Article

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Abstract

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Epithelial Synthesis of Tenascin at Tips of Growing Bronchi and Graded Accumulation in Basement Membrane and Mesenchyme

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The extracellular matrix protein, tenascin, has been proposed as mediator in epithelial–mesenchymal interactions because of its characteristic distribution during embryogenesis. Here we compared the accumulation of tenascin and laminin in the early chicken lung bud. Laminin is deposited in the basement membrane, starting at the tips and increasing along the shafts of growing primary and secondary bronchi. In contrast, tenascin accumulation is highest in basement membranes and mesenchyme at sites where new bronchial branches are formed. By *in situ* hybridization, tenascin mRNA was found to be produced exclusively by the epithelium at sites of active growth of bronchial tubes. © 1991 Academic Press, Inc.

INTRODUCTION

Mesenchymal–epithelial tissue interactions play a key role during morphogenesis of different organs in the embryo. Pioneering studies by Grobstein [1] suggested that induction of epithelial development by mesenchyme can be mediated by the extracellular matrix (ECM)[2] accumulated between the two tissues. Among ECM glycoproteins, fibronectin [2] is a major component of mesenchymal tissues whereas laminin [3] is predominant in basement membranes. Both proteins are important for cell attachment, cell migration, cell differentiation, and morphogenesis [2, 3]. Tenascin, another ECM glycoprotein, has six large, identical subunits which are disulfide-linked to a "hexabrachion" structure (for review see [4]). Tenascin appears in a much more restricted spatial and temporal pattern during development than fibronectin or laminin. For example, tenascin is specifically accumulated in the dense mesenchyme around epithelial organ primordia, often disappears after differentiation, but is reexpressed in the stroma of tumors [5]. It has therefore been postulated that the protein is required for mesenchymal–epithelial interactions during morphogenesis. Corresponding data were provided by studies on developing vibrissae, mammary gland, tooth [5], feathers [6], kidney [7], gut [8], and neural tube [9]. These studies showed that tenascin is predominantly accumulated around actively growing epithelia in basement membrane and mesenchyme. During chick lung development, budding and branching of the epithelial network occur in a similar manner as in other organs [10]. From the third embryonic day onward, a pouch on the pharynx epithelium starts to grow into the presumptive lung mesenchyme, forming the trachea and primary bronchi. Starting at Day 6 the secondary bronchial tree is formed from the primary bronchi by lateral branching and growing of epithelial tubes into the lung mesenchyme. After the 10th day further ramification leads to the formation of the tertiary bronchi. Tenascin has been found associated with basement membranes and dense mesenchyme of lung primordia [6, 11], but its first accumulation and its site of synthesis have not been determined. Here we compared the distribution of tenascin and laminin along newly forming bronchial epithelia. According to a current hypothesis, in other organs tenascin is synthesized in the mesenchyme in response to the inductive influence of the epithelium [8, 12–14]. To see whether this is also the case during early lung morphogenesis, we determined the cellular source of tenascin mRNA by *in situ* hybridization.

MATERIALS AND METHODS

Antibodies. The following monoclonal antibodies (mAb's) were used: M1 against chick fibroblast tenascin [11] and 9F-10 against chick heart laminin. Both mAb's are mouse IgG's, partially purified from ascites fluid by ammonium sulfate precipitation and dialysis against TBS [11]. The antibody 9F-10 was obtained by the injection in a mouse of chick heart laminin partially purified by the method of Paulsson *et al.* [15]. The activated mouse spleen cells were fused with PAI myeloma cells and clones were screened as published [16]. mAb 9F-10 is a laminin-specific antibody according to the following criteria: it reacts with unreduced chick laminin (M_r = 800,000) and a reduced B chain (M_r = 200,000) on immunoblots, as does polyclonal

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[2] Abbreviations used: ECM, extracellular matrix; mAb, monoclonal antibody; PBS, phosphate-buffered saline; BSA, bovine serum albumin; Ig, immunoglobulin.

