Protein Cross-linking Mediated by Tissue Transglutaminase Correlates with the Maturation of Extracellular Matrices During Lung Development

Johannes C. Schittny, Mats Paulsson, Claudio Vallan, Peter H. Burri, Noemi Kedei, and Daniel Aeschlimann

Department of Developmental Biology, Institute of Anatomy, and M. E. Müller-Institute for Biomechanics, University of Bern, Switzerland; Institute for Biochemistry, Medical Faculty, University of Cologne, Germany; Department of Biochemistry, University Medical School of Debrecen, Debrecen, Hungary; and Department of Medicine, University of Wisconsin, Madison, Wisconsin

At birth, the mammalian lung is still immature. The alveoli are not yet formed and the interairspace walls contain two capillary layers which are separated by an interstitial core. After alveolarization (first 2 postnatal weeks in rats) the alveolar septa mature: their capillary layers merge, the amount of connective tissue decreases, and the mature lung parenchyma is formed (second and third week). During the first 3 wk of life the role of tissue transglutaminase (tTG) was studied in rat lung by immunostaining of cryostat and paraffin sections, by Northern and Western blotting, and by a quantitative determination of γ-glutamyl-ϵ-lysine. While enzyme activity and intracellular tTG were already present before term, the enzyme product (γ-glutamyl-ϵ-lysine-crosslink) and extracellular tTG appeared between postnatal days 10 and 19 in the lung parenchyma. In large blood vessels and large airways, which mature earlier than the parenchyma, both the enzyme product and extracellular tTG had already appeared at the end of the first postnatal week. We conclude that tTG is expressed and externalized into the extracellular matrix of lung shortly before maturation of an organ area. Because tTG covalently and irreversibly crosslinks extracellular matrix proteins, we hypothesize that it may prevent or delay further remodeling of basement membranes and may stabilize other extracellular components, such as microfibrils. **Schittny, J. C., M. Paulsson, C. Vallan, P. H. Burri, N. Kedei, and D. Aeschlimann. 1997. Protein crosslinking mediated by tissue transglutaminase correlates with the maturation of extracellular matrices during lung development. Am. J. Respir. Cell Mol. Biol. 17:334–343.**

Transglutaminases are enzymes which Ca^{2+}-dependently catalyze a post-translational modification of proteins. The enzyme reaction leads to the formation of an isopeptide bond either within or between polypeptide chains. It is referred to as R-glutaminyl-peptide, amine-γ-glutamyl transferase reaction (EC 2.3.2.13). The γ-glutamyl-ϵ-lysine crosslinks are formed between the γ-carboxamide group of peptide-bound glutamine residues and most commonly the ϵ-amino group of peptide-bound lysine residues (1, 2). The formation of the transglutaminase crosslink appears to be "irreversible" because it seems that nature has not provided an enzyme for the cleavage of this crosslink in proteins (3). Transglutaminases are abundant enzymes which are involved in a number of different physiologic processes, e.g., plasma transglutaminase factor XIII stabilizes the fibrin-clot during hemostasis, and keratinocyte and epidermal transglutaminase contribute to the formation of the cornified envelope in skin (3, 4).

The physiologic function of tissue transglutaminase (tTG), a monomeric globular protein with an M_r of ~ 77,000, is less clear. Intracellular tTG is involved in crosslinking of cellular proteins during programmed cell death (5). Extracellularly, the enzyme contributes to the stabilization of extracellular matrices as shown for calcifying cartilage (6, 7) and basement membranes (8–10) during matrix assembly and wound healing (10, 11). Several extracellular matrix proteins have been identified as specific glutaminyl substrates for tTG. Among these are fibrin(ogen) (12, 13); fibronectin (14, 15); fibrillin (16–18); vitronectin (19, 20); nidogen/entactin (8, 21); osteonectin (6, 7); osteopontin (22, 23); the collagens II (6, 7), V (24), VII (17), and XI (24); and the N-propeptide of collagen III (25).

At birth, the mammalian lung is functioning but still immature. In rat, the lung consists of smooth-walled channels

(*Received in original form August 5, 1996 and in revised form January 21, 1997*)

Address correspondence to: Johannes C. Schittny, Dept. of Developmental Biology, Institute of Anatomy, University of Bern, Buehlstrasse 26, CH-3012 Bern, Switzerland. E-mail: Schittny@ana.unibe.ch

Abbreviations: bovine serum albumin, BSA; monodansylcadaverine, DNSC; phosphate buffered saline, PBS; Tris-buffered saline, TBS; tissue transglutaminase, tTG.

which end in saccules (saccular stage). True alveoli are not yet formed, and the septa of the parenchyma ("primary septa") are thick and contain a double capillary network. Each leaf of this network is located underneath one of the air-exposed surfaces and they are separated by a core of connective tissue. After a short phase of expansion of the lung, "secondary septa" are lifted off the "primary septa," resulting in the formation of the alveoli and a distinct enlargement of the gas-exchange surface. At about 10 days of age the bulk alveolar formation is completed, and the phase of alveolarization is followed by the phase of microvascular maturation, which lasts until the end of the third postnatal week. During the latter phase, most of the low and thick secondary septa are rapidly turned into high and slender interalveolar walls. The double capillary layers inside the septa are transformed into a central capillary layer with a high meshwork density. The absolute mass of interstitial tissue decreases during this process despite an overall volume gain of the lung of 25%. The final structure of the air/blood barrier is achieved after the fusion of the epithelial and endothelial basement membranes. Normal growth of the lung follows (26–29); for review, *see* Burri (30, 31).

In the present study, we investigated the expression and activity of tTG during lung development. We show that tTG introduces crosslinks into the extracellular matrix of vessels, airways, and interairspace walls shortly before their maturation and, as a result, contributes to the stabilization of these matrices.

Materials and Methods

Animals and Tissues

All tissues were obtained from SIV-Z-50, or Wistar, rats. After abdomen and thorax were opened, the lungs were perfused with phosphate-buffered saline (PBS = 10 mM sodium phosphate, containing 127 mM sodium chloride, pH 7.4) containing 5 units/ml heparin, 10 mg/ml procaine, and 10 mM EDTA (Fluka Chemie AG, Buchs, Switzerland) via the right ventricle and filled with PBS containing 4% polyvinylpyrrolidon (Merck, Darmstadt, Germany) by instillation via tracheostomy until the midrespiratory lung volume was reached. The tissue was immediately removed, briefly rinsed, and frozen in liquid nitrogen for cryostat sectioning. For extraction of tissues, the lungs were only perfused. Fetuses were obtained from timed pregnant animals. Whole fetuses were frozen either in liquid nitrogen or on a dry-ice-cooled plate. Fetal homogenates were obtained from fetal lungs without perfusion or filling.

Immunochemical Reagents

Antisera to guinea pig tTG (8), to γ-glutamyl-ε-lysine crosslink [γ-glutamyl-ε-lysine dipeptide-hemocyanin conjugate (7)], and to the dansyl group [dansylated (hapten) hemocyanin (6)] were raised in rabbits and affinity purified when needed (7, 8). In addition to the described characterization of the anti-tTG antibody (6–8), we tested for cross-reactivity to rat factor XIII using Western blots and were not able to detect any (data not shown). The affinity-purified antibodies were diluted in the required buffers as follows: anti-tTG 1:25–1:30 for immunohistochemistry and 1:100 for immunoblotting, anti-γ-glutamyl-ε-lysine crosslink 1:10, anti-dansyl 1:100 (negative control of anti-γ-glutamyl-ε-lysine crosslink), or 1:300 (*in situ* staining of transglutaminase activity).

