Antibodies against Domain E3 of Laminin-1 and Integrin α6 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 α1 chain and two smaller chains (β1 and γ1). Immunofluorescence revealed a transient expression of laminin α1 chain in the epithelial basement membrane during early stages of branching morphogenesis. Other laminin-1 chains and α6, β1, and β4 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 α1 chain, the E3 domain. An inhibition of branching was also seen with a mAb against the integrin α6 subunit. The antibodies against laminin α1 chain and integrin α6 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 α6 integrin subunit seemed to leave the basement membrane intact. We suggest that the laminin E3 domain is involved in basement membrane formation, whereas α6β1 integrin binding to laminin-1 may elicit differentiation signals to the epithelial cells.

IN 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.

Lamins are large glycoprotein complexes of basement membranes (Engel, 1993). The first described laminin was previously called EHS (Engelbreth-Holm-Swarm)-laminin with the chains αe, Blε, and B2ε (Timpl, 1989; Tryggvason, 1993), but it is now called laminin-1 with the chains α1, β1, and γ1 (Burgeon 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 α6β1 (Hall et al., 1990), integrin αβ1 (von der Mark et al., 1991; Kramer et al., 1991), and possibly also by integrin α3β1 (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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cells was perturbed by application of antibodies against domain E3 and E8 (Klein et al., 1988; Sorokin et al., 1992). An antibody against integrin α6 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 α6β1 (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 (Schügerl 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 (Bernfeld et al., 1984; Ogawa and Takahashi, 1991; Takahashi and Ogawa, 1991). We recently described that both laminin (Kadoya and Yamashina, 1989, 1991) and integrin α6 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 α6 integrins, as well as the mesenchymal proteins tenasin-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 α6β1 in the expected locations, but no tenasin-C was found during early stages of morphogenesis. We therefore tested whether blocking antibodies against epimorphin, laminin-1, or integrin α6β1 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 α6 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 tenasin-C (MTn 15; Außerheide and Ekblom, 1988) were purified from serum-free hybridoma culture media by Bacteroid ABx column (J. T. Baker, Inc., Phillipsburg, NJ; Außerheide 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 α6 integrin subunit (Sommerberg et al., 1990; Sorokin et al., 1990), was purchased from Immunotech (Mar- seille, France; lot No. 10) and Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands, lot No. 1566-08-00). Antiepimorphin mAb (MC-1) (Hirai et al., 1992), polyclonal anti-β1 integrin antibodies (Güllberg et al., 1989), anti-β4 integrin serum (SP 90395; Dr. P. C. Marchisio, Torino University, Torino, Italy), Fab fragment of antiuvomorulin/E-cadherin rabbit antibody (Vestweber and Kemler, 1985), and purified anti-β1 integrin monoclonal antibody (9EG7; Lenter et al., 1993) were gifts from these authors. Rat anti-mouse tenasin-C mAb (MTn12; Außerheide and Ekblom, 1988), polyclonal antiamnin antibodies (ISH-1; Klein et al., 1988), and antinidogen antibodies (Paulsson et al., 1988; 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 × 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 Nucleopore filters (pore size = 0.05 μm) 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 μg/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 μg/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 μm) were fixed with acetone for 2 min, treated with 5% BSA in PBS for 20 min, and incubated with the following antibodies: GoH3 (2 μg/ml), mAb 200 (20 μg/ml), mAb 198 (10 μg/ml), anti-β1 integrin (4 μg/ml), anti-β4 integrin (5 μg/ml), and anti-E-cadherin (6.5 μg/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 (Axioskop; 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-μm-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 α1 chain, 5'-GCCCTTCCTTTAATTGATATCTCTGACCTGTCAGCCATGCTACATC-3', complementary to position 8452-8501, which encodes part of the domain E3 (Sasaki et al., 1988), was analyzed against the Entrez data base (Release 60; 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 (Durbè et al., 1993) with the following modification: after hybridization with 32P-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.
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 according to the manufacturer's instructions. Briefly, the RT reaction was carried out with 1 μg 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 α6 integrin subunit) or 35 (for laminin α1 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 α1 chain, we used sense primer 5'-ACCTGGTGCTCCCTCTGATC-3' (position 8243-8263) and antisense primer 5'-ATGGGCTTGCGTTGCTTGGTGG-3' (position 9021-9041) corresponding to a part of domain E3 sequence (Sasaki et al., 1988), and for α6 integrin subunit, we used sense primer 5'-GTCAGGCTGCTGAACATGACG-3' (position 2975-2995) and antisense primer 5'-CTGGGAAAATAAGGGCGGC-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 α1 Chain in the Developing Submandibular Gland

