Shift in Collagen Type as an Early Response to Induction of the Metanephric Mesenchyme

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ABSTRACT Conversion of the nephrogenic mesenchyme into epithelial tubules requires an inductive stimulus from the ureter bud. Here we show with immunofluorescence techniques that the undifferentiated mesenchyme before induction expresses uniformly type I and type III collagens. Induction both in vivo and in vitro leads to a loss of these proteins and to the appearance of basement membrane components including type IV collagen. This change correlates both spatially and temporally with the determination of the mesenchyme and precedes any morphological events. During morphogenesis, type IV collagen concentrates at the borders of the developing tubular structures where, by electron microscopy, a thin, often discontinuous basal lamina was seen to cover the first pretubular cell aggregates. Subsequently, the differentiating tubules were surrounded by a well-developed basal lamina.

No loss of the interstitial collagens was seen in the metanephric mesenchyme when brought into contact with noninducing tissues or when cultured alone. Similar observations were made with nonnephrogenic mesenchyme (salivary, lung) when exposed to various heterotypic tissues known to induce tubules in the nephrogenic mesenchyme.

The sequential shift in the composition of the extracellular matrix from an interstitial, mesenchymal type to a differentiated, epithelial type is so far the first detectable response of the nephrogenic mesenchyme to the tubule-inducing signal.

In the embryonic kidney rudiment, an inductive interaction between the epithelial component and the mesenchymal blastema is required for differentiation (9). This reciprocal interaction leads to the orderly branching of the ureter bud and to the determination of the mesenchyme. The mesenchymal cells then aggregate into condensates, and these convert into epithelial tubules (22). The induction of the mesenchyme can be precisely timed and correlated with the morphological and biological events by culturing the interacting components on opposite sides of a filter membrane (10). In this particular system with a heterotypic inductor (embryonic spinal cord), a 24-h transfilter contact is necessary for the full response in the metanephric mesenchyme (23). The first pretubular cell aggregates develop in the mesenchyme ~12 h later, and tubular structures are seen after another 12 h, on day 2. Antigens specific for the luminal side of the tubules appear on day 4 of culture (6).

In the kidney model system, the kinetics of the induction and the morphogenetic events following determination are thus well known. However, changes during and after induction before cell aggregation are poorly understood at the molecular level. Advances in the characterization of extracellular matrix proteins have made immunohistological localization of these proteins possible (18, 27, 29, 30). By this technique we recently showed the appearance of one basement membrane protein, laminin, in the kidney mesenchyme during the 24-h induction period (5, 29). This suggests that the response of the mesenchyme to induction involves a change in the matrix composition, which may also include several of the known isotypes of collagen (2). Previous studies (reviewed in references 2 and 30) have established that type I and III collagens are mainly found in interstitial connective tissue where they often show extensive codistribution and that type II collagen is a major component of hyaline cartilages, whereas type IV and perhaps type V collagens are unique for basement membranes. We have therefore studied the expression of the different collagen types...
during and after induction. Whole kidney anlagen of early developmental stages and isolated mesenchymes induced in transfilter cultures were examined by immunofluorescence using antibodies against type I collagen, type III procollagen (18, 30), and type IV collagen (27).

**MATERIALS AND METHODS**

**Kidneys**

Hybrid mouse embryos BALBc X CBA were used throughout the study. The age of the embryos was determined from the vaginal plug, the appearance of

![Figure 1](image)

**FIGURE 1** Localization of type I collagen and type III procollagen in 11- to 12-d-old embryonic kidney rudiments, by indirect immunofluorescence (IFL). (a) Phase-contrast micrograph of live 11-d kidney. No branching of the ureter bud (u) to the mesenchyme (m) has occurred. (b) Phase-contrast micrograph of live 12-d kidney, showing the beginning of condensation of mesenchymal cells around the tips of the branched ureter bud (u). (c) Type I collagen in an 11-d kidney. The mesenchyme (m) expresses type I collagen uniformly. Prominent staining is seen around the ureter (u). (d) Type III procollagen in an 11.5-d kidney. The ureter bud has grown into the mesenchyme and branched. Dark areas not expressing procollagen type III can be seen around both tips of the branched ureter. (e) Type I collagen in a 12-d kidney. Larger magnification of the epitheliomesenchymal interphase, showing the loss of type I collagen in the mesenchyme (m) surrounding one branch of the epithelial ureter (u). (f) Phase-contrast micrograph of e, showing the beginning of the condensation around the ureter. Bars, 70 µm. a–d, X 100; f, X 300.
Transfilter Cultures

