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Deposition of an intermediate form of procollagen type III (pN-collagen) into fibrils in the matrix of amniotic epithelial cells

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We have followed the deposition and maturation of the pericellular matrix of amniotic epithelial cell cultures for up to eight weeks using metabolic labeling and immunoelectron microscopy. This matrix contains mainly collagen type III and fibronectin. Cleavage of the carboxypropeptide occurred after secretion of the procollagen molecules into the medium but was not accompanied by a significant release of the aminopropeptide. The early matrix, as isolated from the cultures by a deoxycholate procedure, contained collagenous proteins predominantly composed of pNα1(III) chains, which still possessed the aminopropeptide, and only little material in the form of α1(III) chains. The relative amount of α1(III) chains increased during subsequent days of culture. Electron microscopy showed two types of structures in the matrix: thin fibrils, ranging from 10 to 30 nm in diameter, with no apparent cross-striation, and 50–500 nm thick bundles composed of filamentous and amorphous material. In the fibrils, immunoferritin electron microscopy showed a regular staining for the aminopropeptide of procollagen type III with a periodicity of 71 nm. These collagenous fibrils did not stain for fibronectin which was found in the bundles. Since most of the aminopropeptide in the matrix appeared covalently linked as pN-collagen, we conclude that the deposition of this intermediate form of procollagen is a general mechanism in collagen type III fibrillogenesis.

Key words: fibrillogenesis/fibronectin/immunoelectron microscopy/metabolic labeling/procollagen aminopropeptide

Introduction

Most of the interstitial compartments of the body contain numerous collagen fibrils which are composed of type I and/or type III collagens. The latter protein is particularly found in regions rich in thin fibrils as indicated by immunofluorescence (Nowack *et al.*, 1976a; Timpl *et al.*, 1977) and immunoelectron microscopy (Fleischmajer *et al.*, 1980; 1981). It has been proposed that fibrillogenesis of interstitial collagens is preceded by the conversion of precursor molecules into collagen (Fessler and Fessler, 1978). A rather slow processing of procollagen type III was, however, observed in organ cultures of chick embryo blood vessels (Fessler *et al.*, 1981). After a rapid release of the carboxypropeptide an intermediate form of the precursor, pN-collagen III, which still possessed the aminopropeptide persisted for a long period in the tissue. This slow processing of pN-collagen was consonant with other data obtained by *in vivo* labeling (Limeback and Sodek, 1979; Robins, 1979) or by characterizing medium proteins in a variety of cell cultures (Goldberg, 1977; Fessler and Fessler, 1979; Krieg *et al.*, 1979; Alitalo *et al.*, 1980a). In human skin the aminopropeptide of procollagen type III appeared closely associated with 20–40 nm thick collagen fibrils as shown by immunoelectron microscopy (Fleischmajer *et al.*, 1981). Together the data suggested that pN-collagen III is a structural component of thin fibrils and perhaps involved in regulating the growth of these fibrils.

Amniotic epithelial cells in primary culture produce and secrete two major components, procollagen type III and fibronectin, together with small amounts of basement membrane proteins (Alitalo *et al.*, 1980a). The same proteins are deposited in a pericellular matrix resembling the acellular layer found underneath the amniotic epithelium *in situ*. The cells failed to produce significant amounts of procollagen type I which, due to its abundance, often obscures studies on other collagenous proteins. Thus, amniotic epithelial cells offered a unique opportunity for correlating processing and deposition of procollagen type III.

Results

Processing of procollagen type III

Analysis of radioactive polypeptides in cultures of amniotic epithelial cells labeled for 24 h with glycine and proline revealed three major polypeptides in the medium. These bands were identified by immunoprecipitation (Figure 1) as fibronectin chains (M_r = 220 000) and as two chains of procollagen type III (M_r = 145 000 and 120 000, based on collagenous standards) due to their reactivity with antibodies against the aminopropeptide. According to their relative mobilities (Fessler *et al.*, 1981) the latter bands resemble proα1(III) chains of unprocessed procollagen and pNα1(III) chains of pN-collagen III. As expected, fibronectin could be bound to gelatin-Sepharose while the procollagens were selectively degraded by bacterial collagenase (not shown). The medium contained no or only small amounts of a collagenase-sensitive polypeptide which migrated with the mobility of the α1(III) chain of collagen type III. Pepsin treatment converted all the procollagen chains into α1(III) chains (Alitalo *et al.*, 1980a).