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anti-mouse EHS laminin antiserum [15]. Moreover, mAb 9F-10 binds to the same nonreduced protein bands on immunoblots and yields an identical (although more intense) immunofluorescence staining as anti-chick laminin mAb 31 [17]. A polyclonal antiserum against chick heart laminin was obtained by immunizing a rabbit with the nonreduced M_r = 800,000 bands cut out from preparative SDS–polyacrylamide gels. This antiserum has a reactivity very similar to anti-mouse EHS laminin antiserum, but a higher titer against chick laminin (data not shown; D. Brubacher and M. Chiquet, in preparation). The characterization of specific antisera against chick fibroblast tenascin has been published [11].

Immunofluorescence. Fertilized chick eggs were incubated at 37°C in a humified incubator for 5 to $6\frac{1}{2}$ days. The lung primordia were excised from embryos and fixed in 4% paraformaldehyde in PBS, immersed in 25% sucrose in PBS, embedded in Tissue Tek (O.C.T. compound), frozen on dry ice, and cut on a cryomicrotome (Slee) at 10 μm. Dried sections were blocked with 5 mg/ml BSA in PBS for 15 min. Ascites fluid or antisera were diluted 1:100 in blocking solution and applied for 1 h at room temperature. After washing, the sections were incubated for 1 h in rhodamine- or fluorescein-coupled goat anti-mouse or anti-rabbit Ig (Cappel), respectively, diluted 1:100 in blocking solution. To verify the specificity of staining patterns, double labeling of sections was done reciprocally, i.e., either with polyclonal anti-tenascin and monoclonal anti-laminin, or vice versa (Fig. 1). Specimens were photographed on an Olympus immunofluorescence microscope.

In situ hybridization. Chick tenascin cDNA clones were isolated from λgt11 libraries and subcloned into plasmids [18]. Five fragments were selected that span the entire coding region of the 190-kDa tenascin variant [18]. Gel-purified DNA fragments were mixed in an equimolar ratio, and nick translation was performed with tritiated nucleotides [19]. Paraformaldehyde-fixed cryostat sections of 10 μm were incubated with labeled cDNA probe, washed, and autoradiographed as described by Baumgartner *et al.* [19]. Sections were photographed under dark or bright field illumination on a Zeiss microscope.

As a control for specificity, a 330-bp-long cDNA probe was prepared from the extra fibronectin type III repeats which are present only in the 230-kDa splicing variant of tenascin [18]. This probe had no sequence overlap with the one described above, yet showed an identical hybridization pattern in the lung bud (not shown).

RESULTS

The distribution of tenascin and laminin was investigated by immunofluorescence during growth of primary and branching of secondary bronchi in the chick embryo from 5 to $6\frac{1}{2}$ days of development. In addition, the site of tenascin mRNA expression was determined by *in situ* hybridization with a nick-translated, ^{3}H-labeled cDNA probe specific for tenascin. Staining with anti-tenascin antibody was found in the dense mesenchyme surrounding the ingrowing primary bronchial buds at 5 days. In addition, the epithelial basement membrane at this site stained intensely for tenascin, with a gradual loss of labeling toward more proximal, older regions of the epithelial tubes (Fig. 1A). In contrast, antibodies against laminin outlined not only bronchial but also gut and coelomic basement membranes (Fig. 1B) which were negative for tenascin (Fig. 1A).

A similar immunofluorescence staining pattern was observed at $6\frac{1}{2}$ days when the secondary ecto- and entobronchi branch off. Again, bronchial basement membranes and dense mesenchyme were positive for tenascin (Fig. 1C). At the tips of newly forming entobronchi, there was faint but specific tenascin staining visible within the epithelial layer (Fig. 1C, asterisk). Anti-laminin labeled the epithelial basement membranes of secondary bronchi, however, in a graded fashion. Basement membranes at the apex of growing epithelial buds stained only weakly for laminin (Fig. 1D, asterisk). Thus, distribution of the two molecules seemed to differ along the basement membrane of growing bronchial branches: Distally, staining was strong for tenascin and weak for laminin.

