

# Antibodies against Domain E3 of Laminin-1 and Integrin $\alpha 6$ Subunit Perturb Branching Epithelial Morphogenesis of Submandibular Gland, but by Different Modes

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**Abstract.** Branching epithelial morphogenesis requires interactions between the surrounding mesenchyme and the epithelium, as well as interactions between basement membrane components and the epithelium. Embryonic submandibular gland was used to study the roles of two mesenchymal proteins, epimorphin and tenascin-C, as well as the epithelial protein laminin-1 and one of its integrin receptors on branching morphogenesis. Laminin-1 is a heterotrimer composed of an  $\alpha 1$  chain and two smaller chains ( $\beta 1$  and  $\gamma 1$ ). Immunofluorescence revealed a transient expression of laminin  $\alpha 1$  chain in the epithelial basement membrane during early stages of branching morphogenesis. Other laminin-1 chains and  $\alpha 6$ ,  $\beta 1$ , and  $\beta 4$  integrin subunits seemed to be expressed constitutively. Expression of epimorphin, but not tenascin-C, was seen in the mesenchyme during early developmental stages, but a

mAb against epimorphin did not perturb branching morphogenesis of this early epithelium. In contrast, inhibition of branching morphogenesis was seen with a mAb against the carboxy terminus of laminin  $\alpha 1$  chain, the E3 domain. An inhibition of branching was also seen with a mAb against the integrin  $\alpha 6$  subunit. The antibodies against laminin  $\alpha 1$  chain and integrin  $\alpha 6$  subunit perturbed development in distinct fashions. Whereas treatment with the anti-E3 resulted in discontinuities of the basement membrane at the tips of the branching epithelium, treatment with the mAb against  $\alpha 6$  integrin subunit seemed to leave the basement membrane intact. We suggest that the laminin E3 domain is involved in basement membrane formation, whereas  $\alpha 6\beta 1$  integrin binding to laminin-1 may elicit differentiation signals to the epithelial cells.

**I**N most tissues, epithelial sheets develop as a result of interactions between mesenchyme and epithelium. Basement membranes are located between the tissue compartments from the onset of development. Although basement membranes have been shown to be important for development of epithelial sheets during glandular development (Bernfield, 1984; Ekblom, 1993), the exact role of the various basement membrane components and their domains is still not understood very well.

Laminins are large glycoprotein complexes of basement membranes (Engel, 1993). The first described laminin was previously called EHS (Engelbreth-Holm-Swarm)-laminin

with the chains Ae, Ble, and B2e (Timpl, 1989; Tryggvason, 1993), but it is now called laminin-1 with the chains  $\alpha 1$ ,  $\beta 1$ , and  $\gamma 1$  (Burgeson et al., 1994; Timpl and Brown, 1994). Several biological active domains of laminin-1 have been identified. Domain P1 of mouse laminin-1 has a cryptic cell-binding site (Nurcombe et al., 1989; Aumailley et al., 1990) and a nidogen-binding site (Mayer et al., 1993). Domain E3 was initially defined as a heparin-binding site (Ott et al., 1982; Edgar et al., 1984), but a cell-binding site has also been recently mapped to it (Sonnenberg et al., 1990; Sorokin et al., 1992). Domain E8 possesses major cell binding sites recognized by the integrin  $\alpha 6\beta 1$  (Hall et al., 1990), integrin  $\alpha 7\beta 1$  (von der Mark et al., 1991; Kramer et al., 1991), and possibly also by integrin  $\alpha 3\beta 1$  (Elices et al., 1991). The binding activities of the domains of laminin-1 were established in cell attachment studies, but it is likely that the domains are also important for organ development.

Studies on organ cultures of mouse embryonic kidneys have suggested a role for domains E3 and E8 of laminin-1 for organ development *in vitro*. In these experiments, formation of polarized epithelial cells from mesenchymal stem

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1. *Abbreviations used in this paper:* EHS, Engelbreth-Holm-Swarm; IMEM, improved minimal essential medium; RT-PCR, reverse transcription PCR.

cells was perturbed by application of antibodies against domain E3 and E8 (Klein et al., 1988; Sorokin et al., 1992). An antibody against integrin  $\alpha 6$  subunit also partially blocks conversion of kidney mesenchyme to epithelium (Sorokin et al., 1990). Hence, it has been proposed that domain E8 acts in the developing kidney by binding to integrin  $\alpha 6\beta 1$  (Ekblom, 1993). It is much more unclear how domain E3 of laminin-1 acts in this system.

The conversion of mesenchyme to epithelium is rather unique for the kidney, and it has been largely unclear whether the domains E3 and E8 are required for early stages of epithelial cell development in other organs. In most other organs, epithelial sheets develop directly from an epithelial bud that grows and branches into the mesenchyme, with no recruitment of mesenchyme to epithelium. Laminin domain P1 has been suggested to be required for branching epithelial morphogenesis of lung (Schuger et al., 1991), and it was suggested recently that laminin E8 domain might be involved in later developmental stages of lung alveolar morphogenesis (Matter and Laurie, 1994). Submandibular gland of mouse or rat embryos is a much-used model to study this form of epithelial development (Bernfield et al., 1984; Nogawa and Takahashi, 1991; Takahashi and Nogawa, 1991). We recently described that both laminin (Kadoya and Yamashina, 1989, 1991) and integrin  $\alpha 6$  subunit (Kadoya and Yamashina, 1993) are widely distributed in developing submandibular gland. We have therefore used this organ to examine the expression and role of laminin domain E3, laminin-binding  $\alpha 6$  integrins, as well as the mesenchymal proteins tenascin-C (Chiquet-Ehrismann et al., 1986; Erickson, 1993) and epimorphin (Hirai et al., 1992), which also have been implicated in epithelial morphogenesis.

Expression studies suggested the presence of epimorphin, laminin-1, and integrin  $\alpha 6\beta 1$  in the expected locations, but no tenascin-C was found during early stages of morphogenesis. We therefore tested whether blocking antibodies against epimorphin, laminin-1, or integrin  $\alpha 6\beta 1$  could perturb epithelial morphogenesis. We found that antibodies against epimorphin did not perturb branching epithelial morphogenesis in organ culture of early stages of mouse submandibular gland, whereas antibodies against E3 domain of laminin-1 and against  $\alpha 6$  integrin subunit did so. Interestingly, immunohistochemistry and electron microscopy of the developing basement membranes revealed that the effects of the two blocking antibodies were distinct.

## Materials and Methods

### Antibodies

Rat mAbs against fragment E3 of mouse laminin-1 (mAbs 194, 198, and 200; Sorokin et al., 1992) and mouse tenascin-C (MTn 15; Aufderheide, 1988) were purified from serum-free hybridoma culture media by Bakerbond ABx column (J. T. Baker, Inc., Phillipsburg, NJ; Aufderheide and Ekblom, 1988), and they were concentrated by ultrafiltration (Centricon; Millipore Corp., Milford, MA) and 50% ammonium sulfate precipitation. Purified GoH3, a rat mAb recognizing the  $\alpha 6$  integrin subunit (Sonnenberg et al., 1990; Sorokin et al., 1990), was purchased from Immunotech (Marseille, France; lot No. 10) and Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands, lot No. 1566-08-01). Antiepiporphin mAb (MC-1) (Hirai et al., 1992), polyclonal anti- $\beta 1$  integrin antibodies (Gullberg et al., 1989), anti- $\beta 4$  integrin serum (SP 90395; Dr. P. C. Marchisio, Torino University, Torino, Italy), Fab frag-

ment of antiuvomorulin/E-cadherin rabbit antibody (Vestweber and Kessler, 1985), and purified anti- $\beta 1$  integrin monoclonal antibody (9EG7; Lenter et al., 1993) were gifts from these authors. Rat anti-mouse tenascin-C mAb (MTn12; Aufderheide and Ekblom, 1988), polyclonal antilaminin antibodies (ISH-1; Klein et al., 1988), and antinidogen antibodies (Paulsson et al., 1986; Ekblom et al., 1994) were used in immunofluorescence. Purified rat and rabbit IgGs were purchased from Sigma Immunochemicals (St. Louis, MO).

### Tissues and Organ Culture

Submandibular gland or its rudiments and kidneys were dissected from the NMRI or NMRI  $\times$  C57/BL6 hybrid mice. The morning of the vaginal plug was designated as day 0 of embryonic development. For organ cultures, rudiments of submandibular gland with or without sublingual gland from day 13 embryos were placed on Nuclepore filters (pore size = 0.05  $\mu$ m) and cultured in the medium and air interface at 37°C. Improved minimum essential medium (IMEM) (Richter et al., 1972) with or without 1 mg/ml bovine serum albumin (Sigma) was the medium used. Cultures were carried out in serum-free condition supplemented with 50  $\mu$ g/ml transferrin (lot No. 871252; Collaborative Research Inc., Lexington, MA) (Ekblom et al., 1981). Purified mAbs or polyclonal antibodies in physiological saline were added at 10–100  $\mu$ g/ml to the culture medium at onset of culture. When the volume of the antibody solution was >5% of the culture medium, antibody solutions were dialyzed against IMEM, or concentrated amino acids and vitamins for minimum essential medium (Flow Laboratories, Inc., McLean, VA) were supplemented to maintain physiological culture conditions. MC-1 was added in a 10% vol of culture medium (Hirai, Y., personal communication). In control experiments, rat or rabbit IgGs (Sigma), or medium substituted test antibodies. Commercial mAb GoH3 contained BSA, and therefore BSA was also added in some control experiments. After 2 or 3 d in culture, organs were collected for immunohistochemistry and electron microscopy. Appropriate antibody penetration into the cultured organs was confirmed by direct immunofluorescence.