In vitro transfilter experiments were performed according to techniques described in detail in previous reports (22, 23, 25). Separated metanephric mesenchymes of 11-d embryos were placed on a Nuclepore filter with nominal pore size of 1.0 μm (Nuclepore, Pleasanton, Calif.), and fragments of the spinal cords of the same embryos were used as inductors and glued on the lower surface of the filter (9, 10). To stop transfilter interaction, the inductor was carefully scraped off at different time intervals, and the mesenchymes were subcultured for up to 2 d. Transfilter cultures were fixed and examined for the presence of the different types of collagen at 6-h intervals. The explants were cultured in a Trowell-type organ culture. The medium consisted of Eagle's minimum essential medium supplemented with 10% horse serum and antibiotics.

Control Recombinants

To examine the changes in the collagen distribution in an inductive vs. a noninductive situation, we made various heterotypic recombinants directly on one side of the filter. In addition to the normal inductor (ureter bud), spinal cord and salivary gland mesenchyme (12-d embryo) were combined with the metanephric mesenchyme. These tissues are known to induce the formation of kidney tubules in the nephrogenic mesenchyme (8, 9, 31). As noninducing tissues, lung mesenchyme (11-d embryo) and fetal liver tissues (9, 31) were similarly combined with the nephrogenic mesenchyme. Conversely, the above inductors of tubulogenesis were combined with nonnephrogenic mesenchymes (salivary, pulmonary). These various types of recombinants were cultured as above for 24 to 48 h, fixed, and examined for the distribution of the interstitial collagens.

Immunofluorescence Staining

The tissues were fixed in cold alcohol and processed for immunofluorescence as described (6, 19). Rabbit antibodies against type I bovine collagen and type III procollagen were prepared and rendered specific by immunoadsorption (18, 30). Previously, it was shown that type III procollagen and type III collagen codistribute in tissue sections (18, 30). For most staining experiments, the purified antibodies were used at a concentration of 30-50 μg/ml (type I) and 15-30 μg/ml (type III). Basement membrane collagen was detected with antibodies produced against type IV collagen extracted from a mouse tumor that produces a matrix of basement membrane (27, 28). The antibodies were specific for basement membrane collagen, as they showed no cross reaction with the interstitial collagens (27) and were used at a protein concentration of 20 μg/ml.

Sections were treated with the antibodies for 30 min at room temperature and then washed in phosphate-buffered saline (PBS). Bound collagen antibodies were localized by fluorescein-conjugated goat antibody to rabbit immunoglobulin G (Wellcome Laboratories, Beckenham, U. K.). Pretreatment of the tissue sections with hyaluronidase did not change the staining pattern. Sections treated with purified antibodies against ovalbumin showed no staining.

A Zeiss universal microscope was used (5), and photography was done using Agapan 400 film.

Immunoperoxidase Staining

For immunoperoxidase stainings, tissues were fixed in 3.5% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3, washed in PBS, embedded in Tissue Prep, a paraffin-based embedding compound (Fisher Scientific Co., Fair Lawn, N. J.), and cut into 6-μm-thick sections. After deparaffinization, the sections were treated with 2% H2O2 in methanol at room temperature for 30 min to destroy endogenous peroxidase activity. For localization of type III procollagen by immunoperoxidase methods, sections were treated with purified anti-type III procollagen antibodies (5-15 μg/ml) for 30 min at 37°C, washed thoroughly with PBS containing swine serum when incubated with 1 : 20 swine anti-rabbit IgG serum (Dako-immunoglobulins a/s, Copenhagen, Denmark), washed thoroughly as described above, and treated with peroxidase rabbit antiperoxidase serum (PAP, code no. 113, Dako-immunoglobulins; diluted 1 : 100 with PBS), containing 2% swine serum for 15 min at 37°C. The chromagenic reaction was developed by incubation with 0.075% 3,3'-diaminobenzidine-tetrahydrochloride and 0.05% H2O2 in 50 mM Tris buffer, pH 7.6, for 7 min at 37°C. All appropriate controls as defined by others (4) were included. These did not show the specific dark-brown staining.