In situ hybridization with a cDNA probe for tenascin revealed that during these stages the expression of its mRNA is restricted to the tips of budding bronchial epithelia and is absent from older epithelia and dense mesenchyme (Figs. 2A and 2B). High power bright field images of such budding epithelia showed that the tenascin mRNA is concentrated at the tip of bronchial tubes and is lost gradually toward their shafts (Fig. 2C). The lung mesenchyme was devoid of silver grains. In contrast, the gut smooth muscle mesenchyme, but not the gut epithelium, expresses tenascin mRNA at this point of development (Fig. 2B).

DISCUSSION

In earlier studies, tenascin expression has been shown to be induced in the mesenchyme in response to epithelial–mesenchymal interactions. For example, breast carcinomas can turn on tenascin synthesis in the mesenchymal stroma *in vivo* and *in vitro* [12]. In this case, tenascin expression is thought to be induced by factors released from the tumor, such as TGF-β [13], whereas the tumor cells themselves do not synthesize the protein. In other cases like primary budding of the mammary gland [12], tooth and vibrissa development [5], and skin wound healing [20], tenascin is also found associated with epithelial basement membranes and the underlying mesenchyme, but its biosynthetic origin has not been determined. Riou *et al.* [9] have provided experimental evidence that tenascin could be expressed by the neural epithelium upon neural induction *in vitro*. We were nevertheless surprised to find that, in the early lung primordium, the growing epithelium seems to be the only biosynthetic source of tenascin. To reconcile mRNA accumulation with protein localization in this case, one has to assume that tenascin produced by the epithelium diffuses through the basement membrane and is mainly deposited in the surrounding mesenchyme. Diffusion of this huge molecule through a mature, dense basement membrane seems unlikely. The graded staining for laminin at bronchial tips suggests