The deposition of collagenous proteins was studied in cultures continuously labeled for up to three days by subjecting the total cell layer (Figure 2 A–C) including the matrix and cells, the isolated pericellular matrix (D–F), and the medium (G–I) to polypeptide analysis. In the medium the relative amount of the pNα1(III) chain increased at the expense of the proα1(III) chain during the 3-day culture period. Only small amounts of α1(III) chains could be detected at the end of this period. The pericellular matrix contained a distinct pNα1(III) chain band after one day of labeling (D), but negligible amounts of either unprocessed procollagen or α1(III) chains. With prolonged labeling α1(III) chains appeared in the matrix (E) and subsequently exceeded the pNα1(III) chains (F) in amount. A similar change was observed with the total cell layer, which otherwise showed a more complex band pattern. It included small amounts of pro-

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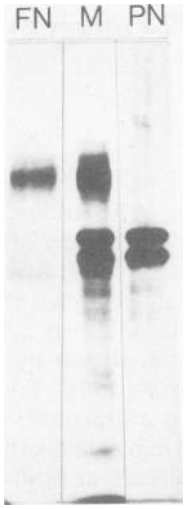

Fig. 1. Immunoprecipitation and SDS-polyacrylamide gel electrophoresis of proteins from metabolically labeled culture medium using anti-human fibronectin serum (FN) or antibodies against the amino-terminal propeptide of procollagen type III (PN). Total culture medium proteins are shown in the middle lane (M).

α1(III) chains presumably derived from intracellular procollagen.

Since the data suggested that conversion of procollagen type III occurred via a pN-collagen intermediate, we analyzed the cell layer in a pulse-chase experiment (Figure 3). A 3-h pulse followed by a 16-h chase showed three collagenous polypeptides, proα1(III), pNα1(III), and α1(III) (lane 1), as identified by immunoprecipitation and collagenase digestion. The proα1(III) chain and later the pNα1(III) chain disappeared completely during a prolonged chase (lanes 1 and 2). Analysis of medium proteins in a shorter pulse-chase experiment showed the initial appearance of proα(III) chains which were later converted to pNα1(III) chains (not shown).

The nature and size of the polypeptides reacting with antibodies against the aminopropeptide was confirmed by immunoblotting using unlabeled cell cultures. The antibodies reacted in the total cell layer with polypeptide bands with the mobility of proα1(III) and pNα1(III) chains while only the latter were detected in the isolated pericellular matrix (Figure 4). The antibodies failed to react with bands in the expected position of the free aminopropeptide after release from pro-

