Cells That Emerge from Embryonic Explants Produce Fibers of Type IV Collagen

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ABSTRACT Double immunofluorescence staining experiments designed to examine the synthesis and deposition of collagen types I and IV in cultured explants of embryonic mouse lung revealed the presence of connective tissue-like fibers that were immunoreactive with anti-type IV collagen antibodies. This observation is contrary to the widely accepted belief that type IV collagen is found only in sheetlike arrangements beneath epithelia or as a sheathlike layer enveloping bundles of nerve or muscle cells. The extracellular matrix produced by cells that migrate from embryonic mouse lung rudiments in vitro was examined by double indirect immunofluorescence microscopy. Affinity-purified monospecific polyclonal antibodies were used to examine cells after growth on glass or native collagen substrata. The data show that embryonic mesenchymal cells can produce organized fibers of type IV collagen that are not contained within a basement membrane, and that embryonic epithelial cells deposit fibers and strands of type IV collagen beneath their basal surface when grown on glass; however, when grown on a rat tail collagen substratum the epithelial cells produce a fine meshwork. To our knowledge this work represents the first report that type IV collagen can be organized by cells into a fibrous extracellular matrix that is not a basement membrane.

The basement membrane is a histological structure found subjacent to all epithelia and surrounding bundles of muscle fibers and nerve cells (Vracko, 1982). A hallmark of basement membranes is that they separate epithelia, muscle, or nerve from the adjacent connective tissue or mesenchyme. Type IV collagen is a major structural component of basement membranes (Timpl et al., 1982).

The other general class of extracellular matrix is associated with mesenchymal connective tissue, which contains predominately collagen types I and III, although the latter is usually less abundant (Eyre, 1980). These two collagen types form the scaffold of fibrous connective tissue found in bone, teeth, dermis, tendon, ligament, and ocular tissue. In addition, collagen types I and III form the fibrous stroma of all visceral organs, blood vessels, peripheral nerves, skeletal muscles, and the fibrous skeleton of the heart (Ham and Cormack, 1979; Eyre, 1980; Bornstein and Sage, 1980). These two general kinds of extracellular matrices are usually considered immutable.

In the present study cells that emerge from embryonic mouse lung explants were examined by immunofluorescence microscopy after detergent permeabilization. The predominant fibrous collagen we observed under these conditions was immunoreactive type IV.

Examination of mesenchymal cell populations showed aligned fibers of type IV collagen. In fact, the heaviest concentrations of type IV collagen fibers were always observed in fields of axially aligned spindle shaped cells. The presence of aligned type IV collagen fibers in regions of mesenchymal outgrowth is consistent with the studies of Harris and his colleagues, who examined the interaction of fibroblasts with type I collagen fibers. The term tractional structuring was used by these workers to describe the process by which the cells aligned the collagen (Harris et al., 1981; Stopack and Harris, 1982). Our data suggest that tractional structuring of type IV collagen occurs as cells emerge from embryonic explants.

The data also show that emergent embryonic epithelial cells produce type IV collagen fibers with a very wide variety of sizes beneath their basal surface when cultured on glass. Contrary to this, epithelial cells resting upon native rat tail collagen (RTC) substrata produce a uniform coherent meshwork of type IV collagen that is probably analogous to its organization within basement membranes.

To our knowledge the present work is the first report that...
type IV collagen fibers exist within the extracellular matrix produced by mesenchymal connective tissue cells. This observation suggests that during embryogenesis the collagens of basement membranes and of connective tissue extracellular matrices may not be as highly segregated as has been believed. In this paper we discuss the possible role of type IV collagen as a structural element in embryonic connective tissues.

MATERIALS AND METHODS

Organ and Cell Culture: Midgestation (12-14 d postcoitum) mouse lungs were removed under sterile conditions, minced into 1-mm² pieces, and placed on glass coverslips that were untreated or coated with native RTC. Explants were cultured without antibiotics in sodium pyruvate-free, high-glucose Dulbecco's modified Eagle's medium (Gibco Laboratories Inc., Grand Island, NY). The medium was supplemented with 10% fetal bovine serum and 50 μg/ml of ascorbic acid (both from Gibco Laboratories Inc.).

