Epithelial–Mesenchymal Interactions in the Developing Kidney Lead to Expression of Tenascin in the Mesenchyme

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Abstract. Tenascin, a mesenchymal extracellular matrix glycoprotein, has been implicated in epithelial–mesenchymal interactions during fetal development (Chiquet-Ehrismann, R., E. J. Mackie, C. A. Pearson, T. Sakakura, 1986, Cell, 47:131–139). We have now investigated the expression of tenascin during embryonic development of the mouse kidney. In this system, mesenchymal cells convert into epithelial cells as a result of a tissue interaction. By immunofluorescence, tenascin could not be found in the mesenchyme until kidney tubule epithelia began to form. It then became detectable around condensates and s-shaped bodies, the early stages of tubulogenesis. In an in vitro culture system, tenascin expression by the mesenchyme is tightly coupled to the de novo formation of epithelia, and does not occur if tubulogenesis is suppressed. The results strongly suggest that the formation of the new epithelium stimulates the expression of tenascin in the nearby mesenchyme. During postnatal development, the expression of tenascin decreases and the spatial distribution changes. In kidneys from adult mice, no tenascin can be found in the cortex, but interspersed patches of staining are visible in the medullary stroma. The results strongly support the view that tenascin is involved in epithelial–mesenchymal interactions. It could therefore be crucial for embryonic development.

Organogenesis is dependent on cell–cell interactions. Most parenchymal organs arise by the interaction of an epithelium with a mesenchyme (Grobstein, 1967; Kratochwil, 1972). In some of these interactions, the epithelium triggers the differentiation of the mesenchyme, but in most tissues the mesenchyme initiates the differentiation of the epithelium (Cunha, 1972; Kratochwil and Schwartz, 1976). A long time ago, Grobstein (1954) suggested that these processes could be mediated by the extracellular matrix located between the interacting tissues, but so far no direct proof of this hypothesis has been presented. There is clear evidence that the interstitial collagens from the mesenchyme that immediately surrounds the new epithelium can occur in the absence of at least type I collagen (Kratochwil et al., 1986).

Kidney development is a good example of the importance of epithelial–mesenchymal interactions. The ureter epithelium induces the mesenchyme to convert into a new epithelium, and the mesenchyme, in turn, induces the branching of the ureter epithelium (Grobstein, 1955; Saxén, 1970). The new, mesenchymally derived epithelium remains surrounded by seemingly undifferentiated mesenchyme, which is assumed to influence the development of the new epithelium (Ekblom, 1984). Previous studies have shown that the differentiation of the mesenchyme into a new epithelium is accompanied by drastic changes in the extracellular matrix composition. Interstitial collagens are lost (Ekblom et al., 1981a), basement membranes are formed, and adhesive proteins appear in the mesenchymally derived new epithelium (Ekblom et al., 1980; Vestweber et al., 1985). A small part of the mesenchyme does not convert into an epithelium and remains positive for the interstitial collagens and fibronectin. It could thus be speculated that these mesenchymal matrix proteins control the development of the new epithelium. None of these matrix proteins, however, selectively appear in the mesenchyme that immediately surrounds the new epithelium (Ekblom and Thesleff, 1985).

A matrix protein which could have such a characteristic distribution has now been described. Tenascin, a mesenchymal matrix protein, can, in several organs, be found in dense mesenchyme that immediately surrounds budding and growing epithelia, but is absent from other parts of the mesenchyme (Chiquet-Ehrismann et al., 1986). This distribution suggested that tenascin is important for either mesenchymal condensation, or for epithelial growth, or both. In the developing kidney, these two steps can be studied separately. In situ, the mesenchyme that immediately surrounds the tips of the ureter condenses as a first step in its conversion to an epithelium. An in vitro model system, using a heterolo-
ment with the hypothesis that tenascin is involved in epithelial-mesenchymal interactions. The development of nearby epithelial cells, and are in agreement but rather in the loose mesenchyme that surrounds the newly formed basement membranes (Vestweber et al., 1985), and could thus be used as a marker for epithelial structures. The monoclonal rat anti-mouse cytokeratin (TROMA-I) antibodies have been described in detail (Brolet et al., 1980). In immunoblots of kidney proteins they recognize a single brand of 52 kD (Fig. 1a). Both monoclonal antibodies were a kind gift of Dr. Rolf Kemler (Friedrich Miescher Laboratory, Max Planck Society, Tübingen, FRG). Polyclonal rabbit anti-mouse fibronectin was a kind gift of Dr. E. Ruoslahti (La Jolla Cancer Research Foundation, La Jolla, CA). Fluorescence labeled second antibodies (FITC conjugated goat anti-rabbit IgG and goat anti-rat IgG) were from Jackson Laboratories/Dianova (Hamburg, FRG). Tissue culture medium and FCS were obtained from Gibco (Karlsruhe, FRG).