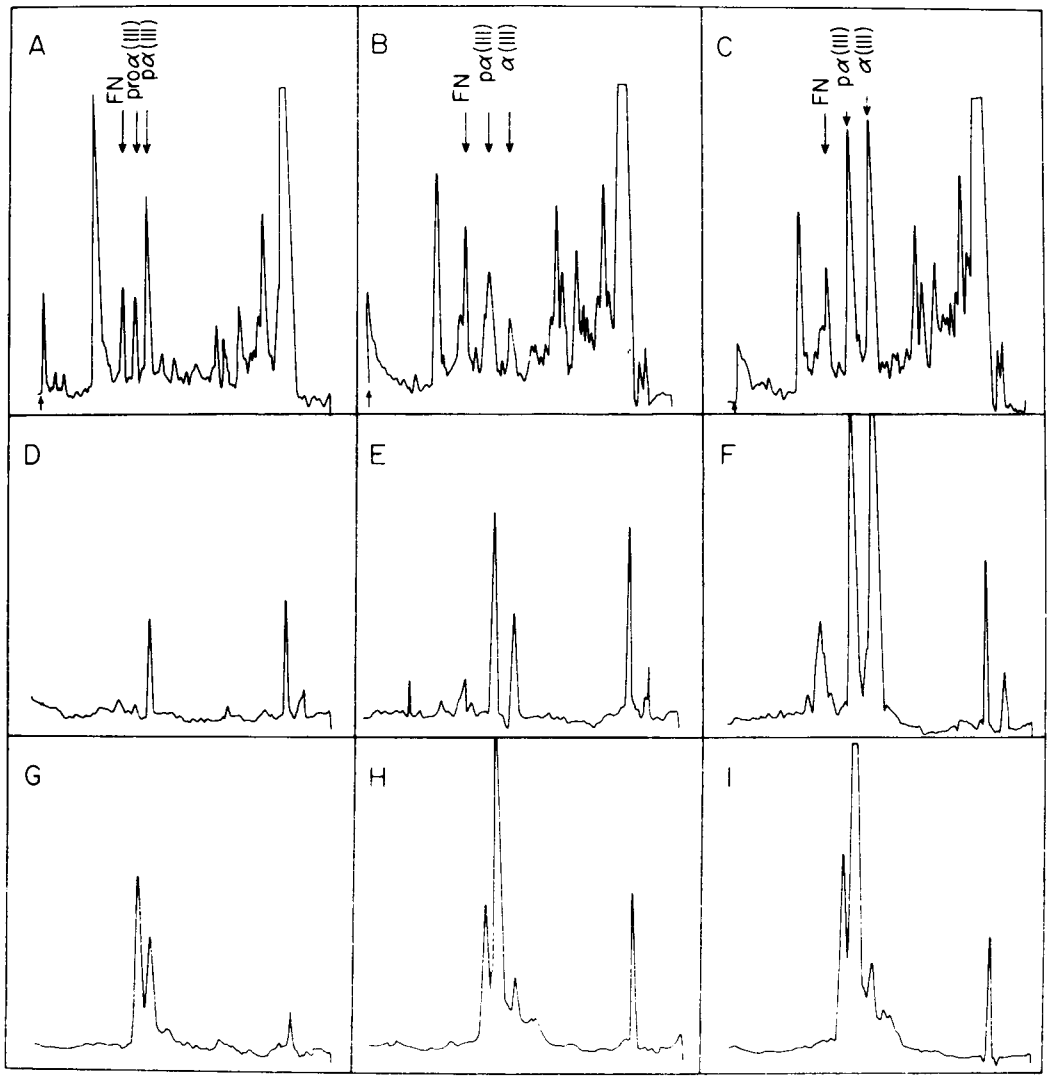

Fig. 2. Densitometric scanning of labeled polypeptides from amniotic epithelial cell layers (**A – C**), isolated pericellular matrices (**D – F**), and culture media (**G – I**). The cultures were labeled for one (**A,D,G**), two (**B,E,H**), or three (**C,F,I**) days with [^{3}H]glycine and [^{3}H]proline and the polypeptides analyzed in different culture compartments by polyacrylamide gel electrophoresis using 5% gels. The arrows indicate the migration positions of fibronectin (FN), procollagen type III chains [proα(III); pα(III)], and collagen type III chains [α(III)].

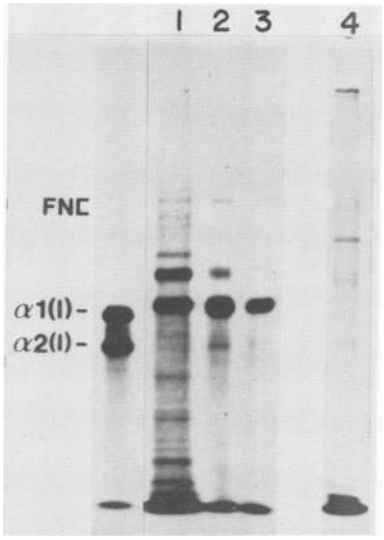

Fig. 3. SDS-polyacrylamide gel (5%) electrophoretic analysis in reducing conditions of procollagen type III processing in cultures of human amniotic epithelial cells. The cells were pulsed with 50 μCi/ml [^{3}H]glycine and [^{3}H]-proline in the presence of 50 μg/ml sodium ascorbate and β-aminopropionitrile fumarate. The markers, radiolabeled type I collagen chains, are shown on the left [α1(I), α2(I)]. Lanes **1**–**4** show proteins of the cell layer after a 3-h pulse followed by chases of 16 h (**1**), 40 h (**2**), 64 h (**3**), or, in the absence of β-apn, 64 h (**4**).

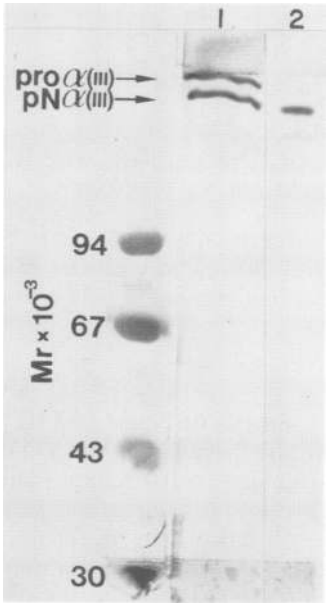

Fig. 4. Immunological identification after electrophoretic transfer of proteins from SDS-PAGE gels (8%) to nitrocellulose sheets. The sheets were treated with antibodies to the amino-terminal procollagen III propeptide followed by anti-IgG peroxidase conjugate and enzyme reaction and fixed with transparant tape. Lane **1** contains the proteins of total cell layer and lane **2** of isolated pericellular matrix. Migration positions of procollagen type III and pNα(III) chains as well as noncollagenous standards are shown on the left with the respective relative molecular weights.

collagen, but reacted in control experiments with an authentic sample of the aminopropeptide.

Ultrastructural localization of pN-collagen III in the pericellular matrix

The polypeptide analyses indicated that a partially processed procollagen is a major structural component of the matrix. Its precise ultrastructural localization was therefore studied at the electron microscopic level. An overview of the distribution was provided by indirect immunofluorescence showing abundant staining for both the aminopropeptide and fibronectin. Both antigens appeared in the growth substratum a few hours after cell seeding and showed codistribution only at places revealed by double-staining in a previous study (Alitalo *et al.*, 1980a). Similarly, in the isolated matrix the procollagen antigen was mainly found in fibrils often grouped in fascicles to form a coarse reticulum (Figure 5a), while fibronectin occurred in a network showing a mossy fibrillar and punctate distribution (Figure 5b).