Preparation of RTC Substrata: Rat tail tendons were dissected free and extracted overnight in 0.1 M acetic acid at 4°C with vigorous stirring. The solution was cleared of debris by centrifugation (10,000 g, 30 min) then made 1.7 M in NaCl. The resulting precipitate was collected by centrifugation, redissolved in 0.1 M acetic acid, dialyzed exhaustively against deionized water, and lyophilized. This solubilization/preparation cycle was repeated three times. Prior to electrophoresis the RTC was dissolved at 1 mg/ml in 0.1 M acetic acid, dialyzed into a high ionic strength phosphate buffer (0.127 M K2HPO4 plus 0.019 M KH2PO4, pH 7.6), and cleared by centrifugation (20,000 g, 30 min). This solution could be kept for months by the addition of 0.01% sodium azide. 35 μl RTC solution on ice was pipetted onto glass coverslips (18 × 18 mm, No. 1, Corning Medical and Scientific, Medfield, MA) and quickly spread with a Teflon policeman. The coverslips were then incubated for 30 min at 37°C in 100% humidity, gently washed with distilled water, and air-dried. The RTC-coated coverslips were sterilized with ultraviolet light.

Purification and Characterization of Antigens: Type I collagen was prepared according to Miller and Rhodes (1982) using differential salt (NaCl) precipitation of rabbit skin extracts. Type IV collagen was purified from the EHS (Englebreth-Holm swarm) mouse tumors according to Orkin et al. (1979). Whole lung explants were extracted with a buffer (1.0 M NaCl), then analyzed by immunoprecipitation. Some samples were previously treated with purified bacterial collagenase. Lane 3, control immunoreaction obtained with 20 μg rabbit nonimmune IgG. Lane 4, immunoprecipitate obtained with 20 μg guinea pig anti-type I collagen antibody. Lane 4 shows the immunoprecipitate obtained with the rabbit anti-type I collagen antibody.

Immunobinding Procedures: Indirect double immunofluorescence microscopy was accomplished as described by Little and Chen (1982), except that the antigen sample was previously treated with purified bacterial collagenase and made permeable by incubation for 30 min 0.4% Triton X-100. In Fig. 3a the explant was fixed as above, incubated in 0.1 M acetic acid for 2 h at 4°C, and then washed with phosphate-buffered saline before immunolabeling. Rhamidine-conjugated affinity-purified goat anti-guinea pig IgG and fluorescein-conjugated goat anti-rabbit IgG were prepared in this laboratory as described by Little and Chen (1982). In brief, this includes extraction of [3H]proline-radioiodinated lung explants with a neutral pH high ionic strength phosphate buffer (0.127 M K2HPO4 plus 0.019 M KH2PO4, pH 7.6, containing 1.0 M NaCl). Radiiodinated polypeptides were dialed into Tris-buffered saline (0.05 M Tris/HCl, pH 7.6; 0.15 M NaCl), then analyzed by immunoprecipitation. Some samples were incubated in bacterial collagenase (purified in this laboratory and used according to Peterkofsky and Diegelmann, 1971) before immunoprecipitation. The method of Laemmli (1970) was used to separate immunoprecipitated polypeptides by SDS PAGE.

Immunotransblots analysis was modified slightly from the method of Towbin et al. (1979). Whole lung explants were extracted with a buffer (1.0 M NaCl) in 0.05 M Tris/HCl, pH 7.6, dialyzed into Tris buffered saline, then subjected to SDS PAGE before electroblotting onto nitrocellulose paper.

Microscopy and Photography: Specimens were observed with a Leitz Ortholux epifluorescence microscope equipped with a Leitz Vario-Orthomat camera using Leitz Fluotar 16/0.45, 40/0.75, and 100/1.32, or Zeiss Plano-63/1.4 objectives. Kodak Tri-X film was developed to 1,600 ASA with Dafine developer. In all paired combinations of immunofluorescent photographic observations (rhodamine/fluorescein) the exposure time was the same for both images (exposure time ranged from 2 to 1 ½ s for various experiments).