Cells and Tissues
Embryonic kidneys were microsurgically isolated from mouse embryos (NMRI x C57 black). The age of the embryos was counted from the day of the vaginal plug, day 0.

Primary cell cultures were obtained from kidneys of 14-d-old embryos by treatment with a solution of trypsin (0.1%) and EDTA (1 mM) in PBS. Ten kidneys were incubated for 3 min in 1 ml of this solution and then dissociated by vigorous pipetting. The digestion was then stopped by the addition of 9 ml of DME with 10% FCS. Large aggregates of cells were allowed to settle down by gravity (25 min at room temperature). Cells in the supernatant were spun down at 800 g for 5 min and seeded at a density of 10⁶ cells per well in a 24-well cluster tissue culture plate (Costar, Cambridge, MA) in DME containing 10% FCS.

Immunoblots
To ensure that the TROMA-I antibody is monospecific in embryonic mouse kidney, as has been shown for mouse trophoderm (Brolet et al., 1980), its reactivity was tested by immunoblotting. For this purpose, 17-d embryonic kidneys were dissolved in sample buffer (Laemmli, 1970) by ultrasonic treatment (90 s) and boiling (3 min). 10 µg of total protein were loaded on one lane of a 12% polyacrylamide gel. After SDS-PAGE, an immunoblot was performed according to Towbin et al. (1979).

Immunoprecipitation
For an identification of mouse tenascin, primary cultures of embryonic kidney cells were metabolically labeled for 24 h. For this purpose, [³⁵S]methionine was added to the medium at a final concentration of 3.7 MBq (0.1 mCi) per ml of medium. Immunoprecipitation of tenascin from these cultures was done as described by Chiquet-Ehrismann et al. (1986), except that Protein A-Sepharose (Pharmacia Fine Chemicals, Freiburg, FRG) was used instead of Protein A-Sepharose.

Organ Culture
To investigate the appearance of tenascin in the absence of an ingrowing epithelium, transfilter experiments were performed as previously described (Grobstein, 1956; Saxén et al., 1985). Briefly, three mesenchymal kidney anlagen, from which the ureter had been removed, were placed on a Nucleopore filter (Nucleopore, Tübingen, FRG). To the lower side of the filter, a piece of spinal cord, a heterologous inducer of tubulogenesis, had been attached with agar. Tissues were then cultured for 72 or 96 h on a Trowell type stainless steel grid. The culture medium consisted of improved Eagle's MEM (Richter et al., 1972) supplemented with 50 µg/ml human transferrin (Collaborative Research Inc., Waltham, MA) and 4 mM glutamine (Gibco) (Ekblom et al., 1981b). In some experiments, heparin (No. H 8514, Sigma Chemical Co.) was added to the cultures at a final concentration of 200 µg/ml.

Cryostat Sections
Tissues were embedded in O.C.T. compound (Miles Scientific, Naperville, IL), placed in liquid nitrogen, and stored at −70°C for up to 1 wk or at −20°C for up to 2 mo. Sections (5 µm thick) were then cut on a cryostat (Reichert-Jung, Austria).