Immunohistochemistry

Sections of 8–10 μm were cut on a Leica cryostat-microtome 1720 (Leica AG, Zürich, Switzerland) at −18°C, transferred onto siliconized (aminopropyl-trimethoxy-silane) microslides, and air-dried overnight at room temperature.

Immunofluorescence staining of unfixed cryostat sections. The sections were rehydrated in PBS (5–10 min), blocked with PBS containing 1% bovine serum albumin (PBS/BSA) for 30 min, and incubated with the first antibody, which was diluted in PBS/BSA, for 1–2 h. Incubation was followed by three changes of PBS/BSA before (15 min) and after (30 min) treatment with rhodamine-labeled secondary antibody (goat antirabbit IgG; Cappel Research Products, Organon Teknika Co., Durham, NC; 1 mg/ml diluted 1:60 in PBS/BSA, 30–60 min). Sections were embedded in Mowiol [PBS containing 15% Hoechst 4.88 (Hoechst AG, Frankfurt a.M., Germany) and 50 mg/ml 1,4-diazabicyclo(2.2.2)octane (Merck)].

Immunoperoxidase staining of fixed cryostat sections. Sections were fixed for 10 min either in acetone at −20°C, methanol, or in Tris-buffered saline (TBS) (50 mM Tris/HCl, pH 7.4, containing 100 mM sodium chloride) containing 4% paraformaldehyde (freshly prepared). The kind of fixation used had no influence on the result of the staining with anti-tTG or anti-γ-glutamyl-ε-lysine crosslink. The fixative was removed by rinsing with TBS and the endogenous peroxidases were quenched by incubation in methanol containing 1% hydrogen peroxide. After an additional washing step (TBS, 3 × 5 min followed by 1 × TBS containing 1% BSA; TBS/BSA) the sections were incubated for 1–2 h with antibody diluted in TBS/BSA. Before and after the application of peroxidase-conjugated swine antirabbit IgG (diluted 1:100 in TBS containing 1% BSA; Dakopatts, Glostrup, Denmark), sections were washed 3 times with TBS. The slides were developed with 3-amino-9-ethylcarbazole (Sigma Chemicals Company, St. Louis, MO)/H_2O_2 (6) and embedded in Entellan (Merck).

Detection of transglutaminase activity in situ. Unfixed cryosections were rehydrated in TBS, blocked with TBS/BSA, incubated with 0.1 M Tris/HCl (pH 8.3) containing 5 mM $CaCl_2$ and 15 nM monodansylcadaverine (DNSC; Serva, Heidelberg, Germany), either with or without 2.7 μg of exogenous tTG purified from guinea-pig liver (8), and further processed for immunofluorescence staining using the anti-dansyl antibody as described above. The enzyme activity of the transglutaminases was inhibited by addition of 25 mM EDTA to the incubation buffer (8).

All steps were carried out in a moist chamber at room temperature. Negative controls were performed with nonspecific rabbit IgG or anti-dansyl antibodies, and by a preincubation of anti-tTG antibodies with purified tTG. No or little nonspecific background was observed in all negative controls. The samples shown were taken from central parts of the lung. The immunohistochemical methods used were not sensitive enough to show significant differences between central and peripheral regions of the lung. The stained sections were examined and photographed with filters appropriate for rhodamine staining or interference

contrast (when required). All sections needed for one figure were processed in parallel. The same exposure settings were used for all immunofluorescence images.

Northern Blots

Total RNA was prepared using the guanidine thiocyanate extraction method (32) and enriched for poly-(A)-RNA according to Maniatis and colleagues (33). Poly(A)-enriched RNA, 5 μg, was denatured with glyoxal and separated on 1% agarose gels before blotting onto nitrocellulose. Membranes were prehybridized for 4 h in 50% formamide, 4× saline sodium citrate, 5× Denhardt's solution, 100 μg/ml denatured salmon sperm DNA, 0.2% sodium dodecyl sulfate, and 0.1% sodium pyrophosphate, and hybridized in the same solution with randomly primed ^{32}P-labeled oligonucletides corresponding to the tissue-transglutaminase sequence [clone mTg 7.4 (34)] for 18 h at 42°C. Membranes were washed to a final stringency of 1× saline sodium citrate, 0.2% sodium dodecyl sulfate, at 62°C before autoradiography. Before rehybridization with randomly primed γ-actin oligonucletides, membranes were stripped with 80% formamide, 0.1× saline sodium citrate, at 80°C.

Immunoblotting

Lung tissue was homogenized and sequentially extracted at 4°C, first with TBS containing 0.25 M sucrose; followed by TBS containing 0.25 M sucrose and 0.5% Triton-X-100; and third by 4 M guanidine/HCl containing 50 mM Tris/HCl, pH 7.4. In addition, all buffers contained 10 mM EDTA, 2 mM phenylmethanesulfonyl fluoride, 10 mM *N*-ethylmaleimide, 20 mM benzamidine/HCl, and 100 mM 6-aminohexanoic acid as protease inhibitors. Homogenates were centrifuged for extraction and washing at 35,000 × *g* for 20 min at 4°C. After every step of extraction the pellets were washed. Aliquots of the extractions were reduced with 2-mercaptoethanol (1%, vol/vol; Fluka Chemie AG), applied to a 4–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (35), and transferred onto nitrocellulose (36). Immunostaining was done according to the protocol of the ECL Western blot detection reagents (enhanced chemiluminescence; Amersham, Amersham, UK). Primary antibody was diluted 1:100 in TBS containing 5% milk powder, detected by peroxidase-conjugated swine antirabbit IgG (diluted 1:500; Dakopatts), and developed by ECL.

HPLC Determination of γ-Glutamyl-ε-lysine Crosslink

The amount of γ-glutamyl-ε-lysine crosslink was measured in lung tissue homogenates according to Tarcsa and Fésus (37). Briefly, lungs were homogenized in PBS (volume PBS: g wet weight = 4:1) and lyophilized. After rehydration, extensive enzymatic digestion, and derivatization with phenylisothiocyanate, the obtained γ-glutamyl-ε-lysine isodipeptides were first separated on a cation exchange resin and a silica high pressure liquid chromatography (HPLC) column and then quantified after reversed-phase HPLC.

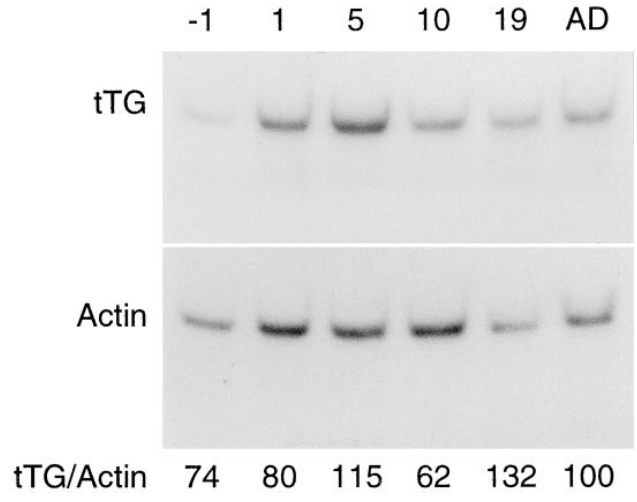

Figure 1. Northern blots using probes for tTG and for actin were done on mRNA from lung homogenates obtained 1 day before the expected birth; on postnatal days 1, 5, 10, and 19; and from adult tissue. The intensities of the tTG bands were normalized to the intensity of the corresponding actin band and compared with adult tissue (both set to 100). Numbers indicate postnatal days; AD = adult.