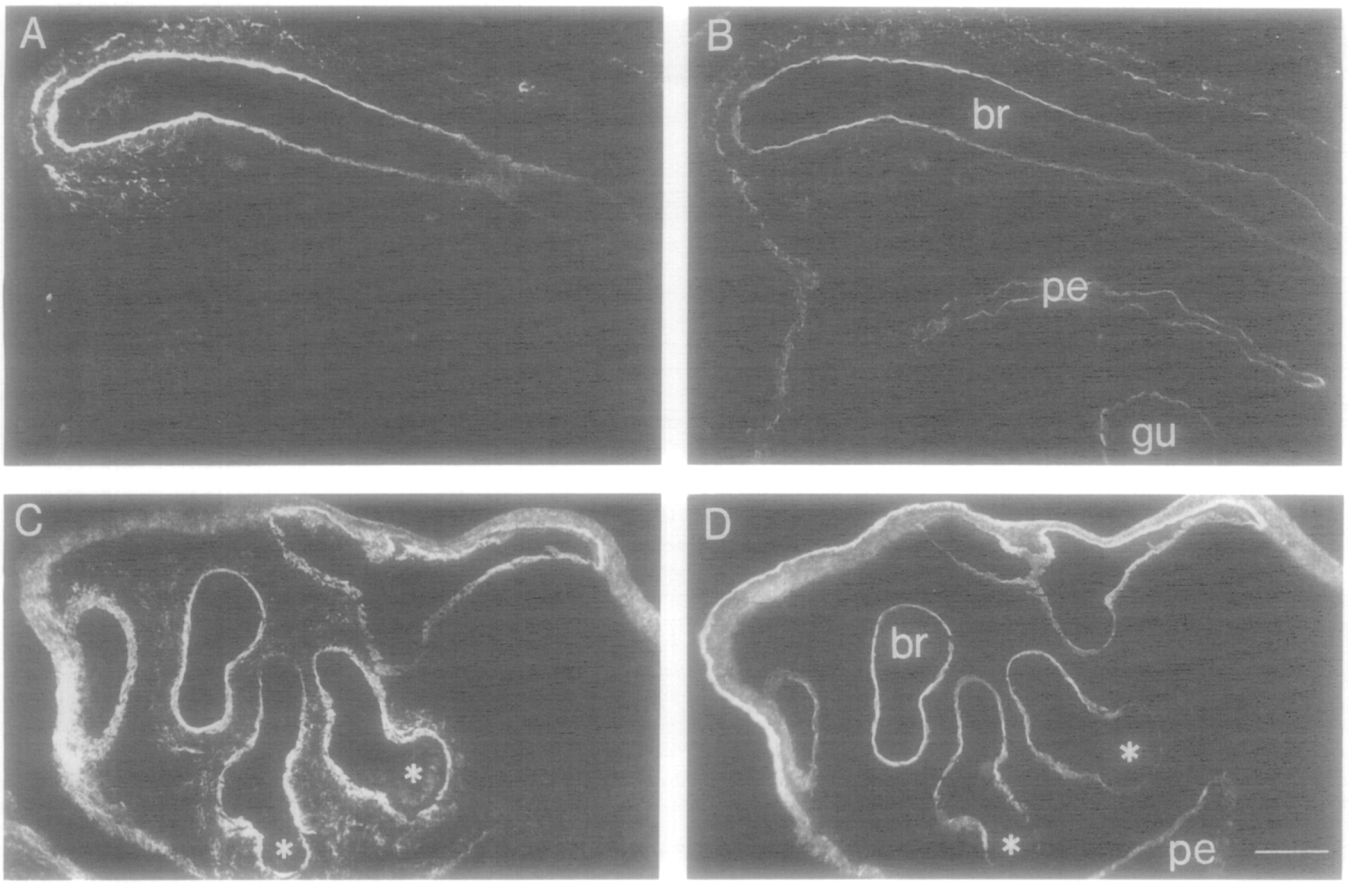

FIG. 1. Immunofluorescence staining of primary (A, B) and secondary (C, D) bronchi with anti-tenascin (A, C) or anti-laminin (B, D) antibodies, respectively. A horizontal section through the lung bud of a 5-day-old embryo (A, B) was double-stained with anti-tenascin mAb M1 (A) and polyclonal anti-chick laminin (B). Consecutive horizontal sections from a $6\frac{1}{2}$-day-old embryo (C, D) were labeled with polyclonal anti-tenascin (C) or mAb 9F-10 against chick laminin (D), respectively. The asterisks in (C) point to growing bronchial tips which show epithelial labeling with anti-tenascin but weak staining with anti-laminin. br, bronchi; pe, peritoneal cavity; gu, gut. Bar, 100 μm.

that basement membranes could change their composition and permeability during differentiation. Whether tenascin is in fact a structural component of immature basement membrane or whether it is temporarily associated with basement membrane cannot be decided yet.

To understand the possible function of tenascin in epithelial–mesenchymal interactions during organ development, its synthetic origin has to be determined in each case. In 6-day-old lung buds, we found expression of tenascin mRNA only in the epithelium. While we prepared this manuscript, a paper by Prieto *et al.* [21] appeared showing that, in 11-day-old chick embryos, cytotactin/tenascin mRNA is still accumulated in the epithelium at the tips of tertiary bronchial branches, but now in addition in the lung mesenchyme as well. On the other hand, we found expression of tenascin in the mesenchyme, not the epithelium, of the gut at 6 days. Thus, in epithelial organs the expression of tenascin might shift from the epithelium to the mesenchyme (or vice versa) during development. Staining of the basement membrane could be an indication for an epithelial synthesis of tenascin. Basement membranes of young somitic vesicles [6], neural tube [9], and the epidermis during wound healing [20] show bright immunoreaction for tenascin. The transient expression pattern of this ECM protein, which might be restricted to small groups of active cells, implies a very potent control mechanism, the nature of which has to be elucidated.