Figure 2

Appearance of type IV collagen in induced 12-d mesenchyme. (a) IFL micrograph of epitheliomesenchymal interphase of 12-d kidney, stained with antibodies against type IV collagen. Fluorescence is seen in the basement membrane of the ureter (u), inside this in the epithelial cells, and, at this stage, also in the mesenchyme (m) as distinct spots. Arrows indicate expression in yet another location, in the developing vasculature. (b) Histological section of kidney in a, demonstrating the condensation of the mesenchymal cells. × 300. Bar, 70 μm.

which was designated as day 0. To visualize the whole kidneys in vivo and the condensation of the mesenchyme, we photographed some kidneys live before fixation (22).
**FIGURE 3** Localization of type III procollagen in 12.5-day kidney by indirect immunofluorescence and immunoperoxidase techniques. (a) Phase-contrast micrograph of liver 12.5-day kidney. The mesenchyme appear as pale areas around the tips of the ureteric tree. (b) Immunoperoxidase micrograph of 12.5-day kidney, demonstrating loss of type III procollagen in the mesenchyme around branches of the ureter (u). (c) IFL micrograph of 12.5-day kidney, showing loss of type III procollagen only around the tips of the branching ureter but not close to the stalk of the ureteric tree. (d) Histological section of c, showing some condensation of the mesenchyme around the tips of the ureteric tree. No signs of tubule morphogenesis are seen at this stage. Arrows denote the stalks of the branching ureteric tree. Bar, 70 μm × 180.

**Electron Microscopy**

For electron microscopy, the cultures were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.6, for 60 min at room temperature. The explants were postfixed in 1% OsO₄ on 0.1 M phosphate buffer, pH 7.2, for 60 min at 4°C and embedded in Epon 812. Thick Epon sections for light microscopy were stained with 1% toluidine blue in 1% sodium tetraborate. Thin sections were stained with uranyl acetate and lead citrate and examined in Jeol Temscan electron microscope.

**RESULTS**

**Differentiation in Vivo**

In 11-d kidney rudiments (Fig. 1a), the undifferentiated mesenchymes expressed uniformly the interstitial collagens type I and type III (Fig. 1c). Type IV collagen was seen in the basement membrane of the ureter bud but not in the surrounding mesenchyme. In vivo, induction by the ureter bud is followed by aggregation of the mesenchymal cells around the ingrowing ureteric tree (Fig. 1b). Before aggregation, the mesenchymal areas corresponding to these “morphogenetic fields” of determined cells could be visualized by changes in the distribution of the interstitial collagens. Staining disappeared around the tips of the ingrowing ureter bud but was still observed in the uninduced parts of the mesenchyme (Fig. 1d).

In 12-d kidneys, a large area of the mesenchyme not expressing the interstitial collagens was evident around the branched ureter bud (Fig. 1e and f). At this stage, some condensation of the mesenchymal cells could be seen around the ureter (Fig. 1f). Type IV collagen could be detected in the mesenchyme at this stage, always in the area not expressing the interstitial collagens (Fig. 2a and b). During the continuous branching of the ureter, the mesenchyme around the tips invariably changed the collagen phenotype from an interstitial type (Fig. 3a and b) to a basement membrane type. This change was exclusively seen around the tips but not around the stalks of the branching ureter or in the remaining mesenchyme (Fig. 3c and d). The distribution of type I collagen and type III procollagen was identical at all stages.

**Differentiation in Vitro**

The freshly separated mesenchymes and those cultured alone expressed the interstitial collagens uniformly (Fig. 4a). In the
FIGURE 4 Loss of type III procollagen from metanephric mesenchyme during transfilter induction with embryonic spinal cord. The filter initially shows yellow autofluorescence, which gradually disappears in areas between the tissues, presumably because of ingrowth of cell processes into the filter, known to be required for induction (23, 24, 35). The upper surface of the filter is therefore marked with a broken white line (a–d). In e, the filter was visualized by staining the whole filter with protein coupled to tetramethylrhodamine isothiocyanate (TRITC). Photographs were then taken by exposing the negative to both TRITC-fluorescence to show filter and fluorescein isothiocyanate (FITC)-fluorescence to show expression of type III collagen (e). (a) Mesenchyme cultured for 6 h without the inductor tissue. A uniform staining with type III procollagen antibodies is seen. (b) Transfilter 6-h culture. The cell layers closest to the inductor tissue have lost type III procollagen. (c) 24-h culture. The inductor was removed at 12 h and the mesenchyme was cultured alone for an additional 12 h. Approximately half of the mesenchyme has lost its type III collagen, whereas the upper part of the mesenchyme still expresses this collagen. The staining is thus similar to that in e and not to that in d. (d) Transfilter 24-h culture. Type III procollagen is seen only in the upper cell layers of the mesenchyme. (e) Transfilter 12-h culture. Approximately half of the mesenchyme has lost its type III collagen, whereas the upper part of the mesenchyme still expresses this collagen. The staining is thus similar to that in e and not to that in d. (f) Transfilter 24-h culture. Type III procollagen is seen only in the upper cell layers of the mesenchyme. (g) Transfilter 12-h culture. Approximately half of the mesenchyme has lost its type III procollagen. m, Mesenchyme; f, filter; sc, spinal cord. Bar, 30 μm. × 400.