By immunoblotting with polyclonal antiserum against laminin-1 reacting equally well with 400-kD α1 chain and 200-kD β1 and/or γ1 chains of EHS tumor material (Fig. 1), we failed to detect any 400-kD α1 chain at any developmental stage that was analyzed. Only 200-kD bands apparently representing laminin β1 or γ1 chains were seen under reducing conditions (Fig. 1). However, an expression of laminin α1 chain in submandibular gland from various stages was noted by RT-PCR analysis (Fig. 2 A). Two splice variants of integrin α6 subunit with different intracellular domains have been detected (Hogervorst et al., 1991, 1993; Cooper et al., 1991). We detected expression of both the α6A and α6B 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 α1 chain was either not expressed or expressed at low levels not detectable by the the immunoblotting procedure. Since the mRNA could be detected, we used immunofluorescence to further investigate whether the laminin α1 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 α1 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 ducts are found. No laminin α1 chain was detected by immunofluorescence at this stage in the basement membrane of the terminal tubules, but weak expression of laminin α1 chain was seen in the basement membrane of the duct epithelium (Fig. 3 A). In contrast, polyclonal anti-laminin-1 antibodies detecting α1, β1, and γ1 chains stained the basement membrane of all parts of the gland epithelium (Fig. 3 B). Staining for laminin α1 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% CaCl2, 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 α1 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 α1 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 antisem 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 α1 chain during early development is shown by Figure 2 A.
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 α6β1 and α6β4 integrins (Sonnenberg et al., 1990). In 13-d-old embryos, the integrin α6 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 β1 subunit was expressed in the same locations as the α6 subunit, but it was also expressed by the mesenchyme surrounding the epithelium (Fig. 4 D). In contrast, the β4 integrin subunit was expressed only weakly in the basal surface of epithelial cells adjacent to the basement membrane. No integrin β4 subunit expression was seen on cells located in the center of the epithelial clusters (Fig. 4 E). In 17-d-old embryos, α6 and β4 integrin subunits were expressed by epithelial cells, but not by mesenchymal cells, whereas the integrin β1 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 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 α1 Chain mRNA during Development of Submandibular Gland**

In small submandibular glands from day 13 embryos with only rudimentary epithelial branches, laminin α1 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 α1 chain (A), laminin-1 (B), α6 integrin (C), β1 integrin (D), β4 integrin (E), and epimorphin (F) in the submandibular gland of day 13 embryos. (A) Laminin α1 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 α6 integrin subunit is found in both submandibular epithelium and capillary endothelium, but not in the mesenchyme. (D) β1 integrin is expressed by both the epithelium and mesenchyme. Note that mesenchymal cells close to the epithelium show stronger staining. (E) β4 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 μm.