### Immunohistochemistry and Transmission Electron Microscopy

Tissues and organ cultures were frozen in Tissue Tek (Miles Laboratories Inc., Elkhart, IN). Frozen sections (7  $\mu$ m) were fixed with acetone for 2 min, treated with 5% BSA in PBS for 20 min, and incubated with the following antibodies: GoH3 (2  $\mu$ g/ml), mAb 200 (20  $\mu$ g/ml), mAb 198 (10  $\mu$ g/ml), anti- $\beta 1$  integrin (4  $\mu$ g/ml), anti- $\beta 4$  integrin (5  $\mu$ g/ml), and anti-E-cadherin (6.5  $\mu$ g/ml). MC-1, ISH-1, MTn12, and antinidogen antibodies were diluted 1:150 (Hirai, Y., personal communication), 1:1000, 1:2, and 1:100, respectively. Bound antibodies were visualized using goat anti-rat IgG conjugated with FITC (Sigma) and goat anti-rabbit conjugated with TRITC secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Slides were examined under a microscope (Axiophot; Carl Zeiss, Inc., Thornwood, NY) equipped with epifluorescence optics. For electron microscopy, cultures were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.5, and processed as described (Kadoya and Yamashina, 1991). 1- $\mu$ m-thick sections were stained with toluidine blue. Ultrathin sections were stained with both lead and uranyl acetate, and they were examined under an electron microscope (CM-10; Philips Electronic Instruments Corp., Mahwah, NJ).

### In Situ Hybridization

A synthetic 50-mer DNA specific for laminin  $\alpha 1$  chain, 5'-GCCTTCCTT-TAATGTATTCTGTCTTGACTGTGTGCCACTTGCCATCACT', complementary to position 8452–8501, which encodes part of the domain E3 (Sasaki et al., 1988), was analyzed against the Entrez data base (Release 6.0; National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD) for coincidental homology with other murine sequences allowing a 30% mismatch. No such homologies were found. In situ hybridization was performed as described (Durbbeej et al., 1993) with the following modification: after hybridization with <sup>35</sup>S-dATP-labeled oligonucleotide probe, sections were washed at 65°C instead of 56°C. Control experiments were carried out with the same amount of labeled probe and unlabeled probe in excess. After a 4- to 9-wk exposure, sections were developed and observed under dark- and bright-field illumination.

## Gel Electrophoresis and Immunoblotting

Tissues were sonicated in solubilizing buffer (10 mM EDTA, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 2 mM PMSF, 0.1% Trasylol, and 0.1% *N*-ethylmaleimide) on ice. After a 1-h incubation on ice, they were centrifuged at 10,000 g for 10 min. Supernatants were dissolved in Laemmli sample buffer containing DTT, boiled for 5 min, and resolved using 5–15% gradient SDS-PAGE (Bio Rad Laboratories, Hercules, CA). Immunoblotting was carried out using antilaminin antiserum diluted at 1:1000 as described (Klein et al., 1988). Protein concentration was determined with the Bio Rad protein assay kit using bovine gamma globulin as a standard.

## Reverse Transcription PCR (RT-PCR)

RNA was purified from the submandibular gland or its rudiment with a Micro-Fast Track Kit (Invitrogen, San Diego, CA) or according to Chomczynski and Sacchi (1987). RT-PCR was carried out with GeneAmp RNA PCR Kit (Perkin-Elmer Corp., Norwalk, CT) according to the manufacturer's instructions. Briefly, the RT reaction was carried out with 1  $\mu$ g RNA using oligo-dT primers. After inactivation of reverse transcriptase at 99°C for 5 min, the reaction mixture was cooled to 5°C for 5 min. The cDNA mixture was then subjected to 25 (for  $\alpha 6$  integrin subunit) or 35 (for laminin  $\alpha 1$  chain) amplification cycles in a Perkin Elmer Cetus thermocycler. Cycles were 1 min denaturation at 94°C, 2 min annealing at 55°C, and 2 min extension at 72°C. The PCR products were analyzed on a 1.2% agarose gel. For laminin  $\alpha 1$  chain, we used sense primer 5'-ACTTGGTGCTCCCTCTGAATC-3' (position 8243–8263) and antisense primer 5'-ATGCGGTGCTTGCTTTTGTGG-3' (position 9021–9041) corresponding to a part of domain E3 sequence (Sasaki et al., 1988), and for  $\alpha 6$  integrin subunit, we used sense primer 5'-GTCAGGTGTGTGAACATCAGG-3' (position 2975–2995) and antisense primer 5'-CTGAAAAATAAGGGGGGGGC-3' (position 3627–3647) (Hierck et al., 1993). The specificity of PCR products was confirmed by restriction enzyme analysis.

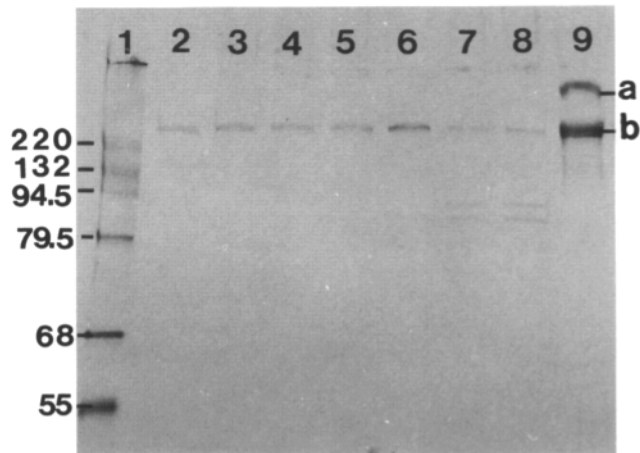
## Results

### Immunoblotting and RT-PCR of Laminin $\alpha 1$ Chain in the Developing Submandibular Gland

By immunoblotting with polyclonal antiserum against laminin-1 reacting equally well with 400-kD  $\alpha 1$  chain and 200-kD  $\beta 1$  and/or  $\gamma 1$  chains of EHS tumor material (Fig. 1), we failed to detect any 400-kD  $\alpha 1$  chain at any developmental stage that was analyzed. Only 200-kD bands apparently representing laminin  $\beta 1$  or  $\gamma 1$  chains were seen under reducing conditions (Fig. 1). However, an expression of laminin  $\alpha 1$  chain in submandibular gland from various stages was noted by RT-PCR analysis (Fig. 2 A). Two splice variants of integrin  $\alpha 6$  subunit with different intracellular domains have been detected (Hogervorst et al., 1991, 1993; Cooper et al., 1991). We detected expression of both the  $\alpha 6A$  and  $\alpha 6B$  variant at all stages that were analyzed (Fig. 2 B). As previously reported, both variants were expressed in the adult gland (Hogervorst et al., 1993). The immunoblots suggested that the laminin  $\alpha 1$  chain was either not expressed or expressed at low levels not detectable by the immunoblotting procedure. Since the mRNA could be detected, we used immunofluorescence to further investigate whether the laminin  $\alpha 1$  polypeptide was at all expressed in the developing submandibular gland. Several recently characterized mAbs against the E3 domain of mouse laminin-1 were used. These mAbs recognize the laminin  $\alpha 1$  chain, but not the other laminin-1 chains (Sorokin et al., 1992).

### Immunodetection of Laminin Chains, Laminin Receptors, and other Extracellular Matrix Proteins

In 17-d-old embryos and newborn pups, submandibular epithelium is highly branched and several terminal tubules and

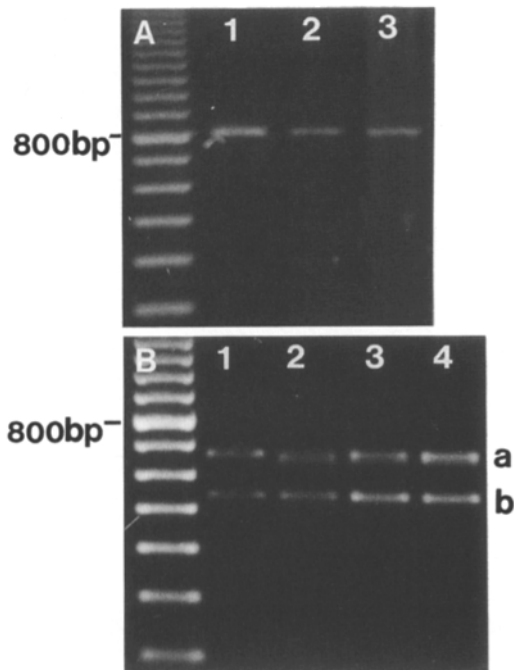


**Figure 1.** Immunoblot analysis of laminin-1 chains during mouse submandibular gland development. 40  $\mu$ g of EDTA extract of submandibular gland from 14-d-old embryos (lane 2), 17-d-old embryos (lane 3), newborn (lane 4), 1-wk-old (lane 5), 2-wk-old (lane 6), 6-wk-old female (lane 7), 6-wk-old male (lane 8), and EHS tumor extract (lane 9) were separated on a 5–15% gradient gel by SDS-PAGE under reducing conditions. After electrophoresis, proteins were transferred to a nitrocellulose filter, and immunoblotting was performed with anti-laminin-1 antiserum, ISH-1. In the sample from EHS tumor, both the 400-kD  $\alpha 1$  chain (a) and the 200-kD  $\beta 1$  and  $\gamma 1$  chains could be blotted (b). Molecular mass markers corresponding to 220, 132, 94.5, 79.5, 68, and 55 kD (top to bottom) are indicated in lane 1. The doublet around 90 kD seen in lanes 7 and 8 is caused by nonspecific staining because the same bands were seen in these samples also by omitting the first antibody (data not shown).

ducts are found. No laminin  $\alpha 1$  chain was detected by immunofluorescence at this stage in the basement membrane of the terminal tubules, but weak expression of laminin  $\alpha 1$  chain was seen in the basement membrane of the duct epithelium (Fig. 3 A). In contrast, polyclonal anti-laminin-1 antibodies detecting  $\alpha 1$ ,  $\beta 1$ , and  $\gamma 1$  chains stained the basement membrane of all parts of the gland epithelium (Fig. 3 B). Staining for laminin  $\alpha 1$  chain in the duct basement membranes was recognized by the higher affinity mAbs 200 and 198, but only faintly with mAb 194, which has only a weak affinity for laminin-1 (Sorokin et al., 1992). Unmasking treatment of the specimens with 0.1% trypsin dissolved in a solution of 0.1% CaCl<sub>2</sub> and 20 mM Tris, pH 7.5, did not increase staining intensity or alter the staining pattern.