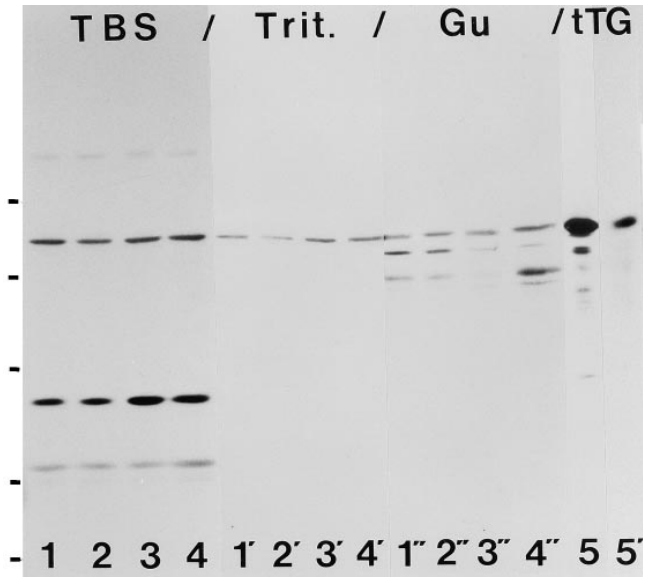

Figure 2. Western blot of lung homogenates obtained 1 day before the expected birth (*lines 1, 1′, 1″*), on postnatal days 5 (*lines 2, 2′, 2″*) and 19 (*lines 3, 3′, 3″*), and from adult animals (*lines 4, 4′, 4″*). Postnatal lungs were first perfused with PBS in order to remove most of the erythrocytes, and then extracted step by step with TBS containing 0.25 M sucrose (*left lanes*/TBS), followed by TBS containing 0.5% triton-X-100 (*central lanes*/Trit), and by 4 M guanidine (*right lanes*/Gu). Proteins separated on a 4 to 20% SDS-PAGE gel were transferred to a nitrocellulose membrane and immunostained with anti-tTG antibodies. Different concentrations of purified guinea-pig tTG were run in *lines 5* and *5′* as a standard. The migration positions of molecular weight markers (96, 67, 42, 30, and 20 kDa) are indicated at the left border of the figure.

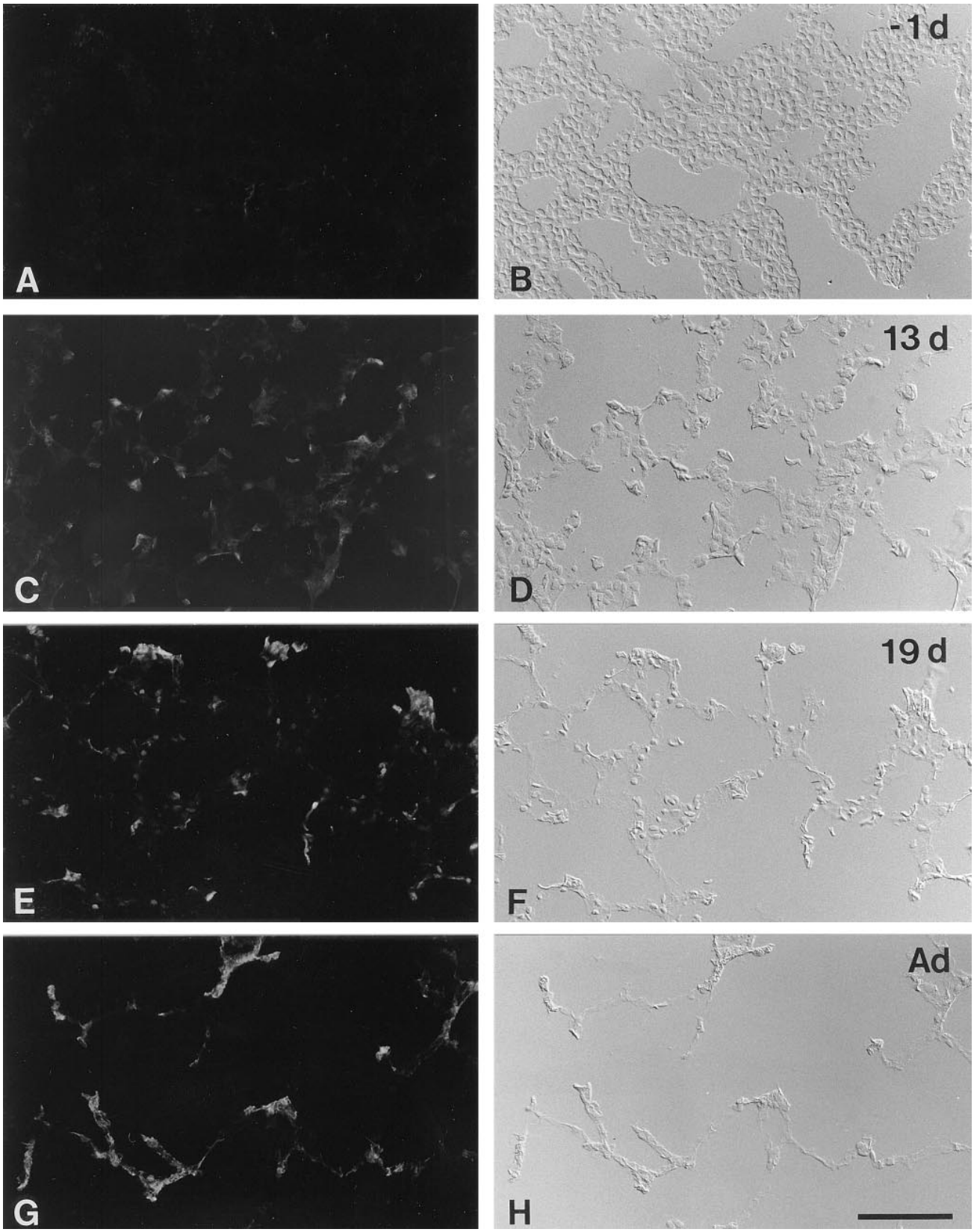

Figure 3. Immunofluorescence localization of tTG on unfixed cryosections of rat lung during organ maturation. The tissue was taken from animals 1 day before the expected birth (*A*, *B*), on postnatal days 13 (*C*, *D*) and 19 (*E*, *F*), and from adult animals (*G*, *H*). During the processing of unfixed cryosections, the cytosolic tTG pool is lost due to its high solubility. The insoluble, extracellular pool of tTG appears to be tightly bound to the matrix and is therefore predominantly retained and detected under these conditions. Staining for matrix-bound tTG was observed in lung parenchyma first around postnatal day 13 (*C*) and increased to levels found in mature tissue (*G*) within 6 days (*E*). *Left panels* show immunofluorescence images; *right panels* show interference contrast micrographs. *Bar*: 200 μm.