FIGURE 5 IFL micrograph showing the appearance of type IV collagen in the nephrogenic mesenchymes in transfilter culture. (a) Transfilter 6-h culture. No basement membrane (type IV) collagen can be detected. (b) Transfilter 24-h culture. Spots of type IV collagen are found in the loose mesenchyme. Compare this punctate fluorescence with the fluorescence of the interstitial collagens (Figs. 4 and 5) in the undifferentiated loose mesenchyme. (c) Transfilter 36-h culture. Inductor removed at 24 h. Collagen type IV is detected in cell aggregates. A continuous fluorescence on the outer side of the elongated and aggregated cells is forming. (d) Phase-contrast micrograph of c showing the cell aggregates. Bar, 30 μm. × 400.

in vitro transfilter cultures with spinal cord, changes in the mesenchyme occurred already after 6-h cultivation: a thin zone devoid of type III procollagen was seen close to the filter (Fig.

4 b). After a further 6 h of cultivation, the lower half of the mesenchyme had lost its interstitial collagen staining (Fig. 4 c and e). The changes were completed in 24 h; the interstitial collagens were then detected only in some areas in a thin superficial layer of the mesenchyme (Fig. 4 d). This complete loss of the interstitial collagens in the mesenchyme required an uninterrupted 24-h exposure to the inductor tissue; when the inductor was removed at 12 h and the mesenchyme examined at 24 h, the staining pattern was similar to that of mesenchymes examined at 12 h (Fig. 4 c). The distribution and disappearance of type I collagen were identical to those of type III procollagen.

The mesenchymes cultured alone did not express type IV collagen. In the transfilter cultures, the staining yielded negative results after 6 h of culture (Fig. 5 a). The first spots of type
IV collagen were detected in 12-h cultures, and by 24 h the still loose mesenchyme showed a uniform punctate pattern of type IV collagen (Fig. 5b). The appearance of type IV collagen in the whole mesenchyme required an uninterrupted exposure of the inductor tissue for 24 h. During the induction period, both interstitial collagens and basement membrane collagen were thus detected in the loose mesenchyme, but with the immunofluorescence technique it could be shown that they did not occur in the same locations at any stage. After a total culture of 36 h, type IV collagen was located around the cell aggregates (Fig. 5c and d).

In electron microscopy, metanephric mesenchyme fixed either immediately after separation or after 24-h transfilter induction appeared the same. The cells were loosely arranged, and some electron-dense, fuzzy material was occasionally seen on the plasma membranes. After 30 h in culture, aggregation of the mesenchymal cells could be observed, and these pretubular aggregates were surrounded by a thin, often discontinuous basal lamina (Fig. 6a, c, and e). After 48 h in culture, the differentiating tubular structures were regularly surrounded by a well-developed basal lamina (Fig. 6b, d, and f).

**Control Recombinants**

Two types of control experiments were carried out. First, various embryonic tissues known to differ in their tubule-inducing capacity were combined with the nephrogenic mesenchyme. In these experiments, combination with tissues capable of triggering tubule formation (ureter bud, spinal cord, salivary mesenchyme) always resulted in the loss of interstitial collagens in the mesenchyme (Fig. 7a). Combination with noninducing tissues (lung mesenchyme, liver) did not have an effect on the distribution of the interstitial collagens. Secondly, tubule-inducing tissues were combined with various non-nephrogenic mesenchymes. In these experiments, we never observed any change in the collagen distribution in the mesenchyme at any stage (Fig. 7b and c).
FIGURE 7 Immunoperoxidase micrographs demonstrating the expression of type III procollagen in direct tissue recombinants. (a) Pieces of 11-d ureter buds (u) placed in contact with nephrogenic mesenchyme and cultured for 24 h. Note that type III procollagen disappears from the mesenchyme around each of the tips of the ureter bud but not around the stalk of the ureter (arrow) or from other parts of the mesenchyme. (b) Pieces of 11-d ureter buds (u) placed in contact with 12-d salivary gland mesenchyme (sm) and subsequently cultured for 48 h. No change in the expression of type III procollagen is seen, even after this prolonged culture. The salivary mesenchyme continues to express interstitial type collagen close to the ureter bud. (c) Three pieces of salivary mesenchyme (sm) placed in contact with a tubule inductor, spinal cord (sc), and subsequently cultured for 24 h. The salivary mesenchyme continues to express type III collagen during the culture period. Bar, 70 μm. × 100.