Immunofluorescent Stainings
Unfixed frozen sections were washed in PBS containing 0.5% BSA for 5 min. Antiserum against tenascin (dilution 1:100) and/or hybridoma supernatants with monoclonal antibodies against laminin or cytokeratin (diluted 1:4) were then applied to the sections. After 60 min of incubation at room temperature, the slides were washed in PBS containing 0.5% BSA and then incubated with secondary antibodies, which were either fluorescein isothiocyanate conjugated goat anti-rabbit IgG or rhodamine conjugated goat anti-mouse IgG. Sections were then washed as above and mounted with Mowiol (Calbiochem-Novabiochem). All mounted sections were left for 72 h at −20°C. Sections were then washed for 15 min in PBS containing 0.5% BSA and then stained for cytokeratin with the monoclonal TROMA-I antibody. Cells were then washed for 15 min in PBS containing 0.5% BSA and then stained for cytokeratin with the monoclonal TROMA-I antibody. Sections were then washed as above and mounted with Mowiol. All mounted sections were left for 72 h at −20°C. Sections were then washed for 15 min in PBS containing 0.5% BSA and then stained for cytokeratin with the monoclonal TROMA-I antibody. To ensure that the TROMA-I antibody is monospecific in embryonic mouse kidney, as has been shown for mouse trophoderm (Brolet et al., 1980), its reactivity was tested by immunoblotting. For this purpose, 17-d embryonic kidneys were dissolved in sample buffer (Laemmli, 1970) by ultrasonic treatment (90 s) and boiling (3 min). 10 µg of total protein were loaded on one lane of a 12% polyacrylamide gel. After SDS-PAGE, an immunoblot was performed according to Towbin et al. (1979).

Figure 1. Demonstration of cytokeratin and tenascin in embryonic kidney by SDS-PAGE. (a) Immunoblot analysis of TROMA-I antigen in embryonic kidneys. 10 µg of total protein from whole 17-d embryonic kidneys were run on a 12% SDS gel under reducing conditions and transferred to nitrocellulose. (b) Immunoprecipitation of tenascin from conditioned medium of primary cultures of embryonic kidney cells. Proteins precipitated by the antibody were run on 7% SDS gels under reducing conditions and revealed by fluorography. Molecular mass standards are shown in kilodaltons (kD) to the right of the gels.
temperature, sections were washed three times in PBS, followed by an incubation with second antibody (dilution 1:1,000), also for 60 min. Sections were again washed three times for five minutes each wash in PBS and then embedded in ELVANOL embedding medium for fluorescent microscopy.

Results

Identification of Mouse Tenascin
To confirm that the rabbit anti-chicken tenascin antiserum recognizes a similar protein in mouse, we analyzed the peptides recognized by the antibodies. As cell cultures from chick and rat have been reported to secrete tenascin into the medium (Chiquet-Ehrismann et al., 1986), we precipitated the protein from conditioned media of metabolically labeled primary cell cultures from embryonic mouse kidney. Fluorography of the precipitate after SDS-PAGE revealed a band of 240 kD and a double band of 190/210 kD (Fig. 1 b), as has been found for chick and rat tenasin.

In Vivo Development
In immunofluorescence, no tenascin was found in the uninduced metanephrogenic mesenchyme of kidneys from 11-d-old embryos. As the double immunofluorescence for cytokeratin (Fig. 2 a) and tenascin shows (Fig. 2 c), the 12-d-old embryonic kidneys were still negative for tenascin, although some condensation of the mesenchyme around the keratin-positive ureter branches (Fig. 2 a) had already occurred. In contrast, fibronectin was strongly expressed in the basement membranes and the mesenchymal matrix at the same time, as shown by double immunofluorescence for fibronectin (Fig. 2 b) and tenascin (Fig. 2 d). It is well known that the first step in the development of the nephrogenic mesenchyme is a binary segregation of the cells into an epithelial and a stromal cell lineage. The conversion of the mesenchyme to epithelium occurs only around the tips of the ureter. The mesenchymal cells around the tip condense, then form a comma-shaped body which gradually elongates to an S-shaped epithelial body. Finally, a tubule and a glomerulus forms from the S-shaped body. Only the cells that do not become induced by the ureter become stromal cells (See Potter, 1965; Ekblom, 1984). Tenascin expression was restricted to certain early stages of the stromal differentiation pathway. In 13-d-old embryonic kidneys, the first faint expression of tenascin could be detected, but it was restricted to the mesenchyme that surrounds the first new epithelial structures. Strong expression was found starting on day 14 of embryonic development.