Results

Analysis of the Expression of mRNA and Protein

The expression of tTG was studied by Northern and Western blotting of lung extracts of staged rat embryos. Both tTG mRNA and protein were already present in homogenates of whole lungs 1 day before term (Figures 1 and 2). A normalization of the mRNA level to the expression of actin revealed two small peaks: one at postnatal day-5 and the second at postnatal day-19. As the observed differences are smaller than 2-fold, we judged them insignificant (Figure 1). The same is true for the expression of tTG at protein level. In all three fractions (TBS, TBS containing triton-X-100, and guanidine), the concentration of tTG did not increase significantly between 1 day before term and adulthood (Figure 2). In both the TBS- and the guanidine-extract, proteolytic degradation of tTG was observed. All extraction buffers contained high concentrations of protease inhibitors. Therefore, it is likely that the degradation had already occurred *in vivo*.

Immunostaining of tTG

As shown for differentiating chondrocytes (6), tTG is present in an intracellular and an extracellular pool. While the intracellular pool is highly soluble, the extracellular pool is

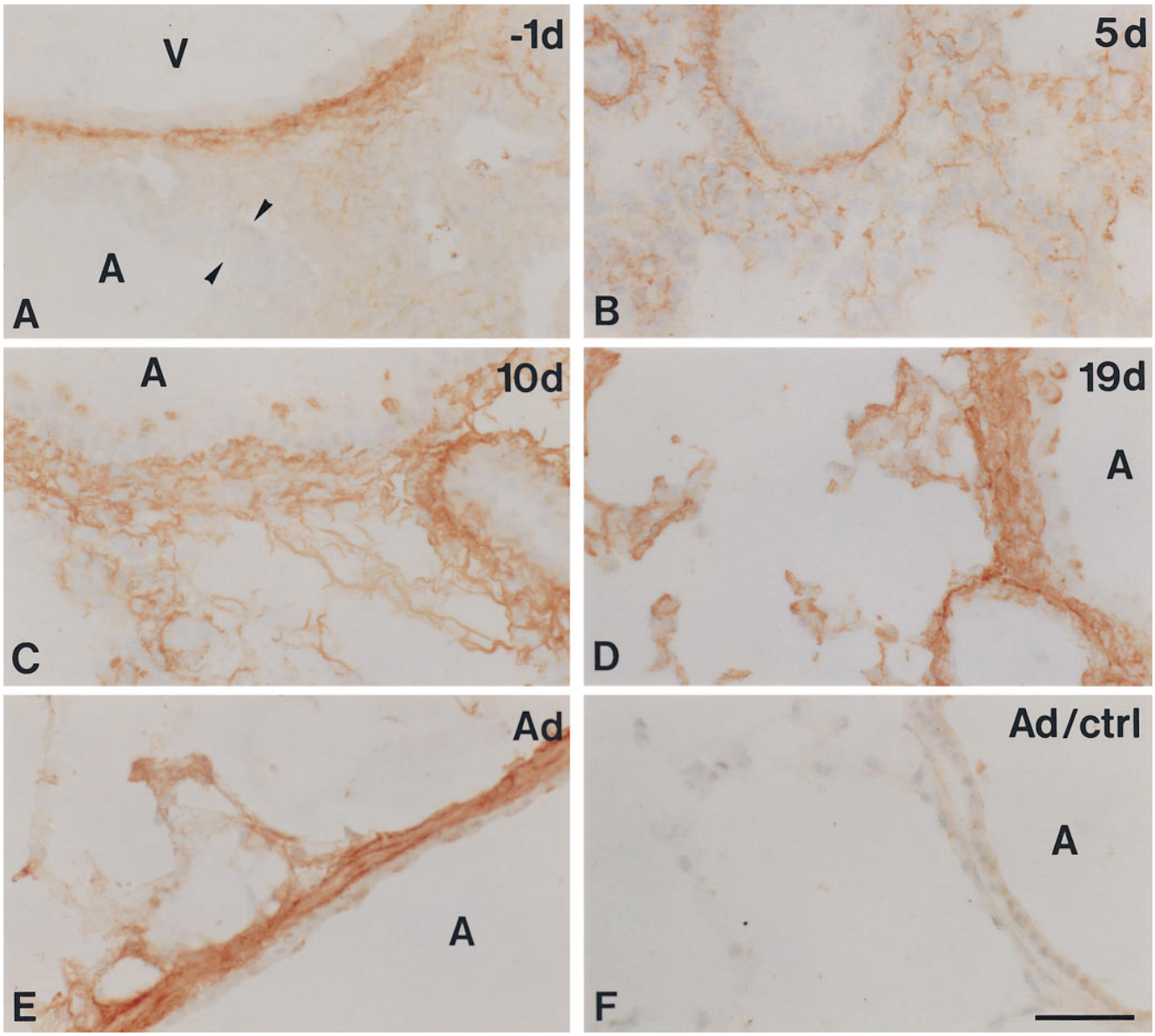

Figure 4. Immunoperoxidase localization of tTG on fixed cryosections. Tissue was obtained 1 day before the expected birth (*A*); at postnatal days 5 (*B*), 10 (*C*), and 19 (*D*); and from adult animals (*E*, *F*). In contrast to Figure 3, the immunoperoxidase staining of fixed cryosections revealed a staining of both the soluble and insoluble pools of tTG. Anti-dansyl antibodies served as negative control (*F*). Immunostaining of the parenchyma and large blood vessels was already observed before term, but the epithelium of large airways was still negative at this time point (*A*). A = large airway; V = large blood vessel; space between *arrowheads* shows epithelium of a large airway. *Bar*: 50 μm

quite strongly bound to the extracellular matrix. Both pools may be distinguished by immunostaining of unfixed cryostat sections with anti-tTG antibodies where the extracellular pool is predominantly detected, and of fixed cryostat sections where both pools are detected. Extracellular tTG first appeared in the lung interairspace walls of the parenchyma around postnatal day-13 (Figure 3A–D) and increased at postnatal day-19 (Figure 3E and F) to levels found in mature tissue (Figure 3G and H). Intracellular tTG appears earlier, and was already detected 1 day before the expected date of birth (Figure 4A). At 1 day before term the enzyme is present in the cells of the interairspace walls and the walls of larger blood vessels, but not in the epithelium of larger airways. Its expression increases gradually in maturing and adult tissue (Figure 4B–E). An apical staining was observed in some epithelial cells of the bronchioles starting at about postnatal day-10 (Figure 4C–E). Investigation of the same sections by interference contrast revealed that these cells belong to the group of nonciliated cells as defined by Jeffery and Reid [(38) and data not shown]. Negative controls were carried out with unspecific rabbit IgG, or with the anti-dansyl antibodies, respectively, replacing the specific primary antibodies. Negative control of the immunofluorescence staining revealed a black