RESULTS

Antibodies were evaluated by immunoprecipitation of collagen types I and IV from radiolabeled extracts of embryonic mouse heart and lung explants. Cultures were incubated for 2 d in 50 μCi/ml of [3H]proline. The yield of counts immunoprecipitable by guinea pig anti-rabbit type I collagen antibodies was ~10%, and the yield for rabbit anti-mouse type IV collagen was ~1.5%. Recovery analysis showed that 85–90% of all [3H]proline counts could be accounted for during the immunoprecipitation procedure.

Immunoprecipitates were examined for specificity by fluorography of a 6% SDS-polyacrylamide slab gels. In Fig. 1A, lane 1 shows the bands containing the α1 and α2 chains characteristic of type I collagen. Lane 4 shows the immunoprecipitate obtained with the rabbit anti-mouse type IV collagen antibodies. The banding pattern shows the high molecular weight forms of this protein at 180–160 kD, and bands at 110 and 70 kD that are characteristic of type IV collagen preparations electrophoresed under reducing conditions (Sage et al., 1979). The absence of α2 chains in lane 4 proves that no detectable type I collagen was immunoprecipitated by rabbit anti-mouse type IV collagen. Lanes 2 and 5 show that
all bands in lanes 1 and 4 are sensitive to digestion with purified bacterial collagenase that is purified free of general protease activity. Therefore, all bands in both lanes 1 and 4 are collagenous and are not fragments of a noncollagenous polypeptide such as fibronectin or laminin. These data show that the antibody preparations specifically precipitate their respective antigens with no cross-reactivity.

Immunotransblot (Western blot) analysis was also used to evaluate antibody specificity. Mouse lung explants were scraped from a tissue culture dish and stirred overnight in a buffer (0.05 M Tris/HCl, pH 7.6; 1.0 M NaCl). The cellular debris was removed by centrifugation, and the resulting extract was dialyzed into Tris-buffered saline. Aliquots of this crude embryonic lung culture extract were mixed with SDS sample buffer and processed for immunotransblot analysis. Staining of 5% polyacrylamide slab gels and the associated nitrocellulose paper showed considerable bands throughout the entire electrophoretic path, which suggested that the crude extracts contained a complex mixture of polypeptides (not shown). Duplicate lanes of nitrocellulose were probed with rabbit anti-mouse type IV collagen antibodies or guinea pig anti-rabbit type I collagen antibodies. The results are shown in Fig. 1 B. Immunoblot analysis with anti-type I collagen antibody revealed several reactive bands (Fig. 1 B, lane 1). Prominent bands include a doublet at ~280 kD, a doublet at ~190 kD, and a band at 100 kD. When an identical strip of nitrocellulose was incubated with anti-type IV collagen a much simpler pattern emerged. A band at ~180 kD represents the only immunoreactive material present in the crude cellular extract. Other lanes, not shown, included controls similar to that in Fig. 1 B, lane 1. A band at ~180 kD represents the only immunoreactive material present in the crude cellular extract. Other lanes, not shown, included controls similar to that in Fig. 1 A: that is, nonimmune antibodies were added, and crude cell extracts were treated with bacterial collagenase. The collagenase treatment removed all immunoreactive material. Incubation with nonimmune IgG did not result in the appearance of any visible bands. The banding pattern in Fig. 1 B, lane 1 is different from that in Fig. 1 B, lane 2. These results strongly support two conclusions: the two antibodies recognize different antigens within the transblotted crude extract, and the immunoreactive material is collagenous.

Immunoprecipitation of native collagens from solution, and recognition of collagenase-sensitive material present in transblotted crude cell extracts show that the two immunoreagents specifically recognize two different collagens. Moreover, the evidence shows that the collagens recognized by the rabbit and guinea pig antibodies are type IV and type I, respectively.