DISCUSSION

Changes in the expression of different collagen isotypes have been described for some cell types during culture in vitro (3, 17) and also for several tissues during embryonic development (1, 14, 15, 26, 33), but the factors regulating these events are poorly understood. In the present study, we demonstrate a shift from an interstitial to a basement membrane collagen pattern in the embryonic kidney mesenchyme as a response to an inductive tissue interaction. Such interactions are major controlling events in differentiation of cells during organogenesis (11, 21), and it is of considerable interest to analyze molecular changes during such processes.

The changes in the composition of the extracellular matrix in the metanephric kidney correlate with the different levels of its determination. The first level before induction by the ureter bud can be considered a level of predetermination. The definite kidney bias of this stage was demonstrated by Grobstein and his collaborators (9, 31), who showed that a variety of embryonic and fetal tissues can trigger tubule formation in the nephrogenic mesenchyme. Conversely, none of the other mesenchymes so far tested respond to an inductor by tubule formation (20). At the predetermined stage, the nephrogenic mesenchyme has lost most, if not all, of its developmental options except for that of becoming kidney tubules. Here we show that this stage of the nephrogenic mesenchyme is characterized by a uniform expression of the interstitial collagens (types I and III).

The second level is reached when the mesenchymal cells are irreversibly destined to become epithelial elements. In transfilter experiments, where this event can be precisely timed, determination is achieved gradually after a 12- to 24-h contact with the inductor (23). This induction correlates closely with the disappearance of the interstitial collagens and with the appearance of basement membrane components (type IV collagen, laminin). The loss of the interstitial collagens starts close to the inductor tissue and proceeds rapidly upward in the transfilter experiments until completed by ~24 h. Shortly after this, the mesenchymal cells aggregate, and the components of the basement membrane become localized around the aggregate and form a continuous basement membrane. When this has been completed, a third level is reached, considered to reflect ter-
minal determination of the cells (7). Molecular markers for all three segments of the nephron can then be demonstrated (6, 7). This sequence of events suggests that the basement membrane is required for the terminal differentiation of the kidney epithelial cells, as suggested for many other cell types (11, 34).

Our heterotypic recombination experiments showed that interstitial collagens disappeared from the kidney mesenchyme when exposed to tubule inducers but not when brought into contact with tissues devoid of an inductive capacity or when cultured alone. Conversely, the above tubule inducers had no effect on the expression of the interstitial collagens when tested against nonnephrogenic mesenchymes. The data suggest that epithelialization is dependent on an appropriate removal of interstitial collagens from the kidney mesenchyme. This loss of the matrix material may allow the cells to aggregate into condensates with subsequent initiation of production of base- ment membrane components.

The disappearance of interstitial collagens from the mesenchyme during induction could be a consequence of an increased production or activation of tissue collagenase. Different collagens are involved in the degradation of interstitial and basement membrane collagens (16, 28). The appearance of type IV collagen in the induced mesenchyme simultaneously with a removal of interstitial collagens by collagenase therefore seems possible. In certain cell cultures, collagenase activation may require close contacts between epithelial and stromal cells (12). Because induction in the kidney model system is also considered to operate through cell contacts (13, 24, 35), the loss of the interstitial collagens in the mesenchyme could be a related phenomenon.

The loss of the original mesenchymal matrix may also be attributable to a replacement of mesenchymal cells by a new cell population not expressing interstitial matrix components. This appears unlikely because the changes started rapidly and were almost completed after 24 h, whereas cell proliferation as judged by thymidine uptake is a later event (32). The early changes detectable by immunofluorescence in transfector experiments offer an experimental opportunity to study the various possibilities in more detail and to approach a better understanding of the molecular aspects of the inductive process.

Received for publication 4 August 1980, and in revised form 20 November 1980.

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