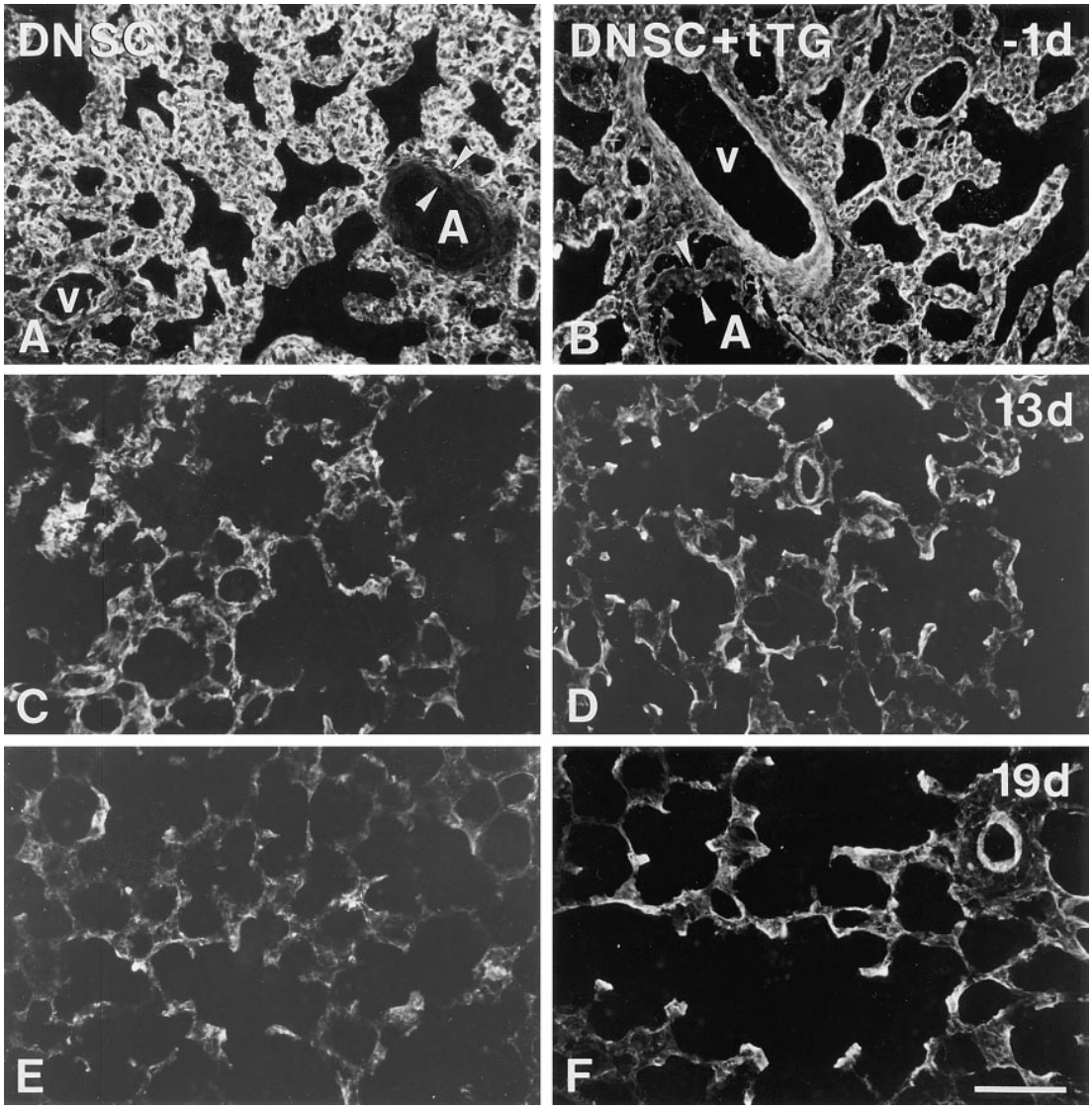

Figure 5. Localization of transglutaminase activity on cryosections of rat lung during organ maturation. Tissues from animals 1 day before the expected birth (*A*, *B*), and at postnatal days 13 (*C*, *D*) and 19 (*E*, *F*) were incubated in calcium and DNSC containing buffer either in the absence or presence of tTG (no tTG present: *A*, *C*, *E*; detection of endogenous transglutaminase activity; tTG present: *B*, *D*, *F*; detection of the presence of substrate proteins). Incorporated DNSC was subsequently detected with antibodies to the dansyl group followed by rhodamine-labeled secondary antibody. While substrate proteins are present in similar amounts at all time points (*B*, *D*, *F*), endogenous transglutaminase activity is highest before birth (*A*) and declines to day 19 (*E*). Before birth the epithelium of the larger airways does not show endogenous transglutaminase activity (*arrowheads* in *panel A*), although substrate proteins are present (*arrowheads* in *panel B*). A = large airway. *Bar*: 200 μm.