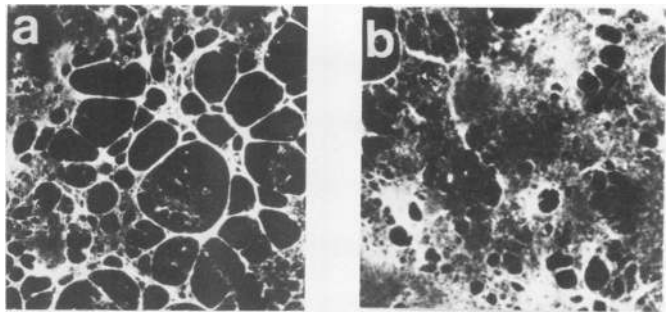

Fig. 5. Immunofluorescence microscopy of isolated pericellular matrix. Fixed matrices were stained in indirect immunofluorescence using antibodies against the amino-terminal propeptide of procollagen type III (**a**) or anti-fibronectin antiserum (**b**). The propeptide is distributed in a coarse fibrillar reticulum and, at places, in mossy distribution. Fibronectin is found primarily in mossy sheets of fibres and aggregates, but also occasionally grouped in larger bundles (x 350).

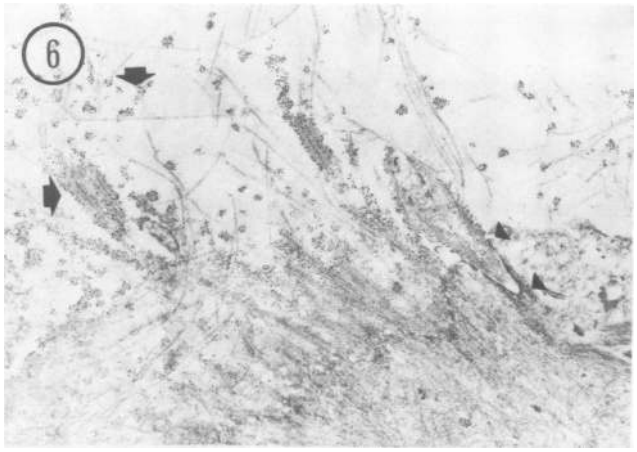

Fig. 6. Immunoferritin transmission electron microscopy of a culture of human amniotic epithelial cells stained (an indirect staining method) for fibronectin. In this oblique section cytoplasmic cortical microfilaments (on the lower right) terminate at the fuzzy zone indicating plasma membrane. Extracellular bundles of filamentous and amorphous material as well as amorphous aggregates (➡) are densely decorated with ferritin markers indicating the presence of fibronectin. At the cell surface the ferritin-decorated bundles are at places in apparent colinear organization with the cytoplasmic microfilament bundles (▶). Extracellular (collagen) fibrils do not themselves bind ferritin markers, but seem to be loosely associated with the fibronectin-containing material. (x 15 000).

Transmission electron microscopy also demonstrated two distinct morphological structures in the matrix: fine fibrils with a diameter of 10–30 nm extending far away from the cells and aggregates and filamentous bundles with a diameter of 50–500 nm. The fibrils showed no cross-striation as usually found in collagenous fibrils except that a faint periodicity resembling the D (= 67 nm) stagger could be observed in some 30 nm fibrils. However, >90% of these fibrils could be densely decorated with a ferritin-antibody conjugate after