The following series of experiments takes advantage of the fact that cells will migrate out of an embryonic lung explant and colonize the surrounding substratum. Fig. 2 A shows a culture prepared such that cells emerging from the lung explant colonized both an RTC layer and glass. The assembly state of type IV collagen differed dramatically depending upon on which of the two substrata the epithelial cells found themselves. The arrow in Fig. 2 designates the edge of the RTC substratum, which is visible by differential interference contrast (DIC) optics.

The immunofluorescent image of type IV collagen (Fig. 2 A) shows that cells initially in contact with glass produce bundles and fibers of type IV collagen between their basal surface and the glass. Cells in contact with the fibrillar RTC substratum produce a delicate meshwork of type IV collagen beneath their basal surface (Fig. 2 A, IV). The Type I collagen immunofluorescent image is sparse and consists mainly of intracellular staining (Fig. 2 A, I).

Emergent epithelial cells on RTC are shown in Fig. 2 B at a higher magnification (by DIC). The corresponding immunofluorescence image shows fine filaments of type IV collagen arranged in a planar meshwork (Fig. 2 B, IV). Juxtanuclear type IV collagen immunofluorescence is evident at a more apical focal plane (Fig. 2 B, IV). Type I collagen immunofluorescence shows some punctate deposits at the level of the substratum, which is in the same focal plane as the meshwork of type IV collagen. Juxtanuclear focus of type I collagen staining are present at a more apical level; this material is slightly out of focus.

Cells with mesenchymal morphology were also examined (Fig. 3). An explant of midgestation lung mesenchyme is shown after 4 d of culture (Fig. 3 A). The low magnification phase-contrast micrograph shows that after the explant attached, cells migrated out and the explant became flattened. The explant was incubated in 0.1 M acetic acid after fixation in order to enhance the immunoreactivity of Type I collagen (Linsenmayer et al., 1983). The immunofluorescent type IV collagen image shows an extensive distribution of fibers throughout the mass of mesenchymal cells. The immunolabeled type I collagen also shows fibers, which for the most part are not completely co-distributed with the type IV fibers. The edge of another lung explant is shown in Fig. 3 B (by DIC). The substratum was covered by both epithelium- and mesenchymelike cells. The emergent mesenchymelike cells produced fibers of type IV collagen that are continuations of type IV fibers within the explant proper (Fig. 3 B, IV). Type I collagen immunostaining shows perinuclear crescents of fluorescence (Fig. 3 B, I).

Mesenchymal outgrowths after 9 d on glass showed linear arrays of intensely stained fibrous type IV collagen (Fig. 4 A). Type I collagen immunostaining showed subcellular planar deposits and juxtanuclear staining.

Under certain conditions we could detect extracellular Type I collagen without incubation in acetic acid before fixation. We observed extracellular deposits of type I collagen in older cultures, in which fields of epithelial cells appeared to have assumed a mesenchymal morphology, engaged in motile activity, and begun tractional structuring. The extracellular type I collagen in the regions of tractional structuring partially co-distributed with fibers of type IV collagen fibrils. Fig. 4 B shows such a field of cells after 9 d of growth on RTC.

DISCUSSION

These data provide direct evidence that embryonic cells can produce fibrous assemblies of type IV collagen. Indeed, mesenchymelike cells form conspicuous connective tissue arrays of type IV collagen in vitro.

The spatial pattern of type IV collagen deposited by epitheliumlike cells was influenced by the type of substratum upon which the cells rested. When the cells rested on glass, fields of epithelial outgrowth produced relatively thick fibrous bundles. When grown on fibrillar RTC surfaces, the epithelial cells produced a fine meshwork distributed in a continuous sheet immediately beneath the basal cell surface. Presumably, the sheet of type IV collagen corresponds to the basement membrane seen in histological section.