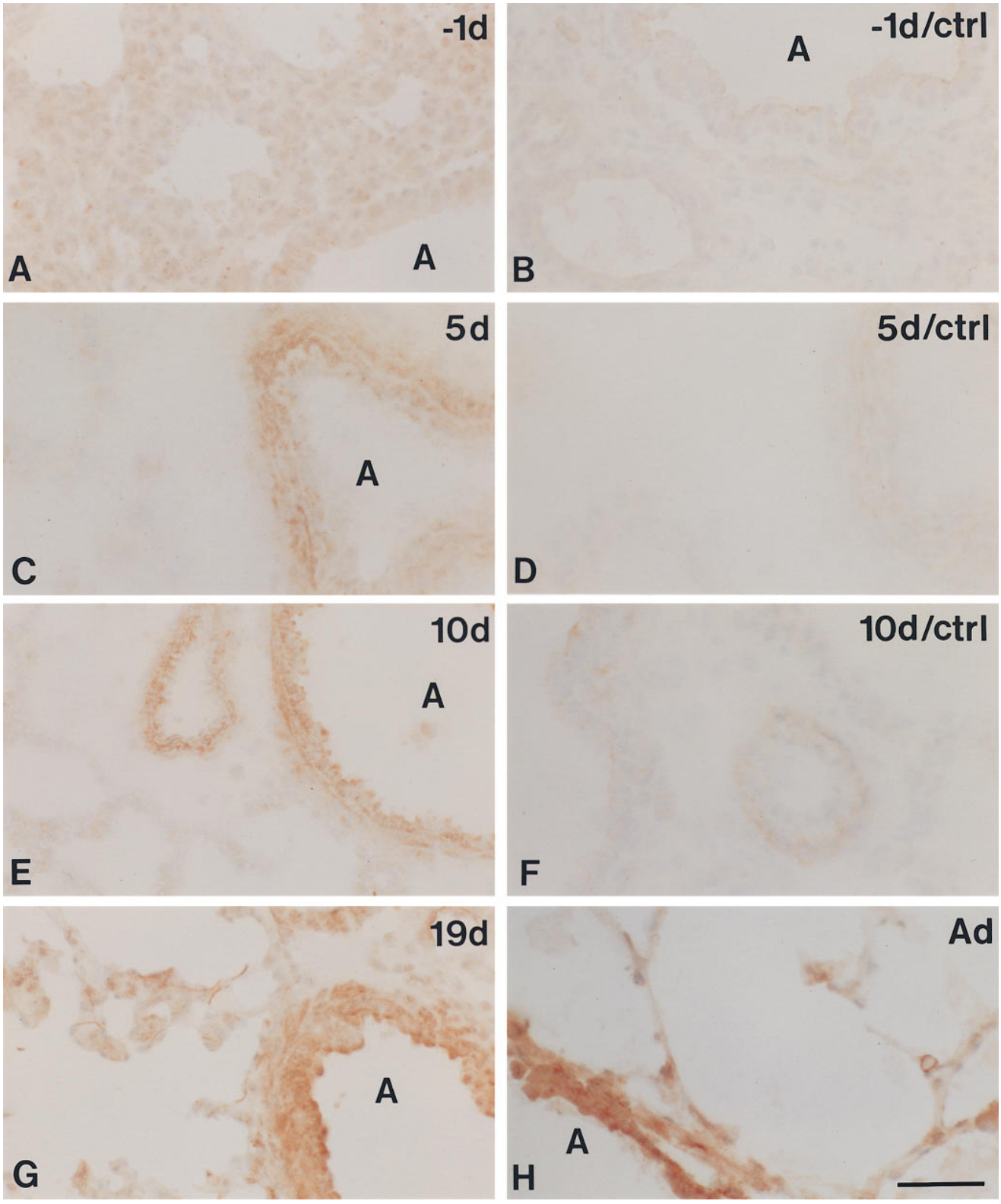

Figure 6. Immunoperoxidase localization of the enzyme product of tTG (γ-glutamyl-ϵ-lysine crosslink) on cryosections. Immunostaining was performed 1 day before the expected birth (*A*, *B*); on postnatal days 5 (*C*, *D*), 10 (*E*, *F*), and 19 (*G*); and on adult tissue (*H*). One day before term (*A*), no significant immunostaining of the γ-glutamyl-ϵ-lysine crosslink was observed. On postnatal days 5 (*C*) and 10 (*E*), only larger airways and blood vessels were γ-glutamyl-ϵ-lysine crosslink-positive, but immunostaining appeared over the parenchyma, as well as over the airways and blood vessels, on postnatal day 19 (*E*) and in adult tissue (*H*). Negative controls using antibodies against the dansyl group are shown in panels *B*, *D*, and *F*. A = large airway; V = large blood vessel. *Bar*: 50 μm

image similar to that shown in Figure 3A, and the ones of the peroxidase staining produced only little unspecific background (Figure 4F).

Detection of Transglutaminase Activity *In Situ*

Endogenous enzyme activity of transglutaminases was detected as incorporation of DNSC into cryosections and by immunofluorescence staining of DNSC with an antibody recognizing the dansyl group, followed by a rhodamine-labeled secondary antibody. One day before term, a strong signal of endogenous transglutaminase activity was already detected in the parenchyma of the lung and in larger blood vessels, but not in the epithelium of larger airways (Figure 5A). At postnatal days 13 and 19 transglutaminase activity was present in all compartments of the lung, but the intensity declined somewhat as compared with the prenatal staining (Figure 5C and E). By the addition of exogenous tTG to the DNSC solution, the distribution of endogenous substrate proteins of tTG can be detected. With two exceptions, the same staining as for endogenous enzyme activity was observed. First, the epithelium of larger prenatal airways was now positive (Figure 5B); and second, the intensity of the staining was the same at every time point investigated (Figure 5B, D, and F). As a control, the enzyme reaction was performed in calcium-depleted buffer (transglutaminases require calcium to be enzymatically active). No fluorescence was detected under these conditions (data not shown).

Detection of the γ-Glutamyl-ε-lysine Crosslink

In a second immunohistochemical approach we determined the distribution of the γ-glutamyl-ε-lysine crosslink, the enzyme product of tTG. Methanol-fixed cryosections were stained with antibodies directed to the crosslink and secondary, peroxidase labeled, antirabbit IgG. Even if the γ-glutamyl-ε-lysine crosslink is produced not only by tTG but also by every transglutaminase, the tissue distribution of γ-glutamyl-ε-lysine crosslink is consistent with the one of extracellular tTG. At 1 day before term we observed only weak, presumably nonspecific, staining (Figure 6A and B). At postnatal days 5 and 10, only the walls of larger airways and vessels are positive for the crosslink (Figure 6C–F). At postnatal day 19 and in adult tissue, all parts of the tissue were stained (Figures 6G and H). In addition, the epithelial cells of the bronchioles, which were tTG positive, were also crosslink positive (Figures 6E–H). As shown in Figure 6B, D, and F, negative controls using the anti-dansyl antibodies revealed only little unspecific background.

Using a highly sensitive HPLC method, the amount of γ-glutamyl-ε-lysine crosslink was determined in tissue homogenates of developing lungs. As shown in Figure 7, the concentration of γ-glutamyl-ε-lysine crosslink per gram of lung (wet weight) increases about 4-fold during postnatal maturation and reaches a 5-fold level at adulthood. Similar values were obtained after normalization to milligram of protein (data not shown).

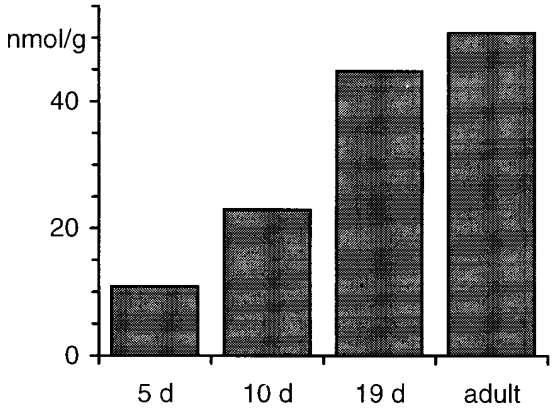

Figure 7. Determination of γ-glutamyl-ε-lysine-dipeptide in lung homogenates. The tissue was obtained from 5, 10, and 19 days old, and from adult animals. The amount of the γ-glutamyl-ε-lysine crosslink was measured using HPLC after a complete digestion of the proteins. The concentration is given as nmol γ-glutamyl-ε-lysine crosslink per g of lung (wet weight).

Discussion

In the present study, we investigated the expression of tTG during postnatal lung development. As shown for developing cartilage, tTG appears in an intracellular, highly soluble pool and an extracellular matrix-bound pool (6). Using unfixed and fixed cryosections we were able to distinguish between these two pools. We found a correlation between both the extracellular expression of tTG and the amount of γ-glutamyl-ε-lysine crosslink, and lung maturation.

During the second and third postnatal weeks, the last step of lung development (phase of microvascular maturation) takes place. During this maturation, the overall thickness of the septa between the newly formed alveoli (alveolar septa) decreases. Especially, the volume of the core of connective tissue in the middle of a septum declines, thus initiating the capillary layers located at both sides of the septum to fuse to one capillary network. In addition, the capillaries get into very close contact with the lung epithelium and the air/blood barrier matures. After the completion of this process, a normal relative proportional growth follows (31). In the lung parenchyma, the first appearance of extracellular tTG (Figure 3) and its enzyme product, the γ-glutamyl-ε-lysine crosslink (Figure 6), takes place at the very end of the second postnatal week, just toward the end of the phase of active interstitial tissue remodeling and septal maturation. This last step of lung development includes a far-reaching reorganization of different extracellular matrices leading to their maturation. Larger airways and larger blood vessels mature earlier than the parenchyma. In these structures, tTG and the γ-glutamyl-ε-lysine crosslink are already present at the end of the first postnatal week (Figures 6C–F).