In 13-d-old embryos, the rudiments of the submandibular gland consist of several large epithelial clusters connected to an epithelial stalk. All epithelial sheets are surrounded by mesenchyme. At this stage, the laminin  $\alpha 1$  chain could be readily detected by immunofluorescence with mAb 200 in basement membranes of both the epithelial clusters and the epithelial stalk. Some, but very weak, expression of laminin  $\alpha 1$  chain was noted in some parts in the mesenchyme, but no expression was seen in the capillary basement membranes (Fig. 4 A). In contrast, the antiserum detecting all three chains of laminin-1 strongly stained both the mesenchymal extracellular matrix, as well as the capillary and epithelial basement membranes (Fig. 4 B).

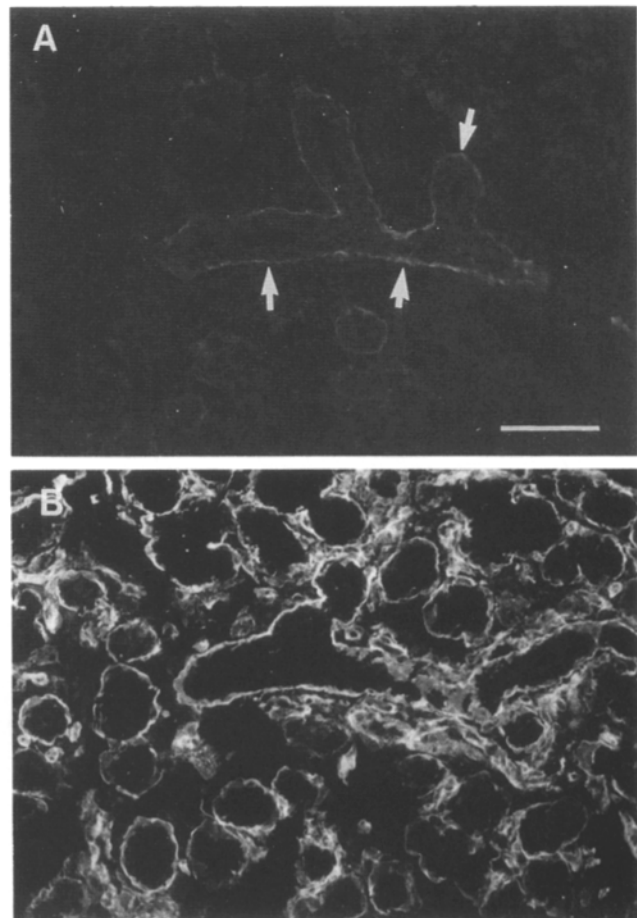
The restricted expression of laminin  $\alpha 1$  chain during early



**Figure 2.** Detection of laminin  $\alpha 1$  chain (*A*) and integrin  $\alpha 6$ A and  $\alpha 6$ B subunit (*B*) mRNAs transcripts by RT-PCR. (*A*) RNA was from submandibular gland from 14-d-old embryos (lane 1), 17-d-old embryos (lane 2), and 2-wk-old pups (lane 3). (*B*) The PCR products for both integrin  $\alpha 6$ A (673 bp; marker *a*) and  $\alpha 6$ B (543 bp; marker *b*) were generated from RNA from submandibular gland from 14-d-old embryos (lane 1), 17 d-old embryos (lane 2), newborn mice (lane 3), and 2-wk-old mice (lane 4). The PCR products were separated on 1.2% agarose gels, and they were stained with ethidium bromide.

stages of submandibular gland development suggested a role for laminin-1 in branching epithelial morphogenesis. It was therefore important to study the distribution of laminin-1 receptors, such as the  $\alpha 6\beta 1$  and  $\alpha 6\beta 4$  integrins (Sonnenberg et al., 1990). In 13-d-old embryos, the integrin  $\alpha 6$  subunit was expressed by endothelial and epithelial cells, and it was enriched on the basal side of the epithelial clusters (Fig. 4 *C*). The integrin  $\beta 1$  subunit was expressed in the same locations as the  $\alpha 6$  subunit, but it was also expressed by the mesenchyme surrounding the epithelium (Fig. 4 *D*). In contrast, the  $\beta 4$  integrin subunit was expressed only weakly in the basal surface of epithelial cells adjacent to the basement membrane. No integrin  $\beta 4$  subunit expression was seen on cells located in the center of the epithelial clusters (Fig. 4 *E*). In 17-d-old embryos,  $\alpha 6$  and  $\beta 4$  integrin subunits were expressed by epithelial cells, but not by mesenchymal cells, whereas the integrin  $\beta 1$  subunit was expressed more uniformly by all cell types.

Two mesenchymal matrix components implicated in epithelial morphogenesis, epimorphin and tenascin-C, were also studied. In submandibular gland rudiments of 13-d-old embryos, weak expression of epimorphin, but not tenascin-C, could be detected in the mesenchyme (Fig. 4 *F*). In the submandibular salivary gland of 17-d-old embryos, strong expression of epimorphin was detected. Some

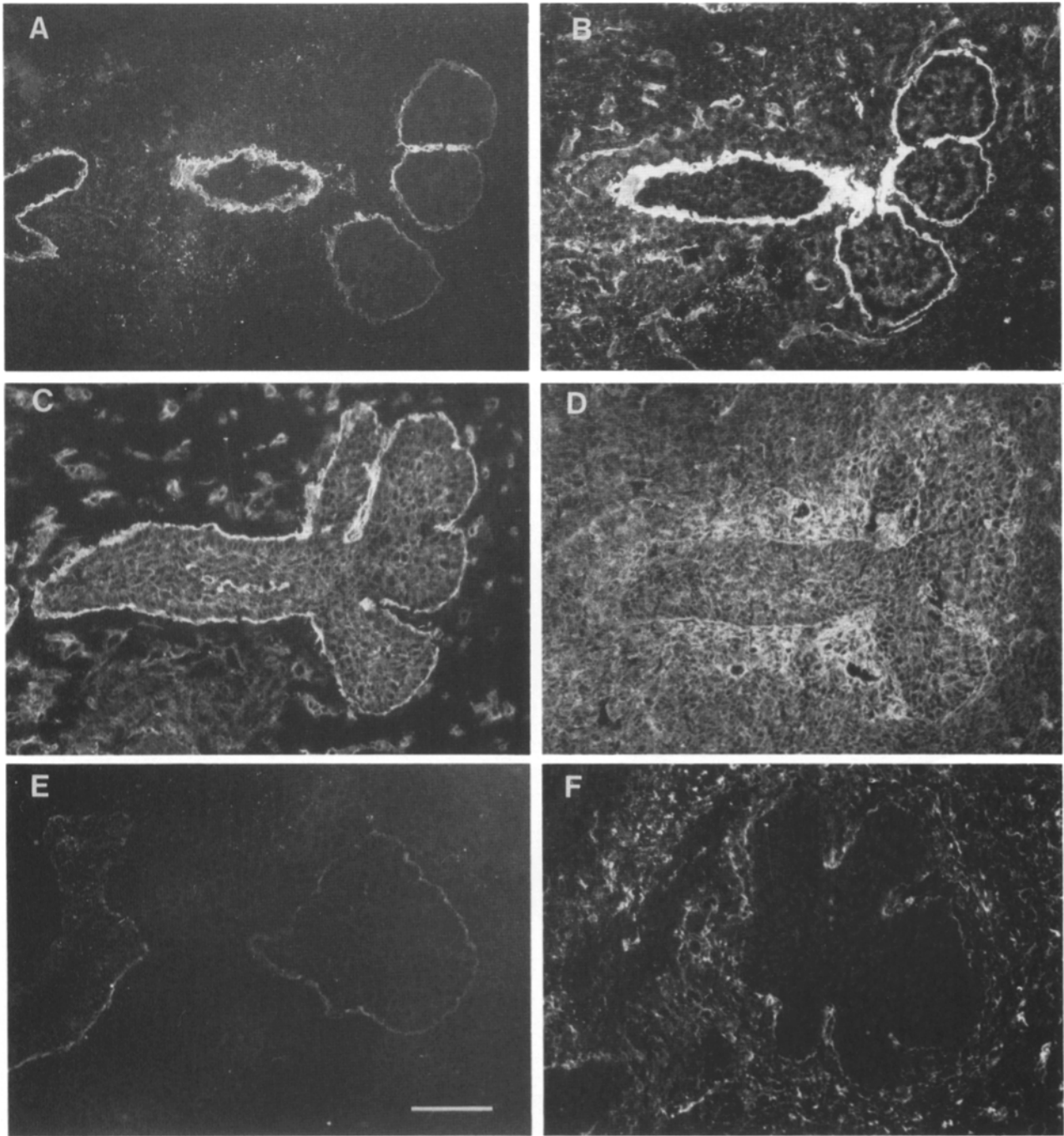


**Figure 3.** Immunolocalization of laminin  $\alpha 1$  chain with mAb 200 (*A*) and laminin-1 with polyclonal ISH-1 antiserum (*B*) in submandibular gland of day 17 embryos. Weak but definite staining for laminin  $\alpha 1$  chain was seen, but only in the basement membrane of the ducts (*A*, arrows). In contrast, polyclonal antiserum against laminin-1 stained basement membranes in all parts of the epithelium (*B*). Bar, 100  $\mu$ m.

tenascin-C was also expressed in the mesenchyme of 17-d-old embryos, but tenascin-C expression was more restricted than that of epimorphin, and it was seen only in the mesenchyme surrounding the duct epithelium (data not shown).