Deposition of type IV collagen by mesenchymal cells was not influenced by glass or RTC substrata. When embryonic
Figure 2  Detergent-permeabilized cells. The micrographs are arranged as vertical panels with a DIC or phase-contrast image on top, the corresponding anti-type IV collagen (IV) image in the middle, and the anti-type I collagen image (I) at the bottom. Each panel shows a paired combination of double-immunolabeled specimens. This first set of micrographs shows an outgrowth of epithelial cells from a midgestation (12-14 d) embryonic mouse lung. The epithelial cells were examined by double immunofluorescence microscopy after 9 d of culture on glass or RTC. (A) A field after 9 d of culture in which some epithelial cells are on RTC and some on glass. The DIC image is focused on the substratum. The arrow indicates the edge of the RTC layer. The appearance of type IV collagen depends upon whether the cells were originally resting upon glass or RTC. The type I collagen immunofluorescence shows subdued fluorescence in plaques and punctate deposits with a detectable degree of immunoreactivity to the RTC. It is possible that some mouse type I collagen associated with the RTC, which then resulted in immunolabeling. Bar, 50 μm. × 450. (B) Epithelial cells grown on a fibrillar rat tail collagen substratum (RTC) shown at high magnification. The DIC image shows a regular epithelial morphology. Notice that some fine RTC fibrils can be distinguished by DIC optics. The type IV immunofluorescent image (IV) shows a delicate uniform meshwork at one focal plane and intracellular fluorescence at a different plane (not in focus). The type I collagen image (I) shows juxtanuclear fluorescence and planar deposits. Bar, 25 μm. × 720.
FIGURE 3  Detergent-permeabilized cells. This set of micrographs, arranged as described in the legend to Fig. 2, shows midgestation (12–14 d) embryonic mouse lung mesenchymal outgrowth. (A) Lung mesenchyme 4 days after placement into culture. The phase-contrast image shows a mound of mesenchyme out of which many cells have emerged. The type IV collagen immunofluorescent image shows abundant fine fibers of type IV collagen (IV). The type I collagen image (I) shows fibers that are longer, less abundant, and distributed in a pattern different than the type IV collagen fibers. Bar, 100 μm. × 200. (B) The edge of an explant after 2 d of outgrowth in vitro. An emergent substratum layer of epithelium covered by cells with a mesenchymal morphology are shown (by DIC). The fibers of type IV collagen (IV) produced by the mesenchymelike cells are continuous with fibers within the explant proper. The type I collagen image (I) shows juxtanuclear crescents of immunofluorescence. Bar, 50 μm. × 400.

cells initially contact a glass surface they produce bright punctate deposits or plaques (planar deposits) of immunofluorescent collagen types I and IV. These deposits appear to lie between the glass and the “ventral” cell surface, as determined from careful focusing with 100× oil emersion objectives. We believe that the plaques may be secretory deposits of nonfibrillar procollagen/collagen that arise as an artifact of growth on planar (glass or plastic) substrata. This possibility is supported by two unpublished observations: first, nonpermeabilized embryonic cell layers incubated in dilute acetic acid show...
FIGURE 4 Detergent-permeabilized cells. This set of micrographs, arranged as described in the legend to Fig. 2, shows embryonic mouse lung mesenchymal outgrowth after 9 d in culture. (A) The DIC image shows a dense overlapping layer of partially aligned cells. Anti-type IV collagen immunostaining (IV) shows bright, axially aligned fibers. The type I collagen immunofluorescent image shows planar substratum deposits with no observable fibers. Bar, 100 μm. × 200. (B) Mesenchymal outgrowth from embryonic lung after 9 d of culture. This is an example of a mesenchymal–epithelial transition region. The cells were apparently engaged in tractional structuring and motile activity. Multiple cell layers and morphologies are present (some out of the focal plane), as are visible strands of the extracellular matrix (by DIC). The type IV collagen immunofluorescence image shows abundant axially aligned fibers (IV). The type I collagen image shows a similar distribution except that extracellular antigen is not present in well-defined fibers (I). Deeper layers of cells display immunoreactive plaques and punctate deposits of collagen types I and IV. Bar, 100 μm. × 160.