At mRNA (Figure 1) and protein (Figure 2) levels, tTG is present 1 day before the expected date of birth and the expression of both increases only insignificantly toward adulthood. As shown by immunostaining of unfixed (Figure 3) and fixed (Figure 4) cryosections, tTG is stored inside the cells until its externalization starts at the very end of the second postnatal week. Even if we were able to demonstrate enzyme activity in parallel to the intracellular

expression of tTG (Figure 5), the enzyme appears to be inactive until its externalization because γ-glutamyl-ϵ-lysine crosslink was immunohistochemically detected in parallel to the extracellular appearance of tTG (Figure 6). Based on these results, we propose a model for lung maturation where tTG starts to appear and to act in the extracellular matrices of the lung shortly before and in parallel to the maturation of these matrices. This model is supported by our findings that the concentration of γ-glutamyl-ϵ-lysine crosslink increases about 4-fold during postnatal lung maturation (Figure 7).

Our results in developing lung are in good agreement with the expression of tTG during the maturation of cartilage. In the latter tissue, tTG expression is strictly regulated and correlates with terminal differentiation of chondrocytes, cartilage calcification in endochondral bone formation, and maturation of tracheal cartilage (6, 7). The mechanism of a step-by-step expression of tTG was also found in these systems. tTG protein is first enriched intracellularly in hypertrophic chondrocytes found in long-bone growth plate and in chondrocytes of trachea. It is subsequently released into the extracellular space upon terminal differentiation, or maturation of chondrocytes, respectively (6). Furthermore, both extracellular transglutaminase activity and the appearance of γ-glutamyl-ϵ-lysine crosslink are predominately associated with the externalization of tTG (6, 7).

A similar stepwise scenario is also seen in wound healing. In patients who received keratinocyte autografts after severe burns, the action of tTG in the dermo-epidermal junction correlates with the clinical stabilization of the grafts. Anchoring fibrils, one of the targets of tTG, appear 1 wk after grafting, but extracellular tTG and γ-glutamyl-ϵ-lysine crosslinking of anchoring fibrils is not seen before 1 mo after grafting (10).

In our lung system we were not able to distinguish between intracellular and extracellular transglutaminase activity. While in lung the overall expression of tTG increases slightly during the first 3 wk of life (Figures 1, 2, and 4), the enzyme activity of transglutaminases declines somewhat during the same time period (Figure 3). Because the assay for enzyme activity is not specific for tTG, any other transglutaminase may contribute to the result as well. It is likely that the high prenatal enzyme activity is caused by a combined action of tTG and of additional members of the family of transglutaminases (3, 4). *In vivo* these additional transglutaminases appear to be silent at least until extracellular tTG starts to act, because the enzyme product (γ-glutamyl-ϵ-lysine crosslink) appears in parallel to extracellular tTG. These results indicate that several members of the transglutaminase family may be differently expressed in lung tissue.

In order to act extracellularly, tTG has to be externalized. It is unlikely that tTG is secreted by the conventional pathway, because it is located in the cytoplasm and it lacks the characteristic features of secretory proteins, such as a signal peptide, glycosylation, and disulfide bonds, even if potential sites for these modifications are found in its sequence (3). The externalization by apoptosis is also unlikely, even if a peak of programmed cell death was observed in the parenchyma at postnatal day-19 (39). First, this peak does not explain the extracellular expression of tTG in larger blood vessels and larger airways. Second, the number of dying parenchymal cells is most likely too small to account for the amount of extracellular tTG (39).

Until now we did not search for the proteins on which tTG is acting in lung. Because fibrillin (16–18) and nidogen/entactin (8, 21) are crosslinked by tTG in other tissues, it is likely that tTG acts on the same proteins in lung. In addition, the microfibril-associated glycoprotein may also be a target (16, 40). Crosslinking of fibrillin and microfibril-associated glycoprotein would result in a stabilization of the microfibrils as shown in cell culture experiments and human skin (17). Because microfibrils represent a building block of the elastic fibers, the latter fibers would also be stabilized. Therefore, one of the functions of tTG may be the stabilization, or maturation, respectively, of fibrilar structures such as the fibrillin-containing microfibrils (16–18, 40).

Nidogen/entactin represents one of the classic basement membrane proteins. In developing tissues, some building blocks of basement membranes (like the laminin-nidogen complex) may be rearranged by reshuffling of non-covalent crosslinks (41). Upon maturation the potential for remodeling appears to be altered because the non-proteolytic extractability of laminin decreases significantly in adult tissues as compared with embryonic ones (42, 43). By crosslinking nidogen/entactin to an unknown partner, tTG may contribute to this alteration. In more general terms, we hypothesize that tTG and/or other transglutaminases contribute to the maturation of basement membranes by crosslinking its building blocks. Remodeling may still be possible, but proteolytic enzymes may now be necessary. Therefore tTG may serve as an agent which contributes to the regulation of basement membrane remodeling and maturation.

Acknowledgments: The authors thank Dr. Laszlo Fesus for helpful discussions, Dr. Peter J. A. Davies for the gift of the tTG-clone mTg 7.4, and Mrs. Marianne Hofstetter for expert technical assistance. This work was financially supported by a grant from the Swiss National Science Foundation (grant numbers 31-36530.92, 31-45831.95, and 31-40499.94). One of the authors (D.A.) is a recipient of a EMBO long-term fellowship (ALTF 97-1994).

References

1. Folk, J. E., and J. S. Finlayson. 1977. The beta(gamma-glutamyl)lysine cross-link and the catalytic role of transglutaminases. *Adv. Protein Chem.* 31:1–133.
2. Lorand, L., and S. M. Conrad. 1984. Transglutaminases. *Mol. Cell Biochem.* 58:9–35.
3. Aeschlimann, D., and M. Paulsson. 1994. Transglutaminases: protein cross-linking enzymes in tissues and body fluids. *Thrombo. Haemostasis* 71:402–415.
4. Greenberg, C. S., P. J. Birckbichler, and R. H. Rice. 1991. Transglutaminases: multifunctional cross-linking enzymes that stabilize tissues. *FASEB J.* 5:3071–3077.
5. Fesus, L., P. J. Davies, and M. Piacentini. 1991. Apoptosis: molecular mechanisms in programmed cell death. *Eur. J. Cell Biol.* 56:170–177.
6. Aeschlimann, D., A. Wetterwald, H. Fleisch, and M. Paulsson. 1993. Expression of tissue transglutaminase in skeletal tissues correlates with events of terminal differentiation of chondrocytes. *J. Cell Biol.* 120:1461–1470.
7. 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.
8. Aeschlimann, D., and M. Paulsson. 1991. Cross-linking of laminin-nidogen complexes by tissue transglutaminase. *J. Biol. Chem.* 266:15308–15317.
9. Martinez, J., D. G. Chalupowicz, R. K. Roush, A. Sheth, and C. Barsigian. 1994. Transglutaminase-mediated processing of fibronectin by endothelial cell monolayers. *Biochemistry* 33:2538–2545.
10. Raghunath, M., B. Hoepfner, D. Aeschlimann, U. Luethi, M. Meuli, S. Alternatt, R. Gobet, L. Bruckner Tuderman, and B. Steinmann. 1996. Cross-linking of the dermo-epidermal junction of skin regenerating from keratinocyt autografts: anchoring fibrils are a target for tissue transglutaminase. *J. Clin. Invest.* 98:1174–1184.