#### *In Situ Hybridization for Laminin $\alpha 1$ Chain mRNA during Development of Submandibular Gland*

In small submandibular glands from day 13 embryos with only rudimentary epithelial branches, laminin  $\alpha 1$  chain mRNA was detected associated with epithelium. In the tip of the epithelium, expression was seen in the basal epithelial cell layer, but in the stalk, no expression was seen in the epithelium itself. Instead, clear expression was seen in the mesenchyme immediately adjacent to the epithelial stalk (Fig. 5, *A* and *B*). In slightly more branched submandibular glands of day 13 embryos, signal intensities were already weak both in the epithelium and mesenchyme. They were still slightly stronger on the tip of the epithelium than in the adjacent mesenchyme (Fig. 5, *C* and *D*). Signals over the background were not seen anymore in glands from day 17

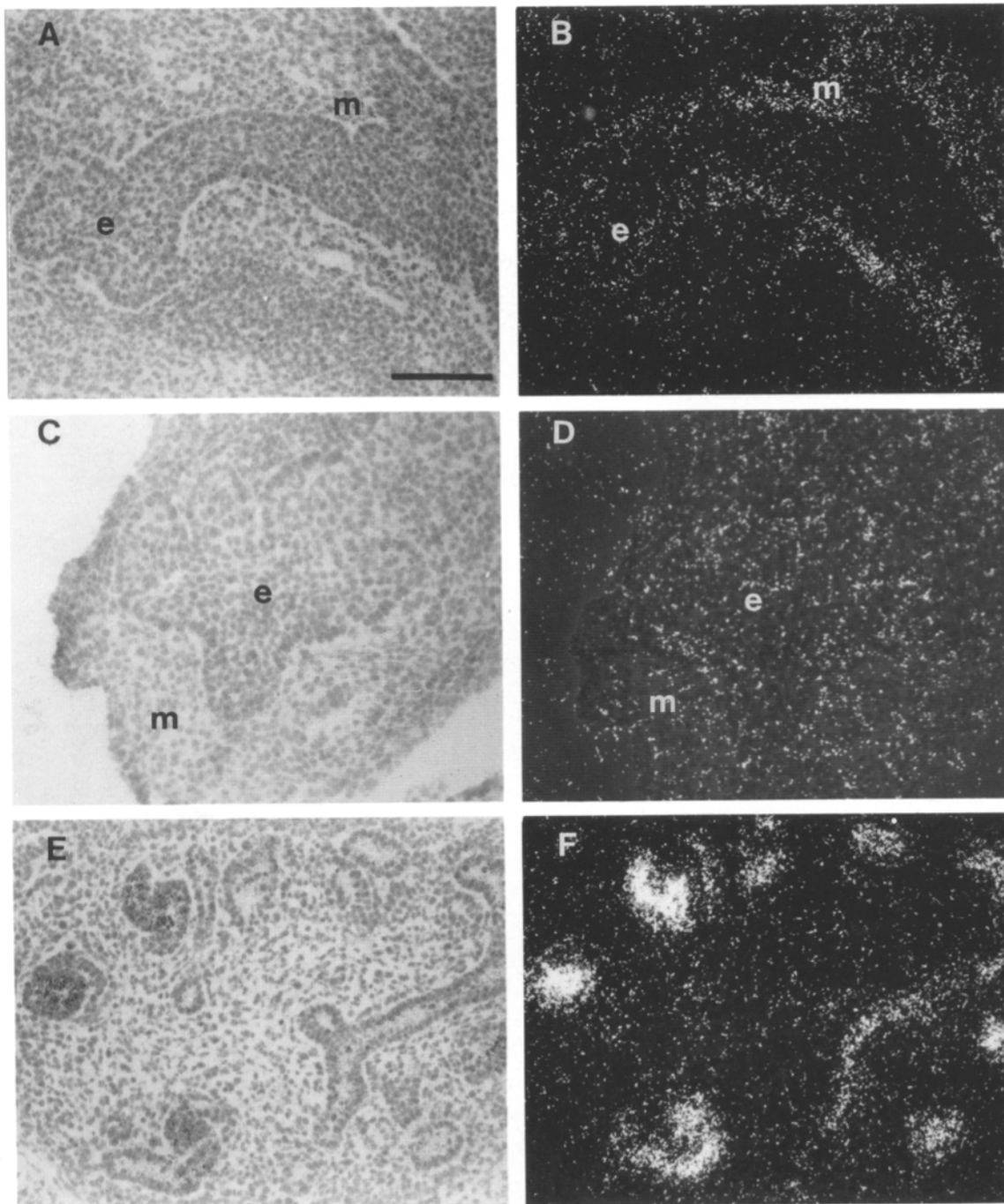


**Figure 4.** Immunolocalization of laminin  $\alpha 1$  chain (A), laminin-1 (B),  $\alpha 6$  integrin (C),  $\beta 1$  integrin (D),  $\beta 4$  integrin (E), and epimorphin (F) in the submandibular gland of day 13 embryos. (A) Laminin  $\alpha 1$  chain is detected only along the basement membrane of the rudimentary epithelium, very weakly in the mesenchyme, but not in the basement membrane of the capillary endothelium. (B) In contrast, anti-laminin-1 antiserum stains the basement membrane of both epithelium and endothelium. Bright punctate staining is also noted between the mesenchymal cells. The epithelial cytoplasm also shows diffuse but distinct staining for laminin-1. (C) Expression of  $\alpha 6$  integrin subunit is found in both submandibular epithelium and capillary endothelium, but not in the mesenchyme. (D)  $\beta 1$  integrin is expressed by both the epithelium and mesenchyme. Note that mesenchymal cells close to the epithelium show stronger staining. (E)  $\beta 4$  integrin is only expressed along the basal surface of the peripheral epithelial cells (F) Epimorphin is expressed in most parts of the mesenchyme, including the epithelial-mesenchymal interface. Bar, 100  $\mu\text{m}$ .

embryos (data not shown). The oligonucleotide that was used reacted in the expected fashion in the embryonic kidney, and it gave strong signals in developing epithelial cells (Fig. 5, E and F), as previously shown with riboprobes (Ekblom

et al., 1990). Hence, the weak signals seen in the embryonic submandibular gland at the later developmental stages resulted from a low expression level rather than from technical problems with the in situ hybridization procedure.