The restricted pattern of expression is demonstrated by a double staining for laminin and tenascin (Fig. 3, a and c) and by a double staining for fibronectin and tenascin (Fig. 3, b and d) in sections from a kidney of a 16-d-old embryo. The monoclonal antibody against laminin detected laminin in basement membranes of the ureter and the S-shaped tubules, and neither the condensates nor the surrounding mesenchyme showed positivity (Fig. 3 a). Double staining of the same sec-
Figure 3. Comparison of laminin (a) and tenascin (c), and fibronectin (b) and tenascin (d) by double immunofluorescence of the cortex and part of the medulla of 16-d embryonic kidneys. Note the strong expression of tenascin around the condensates and the comma- and s-shaped bodies. A comparison of b and d also makes evident that tenascin is restricted to the cortex, while fibronectin is universally expressed. c, condensate; co, comma-shaped body; s, s-shaped body; u, ureter; t, developing tubule; g, developing glomerulus. Bar, 30 μm. For an identification of tubular structures see Ekblom (1984).

Expression showed that tenascin expression was now detectable but it was restricted to the mesenchyme around the condensates and around the S-shaped bodies (Fig. 3 c). No tenascin was visible in the laminin-positive basement membranes (Fig. 3, a and c). The double staining for the two mesenchymal matrix glycoproteins, fibronectin (Fig. 3 b) and tenascin (Fig.
3 d), clearly revealed that tenascin had a much more restricted distribution than fibronectin. While fibronectin was found throughout the kidney in the mesenchyme around epithelial structures both in the cortex and the medulla (Fig. 3 b), tenascin was detected almost exclusively in the cortical regions where the youngest stages of epithelial development are seen (Fig. 3 d). The comma-shaped bodies close to the ureter buds and the young tubules and glomeruli in the upper cortex can be seen in Fig. 3 b as a larger fibronectin-negative area. Only these structures were surrounded by a tenascin-positive mesenchyme (Fig. 3 d). In the medulla, terminally differentiated epithelial tubules are already found in the 16-d-old kidney, and tenascin could no longer be detected around these epithelia (Fig. 3 d).

The gradient of strong expression of tenascin in the cortex and weak expression in the medulla was seen starting on day 14 of embryonic development, and it persisted until the early postnatal stages (Fig. 4 a). It is well known that many new tubules still form in the cortex in newborn kidneys, so that immature stages are abundant in the upper cortex (Potter, 1965) and the data on tenascin expression in the kidneys from newborn mice thus confirmed that expression of tenascin in the mesenchyme invariably tightly correlated with the early stages of epithelial development. In adult kidneys, the pattern was different: cortical regions were devoid of tenascin (Fig. 4 b); most of the medulla was also negative, but some staining was detected in the pyramids and a rather strong staining was seen close to the papillae (Fig. 4 b). To confirm that this gradient was not due to a sectioning artefact in the longitudinal sections through regions of different consistencies, it was also shown in a series of transverse sections. High magnifications of such sections of different regions of newborn and adult kidney are shown in Fig. 4, c–h.

**In Vitro Development**

An experimental in vitro culture system, in which the metanephric mesenchyme is induced by a heterologous inducer, the embryonic spinal cord (Grobstein, 1956), was used to study the interdependence between tubule formation and tenascin expression in more detail. In these transfilter cultures, the first tubules appear on day 2, and on day 3 well-formed polarized epithelia are evident. We found that cytokeratin could be detected in the tubules with the TROMA-1 antibody on day 3 (Fig. 5 a). Thus, the appearance of cytokeratin can apparently not be used as a marker for the early response to induction. We used it here as a marker for the presence of well-developed epithelial tubules.

Tenascin could first be detected around the tubules on day 3 of in vitro development (Fig. 5 b). Tenascin was found around the cytokeratin-positive cells, but some expression was seen also around condensates that did not yet express cytokeratin (Fig. 5 b). Double staining for laminin (Fig. 5 c) and tenascin (Fig. 5 d) demonstrated that the basement membranes of the tubules on day 3 were surrounded by short fibrils of tenascin in the mesenchyme. Tenascin remained present in the mesenchyme on day 4 of in vitro development, and the double staining for laminin (Fig. 4 e) and tenascin (Fig. 4 f) demonstrated expression of tenascin close to the epithelium, but long fibrils of tenascin were also detected several cell layers apart from the basement membrane.