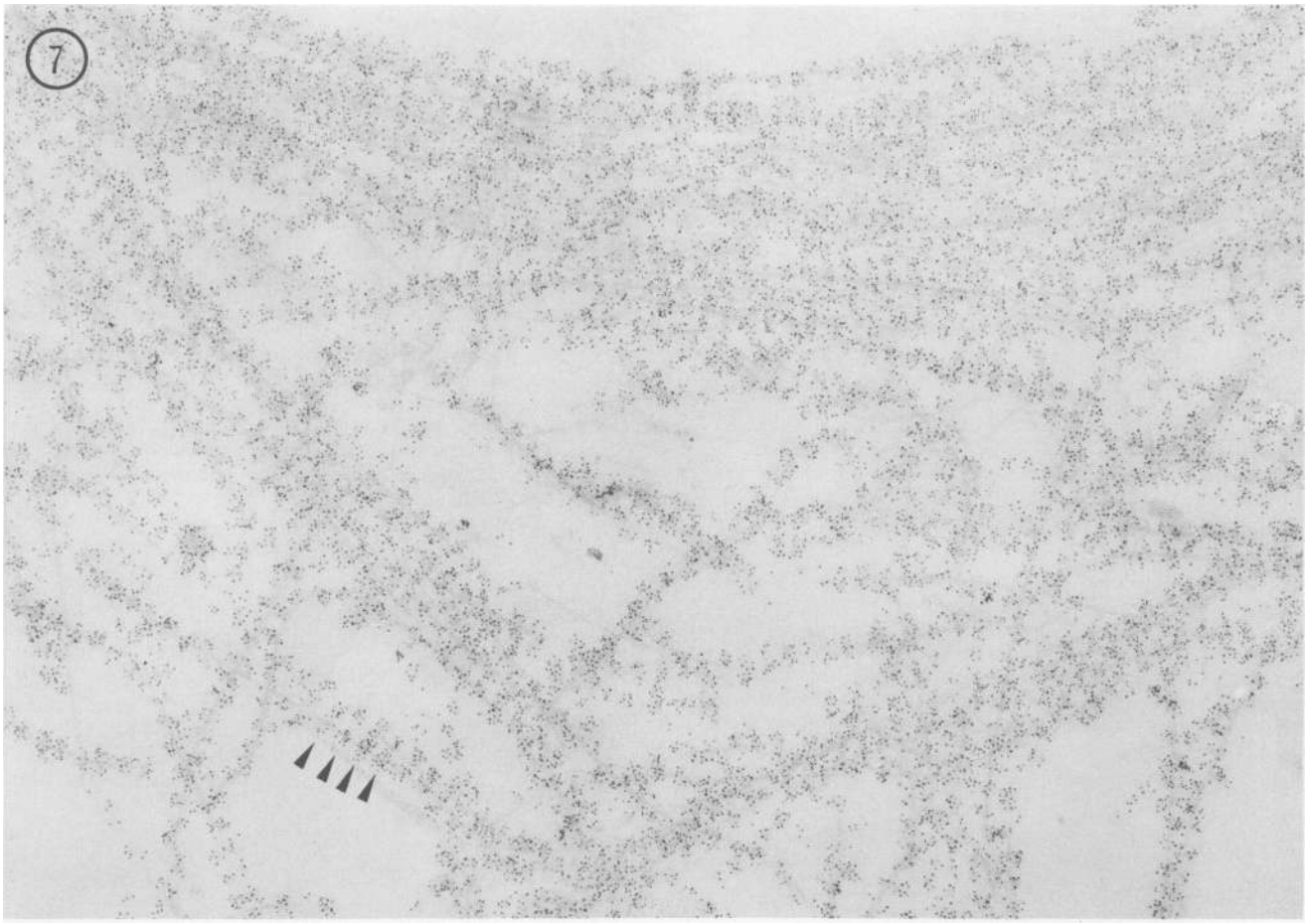

Fig. 7. Immunoferritin transmission electron microscopy staining for the amino-terminal propeptide of procollagen III. Extracellular fibrils bind ferritin markers densely in an apparent periodic pattern (▬). The fibrils are 10–30 nm in diameter with no cross-striated ultrastructure (x 46 000).

treatment with antibodies against the aminopropeptide (Figure 6). Staining appeared in a periodic pattern with intervals of ∿70 nm. No staining was seen in control experiments.

Immunoelectron microscopy showed a different reaction pattern with antibodies against fibronectin (Figure 7). At greater distances from the cells, ferritin markers were found clustered to amorphous patches of 50–200 nm diameter. These patches were occasionally loosely associated with fascicles of unlabeled collagenous fibrils. Closer to the cell surface, fibronectin was found in filamentous and amorphous bundles 50–500 nm in diameter, which appeared at places in close association with cytoplasmic microfilaments (Figure 7).

To visualize the distribution of the pN-collagen III-containing fibrils in three dimensions, immunoferritin-stained cultures were also observed by high-resolution scanning electron microscopy.

After a few days of culture the amniotic epithelial cells formed a strict monolayer. Occasional gaps between the cells, however, allowed visualization of the matrix coated with carbon and gold. Smooth fibrils with a diameter of ∿50 nm (Figure 8, inset) were prominent. Their ultrastructure did not change after treatment with anti-fibronectin or nonimmune rabbit sera (Figure 8, inset). On the contrary, reaction with antibodies against the aminopropeptide produced a uniform knotted appearance of the fibrils (Figure 8). Statistical analysis of the periodic pattern of the knots of non-tilted specimens, using a calibrated TEMSCAN 100 C X microscope, showed a mean distance (± s.d.) of 71.4 ± 2.3 nm. At higher magnification each knot was found to be composed of smaller particles of ∿20 nm in diameter (Figure 8, inset), presumably representing individual gold-coated ferritin particles.