The strikingly different immunofluorescent images of type I collagen in Figs. 3A and 4A are consistent with earlier studies that showed that considerable immunoreactive fibrous type I collagen is present in embryonic chicken fibroblast cultures when frozen sections are examined. However, these diminished plaquelike immunostaining; and second, procollagen and collagen are present in the acetic acid wash of the nonpermeabilized cells.

I collagen in Figs. 3A and 4A are consistent with earlier studies that showed that considerable immunoreactive fibrous type I collagen is present in embryonic chicken fibroblast cultures when frozen sections are examined. However, these
same extracellular fibers are immunologically cryptic when identical cultures are examined in the form of permeabilized whole mounts (Little and Chen, 1982). The image in Fig. 3A shows that incubation in acetic acid renders the cryptic type I collagen fibers immunoreactive.

As the cultures mature, mesenchymal cells from overlapping outgrowths in which individual cells are separated by extensive extracellular matrix material. Streams of uniformly spindle-shaped cells deposit fine interwoven hairlike fibers of type IV collagen aligned in the direction of cell migration (Fig. 4A). In contrast to these overtly mesenchymal cells, other cell populations with a mesenchymal morphology seemed to be derived from populations of epithelial cells, since transitional morphologies were apparent. The type IV collagen produced by transitionally mesenchymal cells appeared to be composed of type IV collagen networks gathered into bundles (Fig. 4B).

Some embryonic epithelial cells must be transiently motile, since epithelial cells are usually the pioneer cells that first inhabit the substratum around an explant. Consistent with this possibility is the work of Greenberg and Hay (1982), who demonstrated that embryonic epithelial cells under appropriate conditions of growth can assume a mesenchymal morphology and motile activity. The nature of cellular outgrowth from embryonic explants precludes the possibility of stating unequivocally whether a given group of cells will remain mesenchymal or epithelial. The fields of cells engaged in tractive restructuring, such as in Fig 4B, exemplify this point.

If fibers of type IV collagen are an integral component of embryonic mesenchyme, what role might these fibers play in connective tissue morphogenesis? Our observations (Figs. 3B and 4B) show that some cellular outgrowths that contain type IV collagen fibers resemble regions of tractive restructuring, as described by Harris et al. (1981) and Stopack and Harris (1982). Thus, organized fields of cells seemed to exert tractive forces on type IV and type I collagen fibers, just as the chick heart fibroblasts isolated by Harris et al. (1981) exert tractive forces on RTC. It is important to note that in areas of tractive restructuring, fields of axially aligned cells always displayed similarly aligned fibers of type IV collagen.

This study raises the possibility that type IV collagen fibers exist in embryos. This suggestion is especially intriguing when considered in light of the work by Schnieke et al. (1983) and Lohler et al. (1984). These investigators showed that a mouse strain deficient in type I collagen production, due to the insertion of a retrovirus into the Type I gene, can proceed successfully through early embryogenesis but dies between the days 12 and 14 of gestation.

We suggest that type IV collagen may form an important part of the fibrous extracellular matrix in early mouse embryos. Furthermore, we speculate that the structure of early connective tissues is in part dependent upon this protein. Later in development when tensile strength is important type I collagen may be required for successful development (see Lohler et al., 1984). In view of this speculation it is interesting that the work of Merlino et al. (1983) shows that gene expression for type I collagen is at very low levels until day 5 of chick development. It is also worth pointing out that embryonic mouse lung explants are not the only embryonic tissues in which we observed fibers of type IV collagen. We consistently observed type IV fibers in heart and skin explants from rabbit embryos and also in cultures of differentiated mouse teratocarcinoma.

The present study shows that type IV collagen is a fibrous component of embryonic connective tissues (mesenchyme) as well as basement membranes in vitro. Therefore, we suggest that type IV collagen may be an important constituent of early mesenchymal extracellular matrices in vivo and that fibers of type IV collagen may contribute to the structural integrity of mesenchyme during early morphogenesis.

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