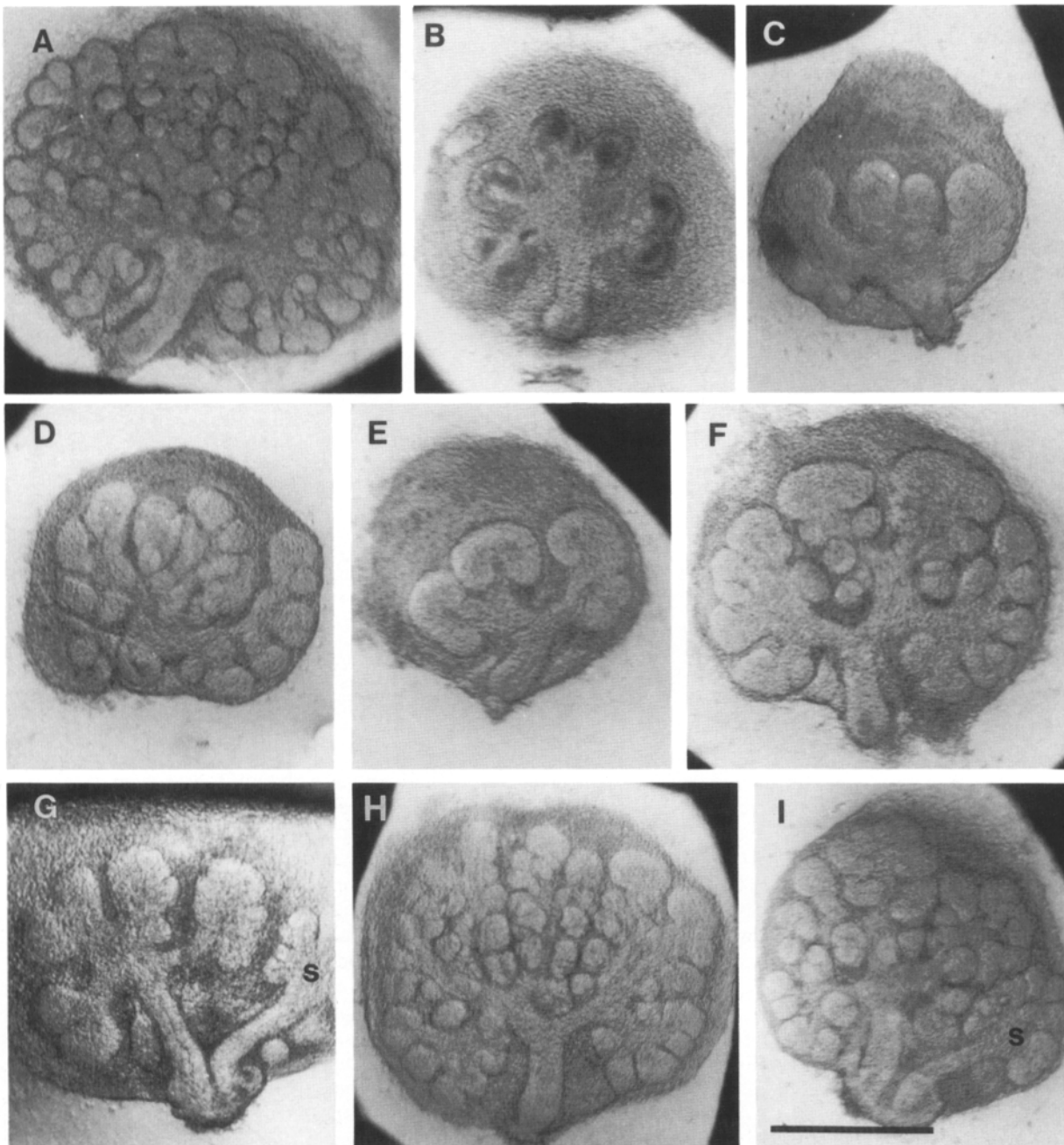


**Figure 5.** Transient expression of laminin- $\alpha 1$  chain mRNA at the epithelial-mesenchymal interphase of the developing submandibular gland. In situ hybridization for laminin  $\alpha 1$  chain was performed on sections from developing submandibular glands of 12.5–13-d-old (*A* and *B*) and 13-d-old embryos (*C* and *D*), and kidneys from 13-d-old embryos (*E* and *F*). Sections were probed with a radiolabeled 50-mer oligonucleotide specific for laminin- $\alpha 1$  chain mRNA, processed for autoradiography, and examined by bright-field (*A*, *C*, and *E*) and dark-field (*B*, *D*, and *F*) illumination. In sections of submandibular gland of embryonic day 12.5–13, silver grains are clustered in mesenchyme (*m*) close to the epithelial stalk. In the epithelial tip grains are found in the epithelium (*e*). In sections of submandibular gland of embryonic day 13, signals are weak both in epithelium and mesenchyme, but slightly stronger signals are seen in the epithelium (*C* and *D*). In sections from kidneys of 13 d-old embryos, strong signals were found in the epithelium (*E* and *F*). Bars, 120  $\mu\text{m}$  in *A*, *B*, *E*, and *F*, and 100  $\mu\text{m}$  in *C* and *D*.

### **Stages of Submandibular Morphogenesis in a Defined Medium**

The function of epimorphin, laminin, and  $\alpha 6$  integrin laminin receptors during branching epithelial morphogenesis

was studied by antibody perturbation experiments. We first optimized the culture conditions and defined the developmental stages in defined medium. Transferrin was found to be essential for the epithelial branching in serum-free culture conditions (Fig. 6*A*). In its absence, the epithelium exhibited



**Figure 6.** Perturbation of branching epithelial morphogenesis with antibodies. Submandibular glands from 13-d-old embryos were cultured for 2 d, and micrographs were then taken with a stereomicroscope. Transferrin (50  $\mu\text{g}/\text{ml}$ ) was supplemented in all cultures except for *B*. (*A*) In positive controls where no antibodies were added, well-developed epithelial structures are evident. (*B*) In explants cultured without transferrin, inhibition of branching morphogenesis is evident, but unbranched epithelial clusters are seen as dark areas, indicating cellular degeneration. In explants treated with 100  $\mu\text{g}/\text{ml}$  of anti-domain E3 mAb 200, branching is perturbed, and the degree of inhibition varied severely (*C*) to moderately (*D*). In explants treated with anti- $\alpha 6$  integrin, mAb GoH3 (40  $\mu\text{g}/\text{ml}$ ) branching morphogenesis was also perturbed, and the degree of inhibition varied from severe (*E*) to moderate (*F*). In explants treated with anti- $\beta 1$  integrin (9EG7, 100  $\mu\text{g}/\text{ml}$ ), inhibition of epithelial branching occurred (*G*). (*H*) Explants treated with anti-tenascin-C mAb MTn 15 at 10  $\mu\text{g}/\text{ml}$  (*H*) and antiepimorphin mAb MC-1 (*I*) show well-branched epithelial structures. In *G* and *I*, rudiments of sublingual gland (*s*) can be seen. Bar, 0.5 mm.

significantly less branching, and the poorly branched terminal clusters appeared as darker structures when viewed live by stereomicroscopy (Fig. 6 *B*).

Submandibular glands from 13-d-old mouse embryos developed as explants in the defined medium as follows: (stage 1) In freshly isolated glands, the epithelium has one central

stalk and one or only a few terminal roundings that form the terminal clusters (Bernfield et al., 1984). (stage 2) The terminal clusters has branched further, and distinct lobules have formed at each of the terminal clusters by distally forming clefts. (stage 3) Lobules have continued to branch and have formed highly branched structures as can be seen in Fig. 6

**Table 1. Effects of Various Antibodies on Branching Morphogenesis of Cultured Submandibular Gland\***

Supplements	Concentration	Days in culture	Number of explants <sup>‡</sup>				Total
			--	-	+	++	
	<i>μg/ml</i>						
Anti-laminin E3, No. 200	100	2	10	2	3	0	15
Anti-laminin E3, No. 200	50	2	1	4	0	0	5
Anti-laminin E3, No. 200	25	2	0	1	3	1	5
Anti-laminin E3, No. 194	100	2	0	0	4	2	6
Anti-integrin α6, GoH3	40	2	6	7	1	0	14
Anti-integrin α6, GoH3	20	2	5	0	0	0	5
Anti-integrin α6, GoH3	10	2	0	0	4	2	6
Anti-integrin β1, 9EG7	100	2	0	4	5	0	9
Anti-tenascin-C, MTn15	100	2	0	0	5	4	9
Antiepipimorphin, MC-1	10% <sup>§</sup>	2	0	1	9	0	10
Antiepipimorphin, MC-1	10% <sup>§</sup>	3	0	0	0	8	8
DME/F12	10% <sup>§</sup>	2	0	1	7	1	9
DME/F12	10% <sup>§</sup>	3	0	0	1	8	9
Normal rat IgG	100	2	0	0	1	9	10
Normal rat IgG	40 <sup>†</sup>	2	0	0	0	5	5
Normal rabbit IgG	100	2	0	0	1	4	5
No		2	0	0	10	16	26

\* Rudiments from day 13 embryos were cultured in IMEM + 50 μg/ml transferrin for 2 or 3 d with or without various supplements.

<sup>‡</sup> Extent of reduced epithelial branching morphogenesis was scored as severe inhibition (--), moderate inhibition (-), delay of branching (+), and advanced development (++). See text for details.

<sup>§</sup> Percent of volume added to medium.

<sup>†</sup> 40 μg/ml normal rat IgG + 200 μg/ml BSA.

A. Based on this staging, we defined the extent of inhibition of branching caused by antibody treatment. Explants were considered severely inhibited (--) when there was complete failure of branching morphogenesis, and the epithelium appeared to stay in stage 1. Moderate inhibition (-) was scored when the epithelium branched once but failed to develop further and seemed to stay in stage 2. Moderate inhibition was also scored when the epithelium branched only marginally and instead formed a few larger abnormal terminal clusters. Delay of branching (+) was scored when explants initially branched normally, but the number of terminal lobules was not as high as seen in Fig. 6 A. Advanced development (++) was scored when explants reached stage 3 (Fig. 6 A) within the examination period.

#### **Perturbation of Branching Epithelial Morphogenesis with Antibodies**

At 50 μg/ml, mAb 200 against the E3 fragment of laminin-1 severely or moderately inhibited branching epithelial morphogenesis, and the effect was slightly enhanced by raising the concentration to 100 μg/ml. Some variations in the size of individual lobules within an explant were noted in the mAb 200-treated explants. In explants grown for 2 d in the presence of mAb 200 at 100 μg/ml, branching was almost completely inhibited (Fig. 6 C) in 10 out of 15 cases (Table I), or it was partially inhibited (Fig. 6 D) in 2 out of 15 explants (Table I). Another mAb with a weak affinity for the laminin E3 fragment, mAb 194, only marginally, if at all, inhibited branching. Control cultures grown in the presence of 100 μg/ml rat or rabbit IgG showed normal branching morphogenesis, and they reached stage 3 of development.