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.
Stages of Submandibular Morphogenesis in a Defined Medium

The function of epimorphin, laminin, and α6 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 μg/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 μg/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-α6 integrin, mAb GoH3 (40 μg/ml) branching morphogenesis was also perturbed, and the degree of inhibition varied from severe (E) to moderate (F). In explants treated with anti-β1 integrin (9EG7, 100 μg/ml), inhibition of epithelial branching occurred (G). (H) Explants treated with anti-tenascin-C mAb MTn 15 at 10 μg/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‡ |
|------------------------------|---------------|-----------------|---------------------|
| Anti-laminin E3, No. 200     | 100 µg/ml     | 2               | 10                  | 2  3  0  15 |
| Anti-laminin E3, No. 200     | 50 µg/ml      | 2               | 1                   | 4  0  0  5  |
| Anti-laminin E3, No. 200     | 25 µg/ml      | 2               | 0                   | 0  3  1  5  |
| Anti-laminin E3, No. 194     | 100 µg/ml     | 2               | 0                   | 0  4  2  6  |
| Anti-integrin α6, GoH3       | 40 µg/ml      | 2               | 6                   | 7  1  0  14 |
| Anti-integrin α6, GoH3       | 20 µg/ml      | 2               | 5                   | 0  0  0  5  |
| Anti-integrin α6, GoH3       | 10 µg/ml      | 2               | 0                   | 0  4  2  6  |
| Anti-integrin β1, 9EG7       | 100 µg/ml     | 2               | 0                   | 4  5  0  9  |
| Anti-tenascin-C, MTn15       | 100 µg/ml     | 2               | 0                   | 5  4  9  9  |
| Anti-epimorphin, MC-1        | 10%§          | 2               | 0                   | 1  9  0  10 |
| Anti-epimorphin, MC-1        | 10%§          | 3               | 0                   | 0  0  8  8  |
| DME/F12                      | 10%§          | 2               | 0                   | 1  7  1  9  |
| DME/F12                      | 10%§          | 3               | 0                   | 0  1  8  9  |
| Normal rat IgG               | 100 µg/ml     | 2               | 0                   | 0  1  9  10 |
| Normal rat IgG               | 40 µg/ml      | 2               | 0                   | 0  0  5  5  |
| Normal rabbit IgG            | 100 µg/ml     | 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.
‡ Extent of reduced epithelial branching morphogenesis was scored as severe inhibition (----), moderate inhibition (---), delay of branching (+), and advanced development (+++). See text for details.
§ Percent of volume added to medium.
¶ 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 15 cases (Table I), or it was partially inhibited (Fig. 6 D) in 2 out 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 1.
Figure 7. Histology of control explants, explants grown without transferrin or in the presence of mAb GoH3 and transferrin. Toluidine blue-stained 1-μm 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 μg/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-α6 integrin mAb GoH3 (40 μg/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-α6 integrin mAb-treated explants (E, arrows). Bars, 100 μm in A, C, and E, and 50 μm in B, D, and F.

Distinct Biological Effects of Antibodies against Laminin E3 Domain and Integrin α6 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 explants 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.
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
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 α6β1 and laminin-1 in branching epithelial morphogenesis. Monoclonal antibodies against integrin α6 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 α6 subunit. Hence, it seems that the E3 domain is involved in some early stages of basement membrane formation, whereas the α6β1 integrin might be involved in signal transduction.

Several control experiments indicated that the antibodies against integrin α6 subunit and laminin E3 domain altered morphogenesis in specific ways. Antibodies against epimorphin, 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 antiepimorphin 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-

![Figure 9](image_url)
The epithelium, but in the stalk areas, it was confined to the mesen-
chymal regions. By immunofluorescence, laminin α1 chain expression was noted in the developing kidney (Ekblom et al., 1990). Other possible cell receptors for laminin chains include dystroglycan (Ervasti and Campbell, 1993), and integrins, such as integrin α6β1 (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 α6β4 complex in the peripherally located cells of the epithelial clusters. The α6β4 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 α6 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...
Differential splicing of the intracellular domains controls the function of α6 integrin subunit during submandibular gland development. It is possible that integrins containing α6 bind to other laminin isoforms or other ligands in the more mature stages, but this remains to be studied.

Splice variants of α6 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 α6 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 α6A and α6B 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 α6 integrin subunit during submandibular gland development. Recently, another alternative splice site on extracellular domains of α6 integrin has been reported (Ziober 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; Ekbom 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 α6 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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