In vitro, tenascin can inhibit the spreading of fibroblasts on fibronectin substrates, causing a rounded cell shape [22]. The expression of tenascin at the tips of growing bronchi suggests that it might be important for the penetration of epithelial tubes into the mesenchyme, perhaps by facilitating the retraction of mesenchymal cells. In early mammalian lung and salivary gland development, other ECM components such as laminin [23], type IV collagen [24, 25], and proteoglycans [26] have been shown to be important for branching morphogenesis. The interplay between these ECM proteins and tenascin remains to be investigated.

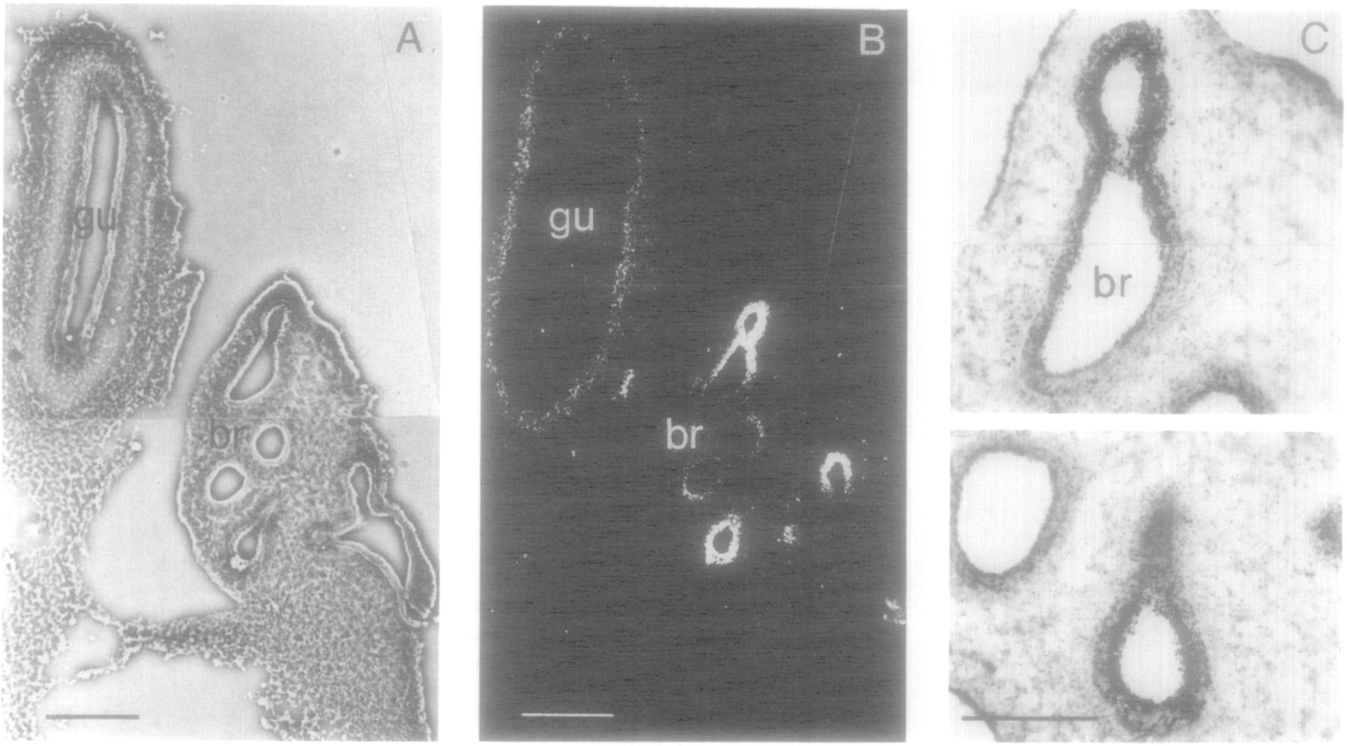

FIG. 2. *In situ* hybridization with tenascin cDNA of a horizontal section through lung primordium and gut dissected from a 6-day-old embryo. (A), phase contrast; (B), dark field illumination; (C), high power bright field image of the same section. br, secondary bronchi, gu, gut. Bars, 200 μm in (A, B) and 100 μm in (C).

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