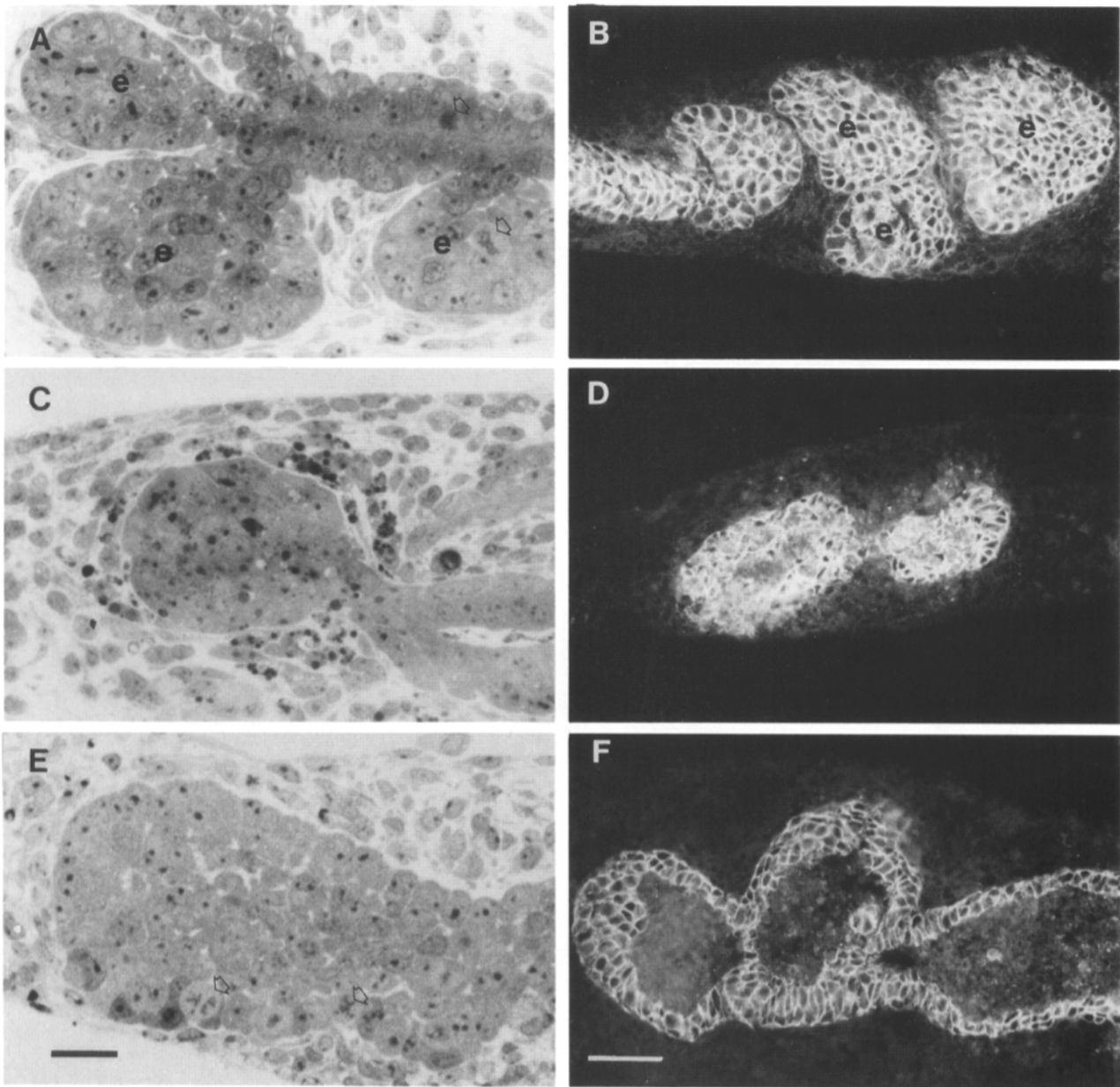
A severe inhibition of epithelial branching was also seen in cultures grown in the presence of mAb GoH3, which

recognizes the integrin α6 subunit. After a 2-d culture in the presence of 20–40 μg/ml mAb GoH3, branching of the epithelial cluster within the submandibular gland cultures was either severely (11/19) or moderately (7/19) inhibited (Fig. 6, E and F). In cultures with a severe inhibition of branching, the epithelial lobules were highly irregular, and the size of the lobules varied greatly. Some parts of the lobules appeared necrotic in the middle. A mAb against the β1 integrin subunit, 9EG7, also moderately inhibited epithelial branching at 100 μg/ml (Fig. 6 G).

The inhibition of branching morphogenesis obtained by treatment with either mAb 200 or GoH3 differed from the inhibition caused by omission of transferrin. Within the few formed lobules in explants treated with either mAb 200 or GoH3, the epithelium appeared to grow, and it formed larger abnormal epithelial clusters (Fig. 6, C and F). In explants grown without transferrin, no such abnormal cluster formation occurred, and the terminal lobules remained small (Fig. 6 B).

In controls that were cultured in the presence of 100 μg/ml of rat or mouse IgG, or mAb MTn15 against tenascin-C, no inhibition of branching morphogenesis was seen (Fig. 6 H). We also investigated the effect of an mAb raised against epimorphin (MC-1), which has been shown to bind the cellular recognition site of this protein and to inhibit epithelial cell polarization in cultured lung rudiments (Hirai et al., 1992; Hirai, 1994). Since the MC-1 solution added to medium was dialyzed against DME/F12, equivalent quantities of DME/F12 were added to control cultures. Although a minimal delay of epithelial branching was evident in MC-1-treated cultures (Fig. 6 I), a similar effect was noted in the control cultures. The effects of the various antibodies are summarized in Table I.



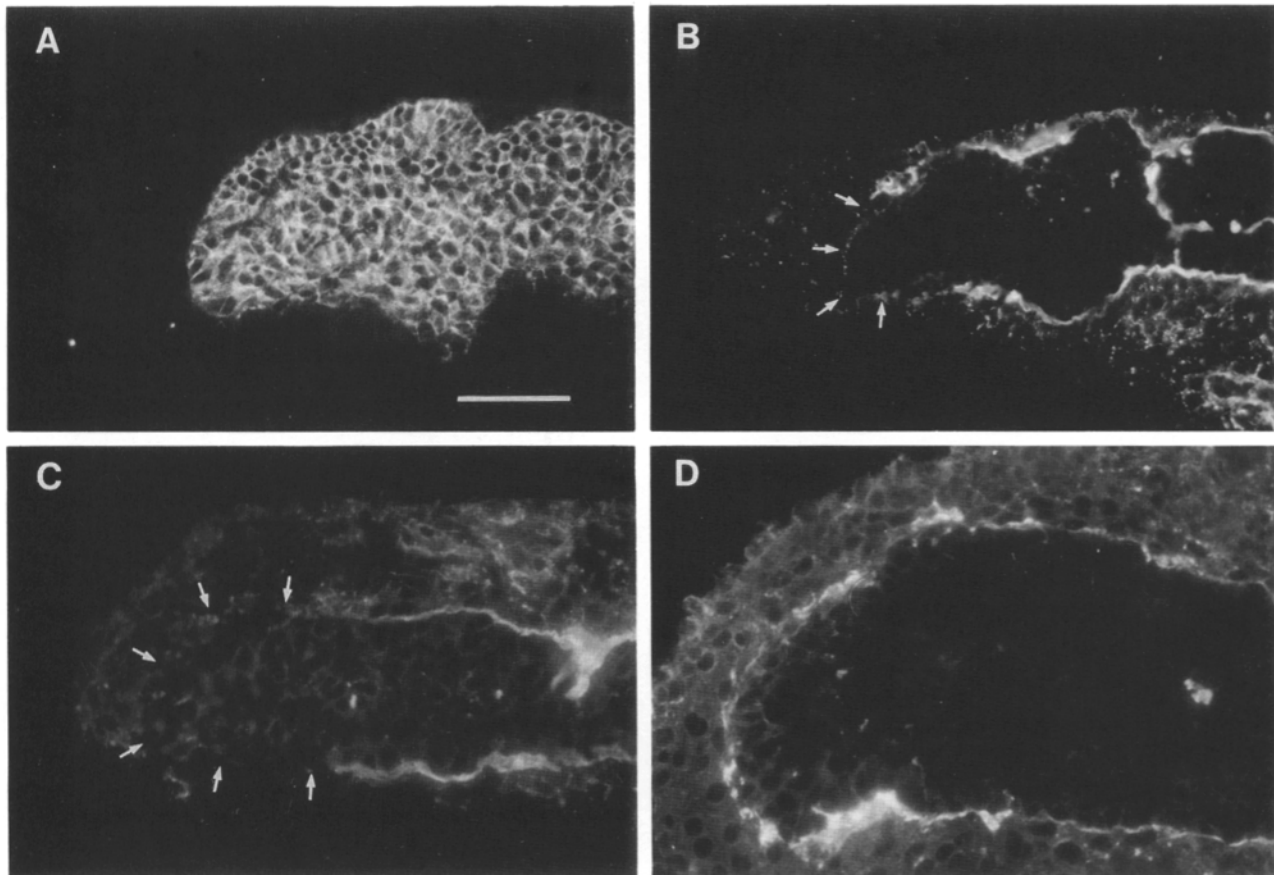


**Figure 7.** Histology of control explants, explants grown without transferrin or in the presence of mAb GoH3 and transferrin. Toluidine blue-stained 1- $\mu$ m thick plastic sections (*A*, *C*, and *E*) and anti-E-cadherin antibody-stained frozen sections (*B*, *D*, and *F*) of 2-d cultured submandibular gland rudiments are shown. In the positive control, culture containing transferrin (50  $\mu$ g/ml) but no antibodies (*A* and *B*), well-branched epithelial clusters (*e*) expressing E-cadherin, an epithelial marker, are evident. In explants cultured without transferrin (*C* and *D*), most of the epithelial cells and mesenchymal cells surrounding epithelium in the explants start to degenerate, and many pycnotic nuclei can be seen (*C*). In explants grown without transferrin, epithelial cells continue to express E-cadherin (*D*). Anti- $\alpha$ 6 integrin mAb GoH3 (40  $\mu$ g/ml) also inhibits epithelial branching morphogenesis (*E* and *F*), but epithelial cells adhering to the basement membranes are intact, and they do not show any sign of degeneration (*E*). In explants treated with GoH3, E-cadherin staining frequently becomes reduced in the innermost area of the abnormal epithelial clusters, although the staining of the peripheral cells close to the basement membranes remains intact (*F*). Note mitotic cells in positive control explants (*A*, arrows) and anti- $\alpha$ 6 integrin mAb-treated explants (*E*, arrows). Bars, 100  $\mu$ m in *A*, *C*, and *E*, and 50  $\mu$ m in *B*, *D*, and *F*.