Addition of heparin has been reported to inhibit kidney tubule formation, although the cells seem to survive rather well (Ekblo et al., 1978). Though, occasionally, tubules were also found in transfilter cultures treated with heparin, it was evident that tenascin did not appear in the large areas of mesenchyme where no tubules could be found (Fig. 5, g and h).

**Discussion**

Tenascin is an extracellular matrix protein expressed by embryonic mesenchyme and by stromal cells of some tumors. It was first identified by a monoclonal antibody against an extracellular matrix protein in chicken muscle and myotendinous junctions (Chiquet and Fambrough, 1984 a, b). Recent evidence (Ericksen and Taylor, 1986) suggests that it is similar to certain other newly described matrix proteins (Bourdon et al., 1983; Ericksen and Inglesias, 1984; Grumet et al., 1985; Kruse et al., 1985). Because tenascin has a restricted tissue distribution during fetal organ development, it was postulated that tenascin is involved in the epithelial-mesenchymal interactions that control epithelial growth (Chiquet-Ehrismann et al., 1986). Our current data on kidney development are in agreement with this view and suggest that expression of tenascin is directly stimulated by epithelial-mesenchymal interactions.

Epithelial-mesenchymal interactions are a common feature in many morphogenetic processes (Grobstein, 1967; Kratochwil, 1972). Grobstein (1954, 1967) suggested that this interaction could be mediated by the extracellular matrix, and since then the matrix has often been implicated in embryonic development and morphogenesis (e.g., Hay, 1981; Bunge and Bunge, 1983; Thiery et al., 1985). Conclusive evidence that cells require a proper substratum for the expression of the differentiated phenotype has come from cell culture systems (Hall et al., 1982; Enat et al., 1984; Hadley et al., 1985; Li et al., 1987). Since the epithelial-mesenchymal interactions involve a local signal transduction from the mesenchyme to the epithelium and vice versa (Kratochwil and Schwartz, 1976; Cooke et al., 1986), an extracellular matrix component secreted and deposited by the mesenchyme would be a natural candidate for a signal transducer. Interstitial collagens and proteoglycans have been implicated (Bernfield et al., 1984), but it seems unlikely that they would be the only crucial factors. In fact, studies with experimentally produced mutants suggest that branching epithelial morphogenesis can proceed well without the presence of type I collagen (Kratochwil et al., 1986). Thus, although type I collagen from the mesenchyme can have a role in epithelial-mesenchymal interactions by modulating the assembly of the epithelial basement membrane (Bernfield et al., 1984), other matrix proteins may be more crucial or can substitute for type I collagen.

The recent studies on mammary, tooth, and vibrissa development strongly suggest that tenascin is an important matrix protein in epithelial-mesenchymal interactions (Chiquet-Ehrismann et al., 1986). In those tissues, the epithelium is of ectodermal origin, and it develops by growth and branching from preexisting epithelial buds. The mesenchymes that surround these epithelia are condensed and it could therefore not be ruled out that expression of tenascin merely is a property of any condensing mesenchyme, rather than a specific response to epithelial-mesenchymal interactions. In the developing kidney, it was possible to distinguish between these
alternatives. In this system, the epithelium is of mesodermal origin, and the formation of a new epithelium from mesenchymal cells can be experimentally followed. Moreover, only the mesenchyme that converts into epithelium condenses, whereas the rest of the mesenchyme that surrounds the new epithelium morphologically remains as a seemingly undifferentiated stroma. The differentiation of the mesenchyme can thus be viewed as a binary separation to an epithelium and to a stroma. Previous studies have demonstrated that the conversion of the mesenchyme into epithelium is accompanied by drastic changes in the extracellular matrix (Ekblom and Thesleff, 1985) but so far, no such changes were known for the differentiation of the mesenchyme to stroma. Both the uninduced mesenchyme and the later stromal cells express interstitial collagens and fibronectin (Ekblom, 1981; Ekblom et al., 1981a). The data presented here show that the uninduced mesenchyme around the new epithelium undergoes a true differentiation process and begins to express a new antigen. Furthermore, tenasin then disappears rather soon and it is thus a stage-specific marker for the stromal cell lineage. This stage restricted appearance of tenasin makes it unlikely that it would serve an organizational function in the extracellular matrix, as has been suggested for embryonic cartilage (Vaughan et al., 1987).