Discussion

The interstitial collagens of type I and III share a similar molecular structure and the ability to form D-staggered fibrils characterized by a unique cross-striation pattern (Kühn and Glanville, 1980). Proper fibril formation may require intracellular packaging of procollagen molecules (Bruns *et al.*, 1979), which then become processed prior to or after their extracellular deposition. The latter possibility was supported by the immunoelectron microscope visualization of aminopropeptides in close association with thin fibrils of human skin (Fleischmajer *et al.*, 1981). The two collagenous proteins differ, however, in certain features such as procollagen processing and maturation of fibrils. Several studies *in vivo* (Limeback and Sodek, 1979; Robins, 1979), in organ culture (Fessler *et al.*, 1981; Fessler and Fessler, 1979), and in cell culture (Krieg *et al.*, 1979; Sage *et al.*, 1979; Alitalo *et al.*, 1980a) indicated a slow conversion of procollagen type III particularly with respect to the release of its aminopropeptide. This agreed with other observations that a particular class of 20–40 nm thick fibrils in skin which are composed of collagen type III (Fleischmajer *et al.*, 1980) could also react with antibodies against the aminopropeptide (Fleischmajer *et al.*, 1981). These studies could not distinguish between the possibilities that the aminopropeptide still belonged to intact

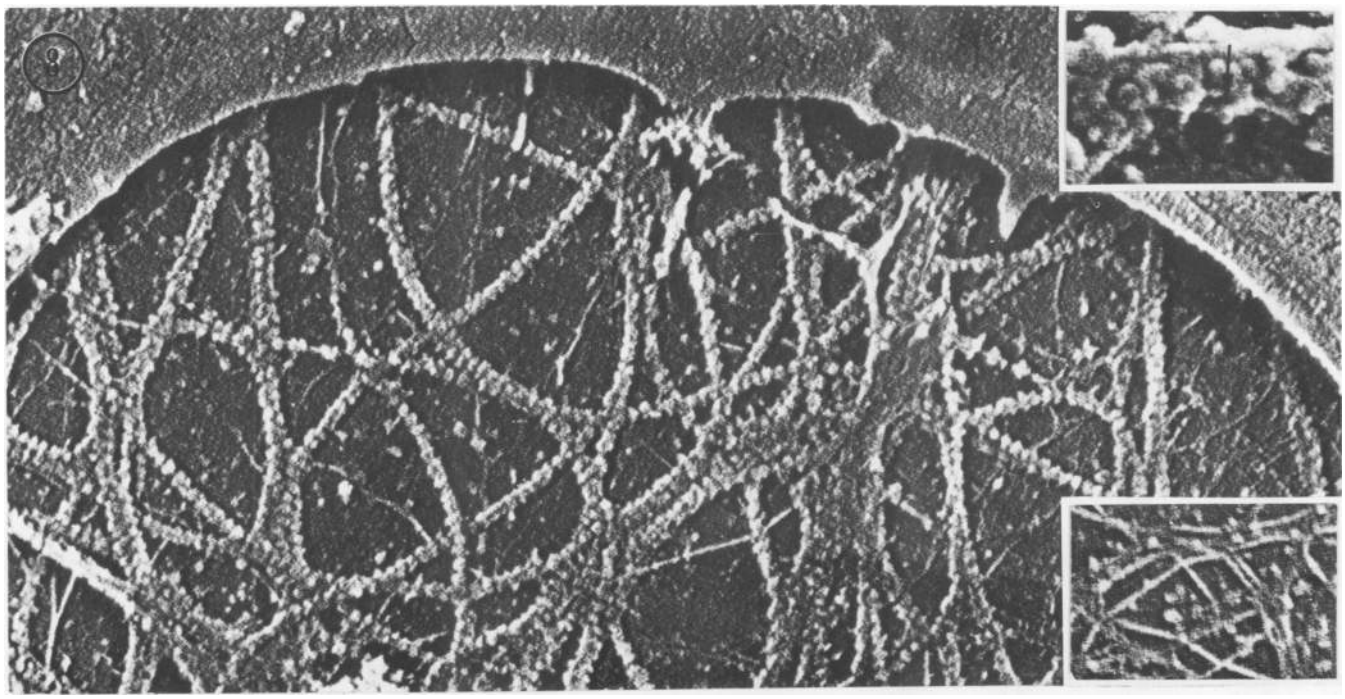

Fig. 8. Immunoferritin scanning electron microscopy of an amniotic epithelial cell culture stained for the amino-terminal propeptide of procollagen III. Beneath the cell edge, growth substratum is covered with a loose network of (collagen) fibrils, which have a periodic dentate or nodular appearance (x 23 500). **Upper inset**, a high magnification (x 115 000) of such a nodular fibril; smaller particles of about 20 nm (⟶) can be resolved. **Lower inset**, (collagen) fibrils from a control sample, treated with non-immune rabbit serum instead of specific antibodies in an indirect immunoferritin method. Fibrils are ∿50 nm in diameter (including carbon and gold coatings) and appear relatively smooth surfaced in ultrastructure.

or processed procollagen molecules as structural components of the fibrils or that it was already released from procollagen but remained non-covalently associated with the fibrils.