### ***Distinct Biological Effects of Antibodies against Laminin E3 Domain and Integrin $\alpha$ 6 Subunit in Organ Culture***

The omission of transferrin from culture medium is known to reduce cell proliferation in organ culture (Ekblom et al., 1981). Stereomicroscopy of the live submandibular gland ex-

plants treated with the different antibodies already suggested that mAbs 200 and GoH3 did not primarily affect cell proliferation. This could be confirmed by comparing the histology of explants grown without transferrin and of explants treated with the antibodies in the presence of transferrin. These studies also revealed that treatment with mAb 200 and GoH3 altered morphogenesis in distinct ways.



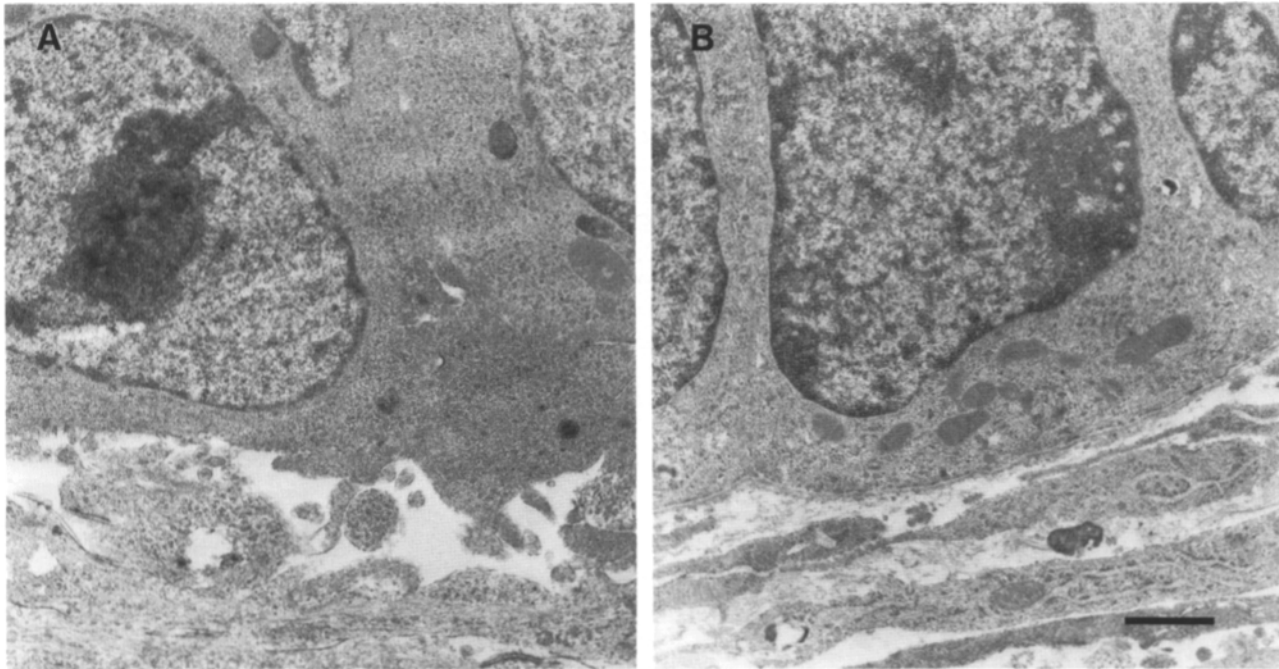
**Figure 8.** Localization of laminin and nidogen, as well as E-cadherin in mAb-treated rudiments. Submandibular rudiments cultured for 2 d in the presence of 100  $\mu\text{g/ml}$  of anti-E3 mAb 200 (*A-C*) and 40  $\mu\text{g/ml}$  of anti- $\alpha 6$  mAb GoH3 (*D*) were stained with rabbit antibodies against (*A*) E-cadherin, (*B*) nidogen, or (*C* and *D*) laminin-1. (*A*) mAb 200-treated explants expressed E-cadherin. Note that there is no accumulation of E-cadherin expression indicative of the formation of the lumen. (*B*) Adjacent section to *A* stained with antinidogen antibodies shows discontinuous basement membrane staining at the tip of an epithelial cluster, and staining with anti-laminin-1 antibodies also shows a similar discontinuity (*C*). In GoH3-treated explants, a continuous staining for laminin-1 can be seen along the epithelial cluster (*D*). Bar, 40  $\mu\text{m}$  in *A* and *B*, and 50  $\mu\text{m}$  in *C* and *D*.

In control cultures grown for 2 d in the presence of transferrin, branching terminal epithelial clusters and well-developed epithelial ducts were evident (Fig. 7 *A*), and E-cadherin was expressed over the entire surface of epithelial cells of both terminal clusters and the stalk (Fig. 7 *B*). In contrast, a large number of pycnotic cells were found in cultures grown for 2 d without transferrin (Fig. 7, *C* and *D*). The reduced cell survival was particularly evident in the area close to the epithelial-mesenchymal interface (Fig. 7 *C*). No such reduced cell survival was evident at the epithelial-mesenchymal interface of the explants treated with GoH3 antibody for 2 d, and some mitotic cells could be seen close to the basement membrane (Fig. 7 *E*). Occasionally, E-cadherin staining was reduced in the innermost area of abnormal epithelial clusters in the GoH3-treated cultures. This may indicate development of the duct lumen. However, we could not see any accumulation of E-cadherin staining, which is characteristic of the luminal plasma membrane surrounding the duct structure.

In explants treated with mAb 200 for 2 d, epithelial cells expressed E-cadherin uniformly over the entire cell surface

(Fig. 8 *A*). The formation of basement membrane in the treated explants was then studied by immunofluorescence for nidogen and laminin-1. In explants treated with mAb 200, nidogen was detected in a continuous fashion along the basal surface of the epithelium of the proximal portion of the poorly branched cluster, but in a discontinuous fashion at the tip of the terminal epithelial clusters (Fig. 8 *B*). A similar lack of laminin-1 staining was seen at the tip of the epithelial cluster in explants treated with mAb 200 (Fig. 8 *C*), whereas no such lack of laminin was apparent in explants treated with mAb GoH3 (Fig. 8 *D*).

Transmission electron microscopy confirmed that treatment with mAb 200 caused a disruption of the basement membrane at the tip of the epithelial cluster (Fig. 9 *A*). Frequent epithelial cytoplasmic protrusions towards the mesenchyme were noted at the basal surface of the epithelium. A similar morphology has been observed in submandibular gland epithelium when the basement membrane was digested by proteolytic enzymes (Kadoya and Yamashina, 1991). Electron microscopy of GoH3-treated explants revealed a smooth contour of epithelial cells adjacent to the



**Figure 9.** Electron micrographs of submandibular gland rudiments treated for 2 d with 100  $\mu\text{g}/\text{ml}$  of mAb 200 (A) or 40  $\mu\text{g}/\text{ml}$  GoH3 (B). No basement membrane can be seen between epithelium and mesenchyme in explants treated with mAb 200, and note epithelial cytoplasmic blebs and projections toward the mesenchyme (A). In contrast, the epithelial base is flat and smooth in explants treated with GoH3, and a continuous basement membrane can be seen adjacent to epithelial cell base (B). Bar, 1  $\mu\text{m}$ .

basement membrane with no sign of basement membrane disruption or the presence of cytoplasmic protrusions towards the mesenchyme (Fig. 9 B).

### Discussion

Branching epithelial morphogenesis is crucial for the development of several organs. Both mesenchymal extracellular matrix proteins such as epimorphin (Hirai et al., 1992), tenascin-C (Chiquet-Ehrismann et al., 1986), and basement membrane components such as laminin-1 and its integrin receptors (Ekblom, 1993) have been implicated in these processes. We therefore studied the role of these proteins during submandibular gland morphogenesis. Both expression studies and antibody perturbation experiments suggest a role for integrin  $\alpha 6 \beta 1$  and laminin-1 in branching epithelial morphogenesis. Monoclonal antibodies against integrin  $\alpha 6$  subunit and laminin-1 E3 domain perturbed branching epithelial morphogenesis in vitro. The blocking antibodies retarded epithelial morphogenesis in distinct fashions. The antibody against E3 domain of laminin-1 blocked basement membrane formation at the tip of the newly forming epithelium, whereas no such effect was seen with the antibody against integrin  $\alpha 6$  subunit. Hence, it seems that the E3 domain is involved in some early stages of basement membrane formation, whereas the  $\alpha 6 \beta 1$  integrin might be involved in signal transduction.

Several control experiments indicated that the antibodies against integrin  $\alpha 6$  subunit and laminin E3 domain altered morphogenesis in specific ways. Antibodies against epimor-

phin, control antibodies against tenascin-C, and nonimmune IgG did not perturb branching epithelial morphogenesis. The absence of tenascin-C at early developmental stages may indicate its stage- or tissue-specific functions. The results with antiepipimorphin antibodies were unexpected. In spite of distinct expression pattern of epimorphin both on days 13 and 17 submandibular mesenchyme, no clear inhibition of branching epithelial morphogenesis was noted with mAb MC-1 against epimorphin. It is possible that the MC-1 concentration required to perturb the function of epimorphin varies from tissues to tissue. Another possibility is that epimorphin is not primarily involved in branching morphogenesis, but in other aspects of epithelial cell development. We measured branching epithelial morphogenesis on days 2 and 3 of submandibular gland organ cultures, whereas Hirai et al. (1992) studied lumen formation on day 4 of lung organ culture. In the presence of MC-1, the epithelium of lung rudiments failed to show polarized distribution of E-cadherin on day 4 of in vitro lung development (Hirai et al., 1992). Epithelial branching morphogenesis and epithelial lumen formation may require distinct mechanisms. It remains to be seen whether epimorphin has a role in lumen formation in the submandibular gland. Although this protein apparently is involved in cell-cell interactions (Hirai, 1994), it might also have other functions. Recently, an alternative function of epimorphin in vesicular transport has been suggested (Pelham, 1993; Hirai et al., 1993).