The expression of tenasin is thus during embryogenesis much more restricted than fibronectin or the interstitial collagens, and we suggest that tenasin expression is stimulated by the development of the new epithelium. It is, furthermore, tempting to speculate that tenasin is participating in the matrix-mediated signal transduction from the mesenchyme to the new epithelium. Similar speculations have been made for collagen type II in signal transduction from epithelium.
Figure 4 (continued)
Figure 5. Comparison of cytokeratin, laminin, and tenascin expression in the in vitro cultures of metanephric mesenchymes induced by a heterologous inducer. The explants were cultured for 3 (a-d) or 4 (e-h) d, stained for the TROMA-1 antigen (a), laminin (c-e), or tenascin (b, d, f, and h). g is a phase-contrast micrograph. Bar, 30 μm. (a and b) Double staining for TROMA-1 antigen and tenascin shows that tenascin is expressed around fully differentiated tubules as well as around tubular structures that do not yet express the TROMA-1 antigen (left). It can thus be concluded that tenascin appears before the TROMA-1 cytokeratin. (c and d) Double staining for laminin and tenascin of an explant cultured for 72 h in vitro. Laminin is found around the tubules (c), and tenascin in the mesenchyme around the tubules (d). Note also tenascin staining in the inducer tissue, the spinal cord (lower right corner in d). (e and f) Double staining for laminin and tenascin of an explant cultured for 96 h in vitro. Note that the expression of laminin (e) and tenascin (f) do not overlap, showing that tenascin...
to mesenchyme in the formation of the cartilaginous neorocranium (Thorogood et al., 1986).

It was recently suggested that chicken cytactin, which is possibly the same protein as tenasin (Erickson and Taylor, 1986), would be located in the embryonic basement membranes (Crossin et al., 1986). Our present study establishes that tenasin, and hence probably also cytactin, rather is located in the mesenchyme that surrounds the epithelial basement membranes. This could particularly convincingly be demonstrated by double stainings for laminin and tenasin.

The in vivo data were confirmed by in vitro studies. The mesenchyme was dissected free from its normal inductor, and the tissue was then experimentally induced in a transfilter culture by using a heterologous inducer (Grobstein, 1956; Ekblom and Thesleff, 1985). No tenasin could be detected in the cells until epithelial tubules formed, but as soon as they appeared, tenasin became detectable in the surrounding mesenchyme. As a marker for the formation of the new epithelium, we used the expression of a keratin type that is recognized by the monoclonal antibody TROMA-1 (Brület et al., 1980), and expression of laminin, which is a marker for the formation of the epithelial basement membrane (Ekblom et al., 1980). In the transfilter cultures, the keratin- and laminin-positive tubules were surrounded by tenasin-positive mesenchymal areas. Inhibition of tubulogenesis by heparin also suppressed the expression of tenasin. Thus the expression of tenasin seems to be tightly coupled to the epithelial morphogenetic process and it does not merely depend on the time of culture.

In view of our results on the fetal kidneys, it was unexpected that some tenasin was also detected in the adult kidney. In the mammary gland, tenasin can be detected only during embryogenesis and in malignant tumors (Chiquet-Ehrismann et al., 1986), and the stroma in adult breast tissue seems to be totally negative for tenasin. This suggested that tenasin is an oncofetal extracellular matrix protein. Our data on the adult kidney show that the presence of tenasin in adult tissue is not invariably a sign of malignancy. It is nevertheless possible that malignant transformation also in the kidney leads to an increased expression of tenasin, to levels usually found only in the embryo. This is supported by our preliminary data of abundant expression of tenasin in two samples of Wilms tumor, a childhood kidney cancer (not shown). It therefore seems that tenasin is not only involved in tissue interactions during kidney embryogenesis but also in neoplastic development of kidney tumors.

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is not a constituent of the embryonic kidney basement membrane. (g and h) Inhibition of tubulogenesis is accompanied by absence of tenasin. The explant was cultured for 96 h in vitro, in the presence of the heterologous inducer with a medium containing heparin (200 μg/ml). It was then examined by phase-contrast microscopy (g) and by staining for tenasin (h).
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