth plate cartilage (Aeschlimann et al., 1993) appears to be osteonectin based on its elution in anion exchange and molecular sieve chromatography and on its immunological similarity (results not shown). Extraction of adult rat tracheal cartilage revealed, in addition to osteonectin monomer, high molecular mass complexes that were resistant to reduction and immunoreactive for osteonectin. The complexes with an apparent molecular mass of 90 and 175 kD probably represent cross-linked osteonectin oligomers since similar complexes were formed upon cross-linking of osteonectin by tissue transglutaminase in vitro. In contrast, a major 65-kD complex gave the NH_2-terminal sequence of osteonectin by Edman degradation, but was not formed by in vitro cross-linking. It is possible that the 65-kD complex results from cross-linking of osteonectin with another component, but the amounts of sample available did not allow an unambiguous identification of such a component.

The proteins cross-linked to osteonectin in situ need to be further characterized and the sequence locations of the cross-linking sites identified. Once available, this information will provide an insight into the physiological interaction partners of osteonectin and into the organization of the calcifying matrix. Osteonectin may bind to collagens (Sage et al., 1989; Mayer et al., 1991) and influence calcium phosphate deposition in the mineralization process in cartilage and bone (Termine et al., 1981*b*; Engel et al., 1987; Maurer et al., 1992). The present demonstration of the transglutaminase cross-link in bone matrix, the isolation of cross-linked osteonectin complexes from long bones and the previously reported presence of transglutaminase activity in newly deposited osteoid (Aeschlimann et al., 1993) indicate a more general role for transglutaminase cross-linking in skeletal tissues, even though the enzymes involved in bone and cartilage may be different members of the transglutaminase protein family (Aeschlimann et al., 1993). Differential posttranslational modification (Kelm and Mann, 1991) may serve to regulate the diverse biological functions proposed for osteonectin in different tissues (Clezardin et al., 1988; Mayer et al., 1991; Sage and Bornstein, 1991) and transglutaminase cross-linking may indeed alter the properties of the protein either by amine incorporation or formation of protein complexes.

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