In another set of control experiments, we found that mAb 200 and GoH3 did not primarily reduce survival of cells. Good survival, growth, and epithelial branching were ob-

tained with synthetic media supplemented with transferrin. Omission of transferrin seriously retarded branching morphogenesis, which we attribute to failure of cells to survive. Both stereomicroscopy and histological analyses showed that explants treated with either mAb 200 or GoH3 did not resemble transferrin-deficient explants. In transferrin-deficient explants, there were clear signs of decreased proliferation at early stages of culture, and the epithelial clusters remained small. In contrast, the epithelium tended to grow into larger clusters, and it appeared that only branching was initially affected in mAb 200- or GoH3-treated explants. Proliferation caused by transferrin and other factors such as EGF and TGF- $\alpha$  (Nogawa and Takahashi, 1991) is important for branching epithelial morphogenesis in the submandibular gland, but it does not seem that the E3 epitopes of laminin or the integrin  $\alpha 6$  subunit are primarily involved in cell proliferation.

Since we suggest a role for the E3 domain of laminin-1, it was important to verify the presence of laminin  $\alpha 1$  chain in the submandibular gland. The E3 domain of laminin-1 is exclusively formed by  $\alpha 1$  chain (Timpl, 1989; Beck et al., 1990), and it is known that the expression of this laminin chain is rather limited in the developing embryo (Cooper and MacQueen, 1983; Klein et al., 1990; Kücherer-Ehret et al., 1990). The  $\beta 1/\gamma 1$  chains of laminin seem to be expressed constitutively in all basement membranes in the submandibular gland throughout pre- and postnatal development, as shown by immunostaining and immunoblotting. In contrast, expression of laminin  $\alpha 1$  chain was more restricted. By RT-PCR, laminin  $\alpha 1$  chain was detected at several stages of submandibular gland development, including the early developmental stages. By immunofluorescence, laminin  $\alpha 1$  chain could be detected clearly only at the early developmental stages. Laminin  $\alpha 1$  chain codistributed with  $\beta 1$  and/or  $\gamma 1$  chains of laminin along the poorly branched epithelial clusters in 13-d-old embryos, suggesting the presence of an intact laminin-1 heterotrimer at this stage. A transient expression of laminin  $\alpha 1$  chain mRNA could also be picked up by *in situ* hybridization. Interestingly, mRNA expression was confined to the epithelium at the tip of the branching epithelium, but in the stalk areas, it was confined to the mesenchyme adjacent to the epithelium. A similar distribution of laminin  $\alpha 1$  chain mRNA was noted in developing lung (Thomas and Dziadek, 1994), whereas a strict epithelial expression was noted in the developing kidney (Ekblom et al., 1990). We emphasize that several observations showed a low expression of laminin  $\alpha 1$  chain also at the early developmental stages in the submandibular gland. We failed to detect laminin  $\alpha 1$  chain by immunoblotting; by *in situ* hybridization, signals were weaker in the submandibular gland than in the kidney, and an antibody with weak affinity for the E3 epitope reacted only faintly in immunofluorescence. It cannot be fully excluded that mAb 200 blocked development by binding to yet unknown laminin chains or proteins with E3-like epitopes. Another possibility is that E3 domains of laminin  $\alpha 1$  chain are developmentally important even when they are present in low amounts.

The data shown here suggest that antibodies against laminin E3 domains perturbed branching epithelial morphogenesis by influencing some step of basement membrane formation. An inhibition of branching morphogenesis was obtained with mAb 200 against E3, and in stereomicroscopy

of live explants, the effect resembled that obtained by the antibody against integrin  $\alpha 6$  subunit. However, electron microscopy and immunofluorescence revealed that only the application of anti-E3 antibody caused a loss of basement membrane at the tips of the branching epithelium, where the rapid remodeling of the basement membranes occurs. Treatment of embryonic submandibular glands with hyaluronidase disrupts the basement membrane and causes a similar delay of branching morphogenesis (Bernfield et al., 1984). Domain E3 has previously been shown to be primarily involved in heparin binding of laminin-1, but not in  $\alpha 6\beta 1$  integrin-mediated cell adhesion to laminin or in laminin self assembly (Yurchenco et al., 1990, 1993; Sung et al., 1993). Our mAb 200 inhibits heparin binding to E3 (Sorokin et al., 1992), and heparin-binding activity of laminin-1 may thus play a role in basement membrane formation. We cannot exclude that mAb 200 acted by interrupting laminin-cell binding. However, only slight inhibition of epithelial branching was obtained with mAb 194, which strongly inhibits E3-cell binding (Sorokin et al., 1992).

The role of a laminin-1-binding integrin in branching morphogenesis was here studied with antibody GoH3. This antibody is known to block interactions between E8 domain of laminin-1 and integrin  $\alpha 6\beta 1$  (Sonnenberg et al., 1990). The observed strong inhibition of submandibular gland morphogenesis with GoH3 suggested that binding of  $\alpha 6\beta 1$  integrin to laminin E8 is important for branching epithelial morphogenesis. This is also suggested by the inhibition of branching by anti- $\beta 1$  integrin monoclonal antibody. These findings do not rule out an involvement of other laminin-binding  $\beta 1$  integrins, such as integrin  $\alpha 7\beta 1$  (von der Mark et al., 1991; Kramer et al., 1991), in this process. We could not see any indications of epithelial detachment from the basement membrane in GoH3 antibody-treated explants. One possible reason for this is the presence of integrin  $\alpha 6\beta 4$  complex in the peripherally located cells of the epithelial clusters. The  $\alpha 6\beta 4$  integrin also seems to bind laminin with yet-undefined domains, and this binding is not inhibited by GoH3 (Sonnenberg et al., 1990). Other possible cell receptors for laminin include dystroglycan (Ervasti and Campbell, 1993), and receptors for other basement membrane components may also be involved in epithelial cell adhesion to basement membranes.

Based on the current study, we cannot exclude that integrins with the  $\alpha 6$  subunit are involved in epithelial differentiation independently of laminin-1. Some integrins are involved in cell-cell interactions rather than cell-matrix interactions. Cell rearrangements in the developing epithelial clusters are important for submandibular gland morphogenesis. In glands from day 13 embryos, only the peripheral cells of the epithelial clusters adhere to the basement membrane. As development advances, the epithelial clusters branch repeatedly, and on day 17 in the embryonic mouse, they form a sheet of acino-tubular structure with a lumen. During the 4-d period, descendants of epithelial cells located in the central part of the cluster separated from the basement membranes should reach the basement membrane (Kadoya and Yamashina, 1989, 1993). Such a rapid epithelial cell rearrangement is a common and essential mechanism for generating epithelial sheets (Gumbiner, 1992), but it has received little attention in previous studies on salivary gland morphogenesis (Spooner and Wessells, 1972; Spooner and

Faubion, 1980; Nogawa and Nakanishi, 1987). The  $\alpha 6$  integrin subunit was expressed in the epithelium of submandibular gland throughout pre- and postnatal development, as shown by immunofluorescence (Kadoya and Yamashina, 1993), indicating that  $\alpha 6$  integrin subunit might be involved also in the cell rearrangements at later developmental stages. Colocalization of  $\alpha 6$  integrin subunit and laminin  $\alpha 1$  chain could no longer be seen at later stages of submandibular epithelial development. It is possible that integrins containing  $\alpha 6$  bind to other laminin isoforms or other ligands in the more mature stages, but this remains to be studied.

Splice variants of  $\alpha 6$  integrin subunit with different intracellular domains have been identified (Tamura et al., 1991; Hogervorst et al., 1991), and they may have distinct functions (Shaw and Mercurio, 1994). Although interesting changes in integrin  $\alpha 6$  splicing pattern occur during preimplantation mouse development and in cell lines in vitro (Hierck et al., 1993), our RT-PCR revealed the presence of the intracellularly different  $\alpha 6A$  and  $\alpha 6B$  variants at all studied embryonic stages of submandibular gland. Both variants have been noted in the adult human salivary gland also (Tamura et al., 1991; Hogervorst et al., 1993), and it is thus unlikely that differential splicing of the intracellular domains controls the function of  $\alpha 6$  integrin subunit during submandibular gland development. Recently, another alternative splice site on extracellular domains of  $\alpha 6$  integrin has been reported (Ziöber et al., 1993). The biological role of this alternative splicing event should be studied further in the future.

The long arm domains E3 and E8 of laminin-1 were previously suggested to be of importance for kidney tubule development, which occurs by conversion of kidney mesenchyme epithelium (Klein et al., 1988; Sorokin et al., 1990), and it was not studied in any detail whether the domains could also be important for branching epithelial morphogenesis. Central cross regions and globular ends of lateral short arms of laminin-1 have been shown to be important for branching epithelial morphogenesis of embryonic lung and kidney (Schuger et al., 1991; Ekblom et al., 1994). Our current study suggests that the long arm domain E3 is involved in branching epithelial morphogenesis of submandibular gland, and that an integrin which can bind to long arm domain E8 also is involved. Domain E8 apparently acts at early developmental stages in the kidney model system also by binding to integrins possessing the  $\alpha 6$  subunit (Sorokin et al., 1990), but it has not been defined how the laminin E3 domain acts in the kidney system. Here, we have provided evidence that domain E3 could be involved in early stages of basement membrane formation. Moreover, we show that two different blocking antibodies perturb epithelial morphogenesis in distinct fashions in one model system. It will now be of great interest to compare the functions of different laminin domains and their receptors in the various systems of branching epithelial morphogenesis.

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