11. Upchurch, H. F., E. Conway, M. K. Patterson, Jr., and M. D. Maxwell. 1991. Localization of cellular transglutaminase on the extracellular matrix after wounding: characteristics of the matrix bound enzyme. *J. Cell Physiol.* 149: 375–382.
12. Achyuthan, K. E., A. Mary, and C. S. Greenberg. 1988. The binding sites on fibrin(ogen) for guinea pig liver transglutaminase are similar to those of blood coagulation factor XIII: characterization of the binding of liver transglutaminase to fibrin. *J. Biol. Chem.* 263:14296–14301.
13. Shainoff, J. R., D. A. Urbanic, and P. M. DiBello. 1991. Immunoelectrophoretic characterizations of the cross-linking of fibrinogen and fibrin by factor XIIIa and tissue transglutaminase: identification of a rapid mode of hybrid alpha-/gamma-chain cross-linking that is promoted by the gamma-chain cross-linking. *J. Biol. Chem.* 266:6429–6437.
14. Fesus, L., M. L. Metsis, L. Muszbek, and V. E. Koteliansky. 1986. Transglutaminase-sensitive glutamine residues of human plasma fibronectin revealed by studying its proteolytic fragments. *Eur. J. Biochem.* 154:371–374.
15. LeMosy, E. K., H. P. Erickson, W. F. Beyer, Jr., J. T. Radek, J. M. Jeong, S. N. Murthy, and L. Lorand. 1992. Visualization of purified fibronectin-transglutaminase complexes. *J. Biol. Chem.* 267:7880–7885.
16. Mecham, R. P., T. Broekelmann, E. C. Davis, M. A. Gibson, and P. Brown-Augsburger. 1995. Elastic fibre assembly: macromolecular interactions. *Ciba. Found. Symp.* 192:172–181.
17. Raghunath, M., T. Baechi, D. Aeschlimann, and B. Steinmann. 1997. Fibrillin, the main component of elastic microfibrils is modified by transglutaminase in cultures of dermal fibroblasts and human dermis. *Eur. J. Ped.* 155:735.
18. Qiam, R. Q., and R. W. Glanville. 1997. Fibrillin molecules in elatic microfilaments are cross-linked by transglutaminase derived isopeptide boands. *J. Biol. Chem.* (In press)
19. Sane, D. C., T. L. Moser, A. M. Pippen, C. J. Parker, K. E. Achyuthan, and C. S. Greenberg. 1988. Vitronectin is a substrate for transglutaminases. *Biochem. Biophys. Res. Commun.* 157:115–120.
20. Skorstengaard, K., T. Halkier, P. Hojrup, and D. Mosher. 1990. Sequence location of a putative transglutaminase cross-linking site in human vitronectin. *FEBS Lett.* 262:269–274.
21. Aeschlimann, D., M. Paulsson, and K. Mann. 1992. Identification of Gln726 in nidogen as the amine acceptor in transglutaminase-catalyzed cross-linking of laminin-nidogen complexes. *J. Biol. Chem.* 267:11316–11321.
22. 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.
23. Sorensen, E. S., L. K. Rasmussen, L. Moller, P. H. Jensen, P. Hojrup, and T. E. Petersen. 1994. Localization of transglutaminase-reactive glutamine residues in bovine osteopontin. *Biochem. J.* 304:13–16.
24. Kleman, J. P., D. Aeschlimann, M. Paulsson, and M. van der Rest. 1995. Transglutaminase-catalyzed cross-linking of fibrils of collagen V/XI in A204 rhabdomyosarcoma cells. *Biochemistry* 34:13768–13775.
25. Bowness, J. M., J. E. Folk, and R. Timpl. 1987. Identification of a substrate site for liver transglutaminase on the aminopropeptide of type III collagen. *J. Biol. Chem.* 262:1022–1024.
26. Burri, P. H., J. Dbaly, and E. R. Weibel. 1974. The postnatal growth of the rat lung. I. Morphometry. *Anat. Rec.* 178:711–730.
27. Burri, P. H. 1974. The postnatal growth of the rat lung. III. Morphology. *Anat. Rec.* 180:77–98.
28. Massaro, D., N. Teich, S. Maxwell, G. D. Massaro, and P. Whitney. 1985. Postnatal development of alveoli: regulation and evidence for a critical period in rats. *J. Clin. Invest.* 76:1297–1305.
29. Randell, S. H., R. R. Mercer, and S. L. Young. 1989. Postnatal growth of pulmonary acini and alveoli in normal and oxygen-exposed rats studied by serial section reconstructions. *Am. J. Anat.* 186:55–68.
30. Burri, P. H. 1984. Fetal and postnatal development of the lung. *Annu. Rev. Physiol.* 46:617–628.
31. Burri, P. H. 1996. Structural aspects of pre- and postnatal development and growth of the lung. *In* Growth and Development of the Lung. J. McDonald, editor. Marcel Dekker, Inc., New York.
32. Chomzynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidine thiocyanate-phenol-chloroform. *Anal. Biochem.* 162: 156–159.
33. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.
34. Gentile, V., M. Saydak, E. A. Chiocca, O. Akande, P. J. Birckbichler, K. N. Lee, J. P. Stein, and P. J. Davies. 1991. Isolation and characterization of cDNA clones to mouse macrophage and human endothelial cell tissue transglutaminases. *J. Biol. Chem.* 266:478–483.
35. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685.
36. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polycrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76:4350–4354.
37. Tarcsa, E., and L. Fésus. 1990. Determination of epsilon (gamma-glutamyl)-lysine crosslink in proteins using phenylisothiocyanate derivatization and high-pressure liquid chromatographic separation. *Anal. Biochem.* 186:135–140.
38. Jeffery, P. K., and L. Reid. 1975. New observations of rat airway epithelium: a quantitative and electron microscopical study. *J. Anat.* 120:295–320.
39. Schittny, J. C., and P. H. Burri. 1995. A peak of programmed cell death appears during postnatal lung development. *J. Cell. Biochem.* 19B:319. (Abstr.)
40. Brown-Augsburger, P., T. Broekelmann, L. Mecham, R. Mercer, M. A. Gibson, E. G. Cleary, W. R. Abrams, J. Rosenbloom, and R. P. Mecham. 1994. Microfibril-associated glycoprotein binds to the carboxyl-terminal domain of tropoelastin and is a substrate for transglutaminase. *J. Biol. Chem.* 269: 28443–28449.
41. Schittny, J. C., and P. D. Yurchenco. 1989. Basement membranes: molecular organization and function in development and disease. *Curr. Opin. Cell Biol.* 1:983–988.
42. Yurchenco, P. D., Y. S. Cheng, and H. Colognato. 1992. Laminin forms an independent network in basement membranes. *J. Cell Biol.* 117:1119–1133.
43. Yurchenco, P. D., and J. C. Schittny. 1990. Molecular architecture of basement membranes. *FASEB J.* 4:1577–1590.