We have now used cultures of amniotic epithelial cells which synthesize predominantly procollagen type III (Alitalo *et al.*, 1980a) as a convenient system to directly compare procollagen processing and the structure of the fibrillar deposit. Intact procollagen could be detected in the medium but was only a short-lived form; it was also present apparently as an intracellular component. At no stage of the cultures were appreciable amounts of unprocessed procollagen found in the matrix. This preponderance of pN-collagen III in the pericellular matrix agrees with the data on blood vessel cultures (Fessler *et al.*, 1981). The final maturation of pN-collagen to collagen seemed to occur in the matrix, presumably in a process catalyzed by a procollagen N-protease specific for type III (Nusgens *et al.*, 1980).

Immunoelectron microscopy demonstrated that pN-collagen III was exclusively associated with thin (10–30 nm) fibrils in the matrix. A periodic staining pattern with a distance of 60–70 nm is expected for such fibrils since the aminopropeptide ought to be located in the hole region of D-staggered pN-collagen molecules (Kühn and Glanville, 1980). The fibrils in the matrix lacked the typical cross-striation pattern of collagenous fibrils. This is a common observation for other thin collagen fibrils such as those found in cartilage (Kühn and von der Mark, 1978). Lack of cross-striation may not necessarily be a characteristic feature of collagen type III fibrils (Fleischmajer *et al.*, 1980; Kühn and Glanville, 1980) and apparently may not be due to the presence of distinct amounts of pN-collagen (Fleischmajer *et al.*, 1981).

These studies do not distinguish between selective deposition of the partially processed form, pN-collagen, and deposition of unprocessed procollagen very rapidly followed by action of the C-protease. Of the procollagen synthesized by amniotic epithelial cells, 80–90% is found in the medium (Alitalo *et al.*, 1980a), as unprocessed and pN-collagen. It is possible that some of the procollagen in the medium gets deposited; however, in fibroblast cultures, according to Goldberg (1977), deposition of type III from the medium is not significant. With time, increasing amounts of α1(III) chains were also found in the matrix. Whether the α1(III) chains identified in the matrix belong to the same fibrils as the pNα1(III) chains is not known but seems likely. Partially processed molecules consisting of both α1(III) and pNα1(III) chains may in fact exist in fetal skin (Timpl *et al.*, 1975). It was recently suggested (Fleischmajer *et al.*, 1981) that a precise regulation between deposition of pN-collagen III and proteolytic removal of the aminopropeptide controls the fibril diameter. Our data are compatible with this possibility.

Immunofluorescence (Bornstein and Ash, 1977; Vaheri *et al.*, 1978) and immunoperoxidase studies (Furcht *et al.*, 1980) of fibroblast matrices indicated a close association between procollagens and fibronectin. Other studies indicated that fibronectin also has the ability to self-assemble into fibrils without collagen (Vuento *et al.*, 1980). The latter possibility seems to be realized in the amniotic epithelial cell matrix, as indicted by a distinct ultrastructural localization of the two proteins. Fibronectin was also found in close contact with cell surfaces and, unlike collagen, in fibronexus-like structures (Singer, 1979). It is tempting to speculate that such structures link the collagen fibrils and epithelial cells.

Materials and methods

Epithelial cells and polypeptide analysis

Primary cultures of amniotic epithelial cells were prepared as described (Alitalo *et al.*, 1980a) from full-term post-partal human placentas. The tryp-

sinized epithelial cells were grown in Petri dishes in Eagle's minimum essential medium supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (50 μg/ml) and sodium ascorbate (50 μg/ml). For radioactive labeling the cultures were incubated with or without calf serum in medium containing 10 μCi/ml of both [2-^{3}H]glycine (23 Ci/mmol; The Radiochemical Centre, Amersham, UK) and L[5-^{3}H]proline (14 Ci/mmol), and 50 μg/ml of both ascorbate and β-aminopropionitrile fumarate. The cells were grown up to eight weeks and labeled media and cell layers were processed separately for polypeptide analysis.

The labeled cultures briefly rinsed with P_i/NaCl (0.01 M phosphate buffer, 0.14 M NaCl, pH 7.4) are referred to as total cell layer. Cell layers were also extracted using a slight modification (Hedman *et al.*, 1981) of the deoxycholate procedure (Hedman *et al.*, 1979) to remove most cellular material leaving behind the isolated pericellular matrix still attached to the growth substratum. This treatment consists of three exposures of 10 min each to 0.5% Na-deoxycholate in 10 mM Tris-Cl buffer, pH 8.0, containing 1 mM phenylmethyl sulfonyl fluorine (PMSF) at 4°C followed by three rinses with 2 mM Tris-Cl, pH 8.0, containing PMSF. Secreted proteins were precipitated from the culture media with ammonium sulfate (281 mg/ml) overnight at 4°C or with 10% trichloroacetic acid (TCA) at 0°C followed by washes with cold 5% TCA, 95% ethanol and absolute diethyl ether as described (Alitalo *et al.*, 1980a).

Purified bacterial collagenase (Form III, Advance Biofactures, NY,) and pepsin (Sigma, St. Louis, MO: 3 x crystallized) were used to digest collagenous or non-collagenous proteins (Alitalo *et al.*, 1980b). The samples were then analyzed by sodium dodecyl sulfate (SDS) 5% polyacrylamide slab gel electrophoresis followed by fluorography and densitometric scanning (Laemmli, 1970; Bonner and Laskey, 1974). Runs were calibrated with non-radioactive (Pharmacia, Uppsala, Sweden) and radioactive (Radiochemical Centre) non-collagenous proteins or collagenous markers covalently prestained according to Griffith (1972).

Immunofluorescence and immunoprecipitation

For immunofluorescence microscopy, the cell cultures or isolated pericellular matrices on glass coverslips were rinsed with P_i/NaCl and fixed with 4% paraformaldehyde (20 min at room temperature) followed by washes in P_i/NaCl. The samples were then stained by indirect immunofluorescence for fibronectin or the aminopropeptide of procollagen type III. The details, with appropriate controls, have been described previously (Vaheri *et al.*, 1978). Antigens were immunoprecipitated from labeled culture media or from the cells solubilized with Na-deoxycholate and Triton X-100 at 4°C (Rowl *et al.*, 1978) by affinity-purified antibodies (Nowack *et al.*, 1976) followed by collagenase or pepsin treatments, when indicated.

Immunoblotting of proteins

The procedure described by Towbin *et al.*, (1979) was used. Polypeptides separated on SDS-polyacrylamide (8%) slab gel electrophoresis were transferred electrophoretically to nitrocellulose sheets (Type HAMP filter; Millipore, Bedford, MA), the protein-binding sites were saturated with bovine serum albumin, and the sheets stained using the immunoperoxidase method. The rabbit antibodies were diluted in 0.05 M Tris-HCl, 0.1 M NaCl, pH 7.5, containing 10% fetal calf serum. The anti-rabbit IgG peroxidase conjugate (γ-chain-specific; Orion Diagnostica, Helsinki, Finland) was used at a dilution of 1:100 in the Tris-buffered saline. Controls included the analysis of authentic pN-collagen III and its isolated aminopropeptide which were purified from bovine skin as described previously (Nowack *et al.*, 1976b).

Transmission and scanning immunoelectron microscopy

Human amniotic epithelial cells were grown on plastic dishes with or without glass coverslips for transmission or scanning immunoferritin electron microscopy, respectively. The cultures were rinsed briefly with P_i/NaCl and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, for 30 min at 4°C. After washing, the cultures were labeled for extracellular fibronectin or procollagen type III using rabbit antiserum prepared against the aminopropeptide of procollagen type III (Nowack *et al.*, 1976a). The rabbit anti-fibronectin serum was absorbed with immobilized fetal calf serum proteins and was found then to be non-reactive with bovine fibronectin. An indirect immunoferritin staining method was used with ferritin covalently conjugated to sheep anti-rabbit IgG immunoglobulins (Hedman *et al.*, 1978; Kishida *et al.*, 1975). After glutaraldehyde postfixation, the cell layers were processed for transmission electron microscopy according to standard procedures and thin sections were made parallel to the growth substratum. For scanning electron microscopy, the fixed glass coverslips were critical point dehydrated from ethanol and coated with gold. A Jeol 100 B transmission electron microscope fitted with a Video Control Amplifier Unit was used at 17 kV, and high resolution scanning electron microscopy studies were carried out with a Jeol 100 CX TEMSCAN electron microscope at an accelerating voltage of 40 kV. The specificity of our immunoferritin staining method has been documented previously (Hedman *et al.*, 1978) and was confirmed in this study by using non-immune rabbit serum instead of the primary antiserum or antibodies. Antibodies against procollagen type I and collagen type II (Nowack *et al.*, 1976a; Timpl *et al.*, 1977) were also used as controls. All these control antisera or antibodies resulted in negligible binding of ferritin to the extracellular structures of the cell cultures. The different labeling patterns observed with anti-fibronectin antiserum and anti-procollagen III antibodies also indicted that the reactions were specific (see **Results**).

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