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RAPID COMMUNICATION

Avian Feather Morphogenesis: Fibronectin-Containing Anchor Filaments

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ABSTRACT During feather morphogenesis anchor filaments, continuous with the basement membrane, extend deep into the dermis. We find that these anchor filaments give intense fluorescence with antifibronectin (FN) antibody, using indirect immunofluorescence and immunoelectron microscopic techniques. These filaments are abundant in the dermis of the developing feather germ, yet are not present in the dermis beneath the epidermis of the elevated feather primordia. We suggest that these FN-containing anchor filaments function in establishing the epidermal placodes of feathers and/or the organization of the feather dermis.

The role of extracellular matrices in morphogenetic and differentiative events is of great interest to developmental biologists. The ability of fibronectin (FN) to bind extracellular matrix components such as the collagens (Engvall et al., '78; Gold and Pearlstein, '80), some of the glycosaminoglycans (Ruoslahti and Engvall, '80; Yamada et al., '80), and other FN molecules (Yamada et al., '77), as well as to bind to cell surfaces establishes FN as an adhesive glycoprotein. The distribution of FN can be related to particular stages of morphogenesis and differentiation in several systems (Kurkinen et al., '79; Thesleff et al., '81; Wartiovaara et al., '76).

Anchor filaments are an intriguing structural characteristic of the feather-forming skin in chick embryos. The prominence of these fibrous processes in the early feather germ and absence from the elevated feather germ have led investigators (Wessells, '65; Kallman et al., '67; Kischer and Keeter, '71) to suggest that they are associated with some aspect of feather morphogenesis. However, the ability to propose a more specific role for them has been limited by the lack of information concerning their biochemical nature.

We have now determined that FN is a component of these dermal anchor filaments and may, in fact, constitute the filaments entirely. That the spatial arrangement of feathers is controlled by the dermis (Linsenmayer, '72; Novel, '73), suggests that these FN-containing anchor filaments, being organized by the dermis, may somehow function in establishing the sites of epidermal placode formation. Additionally, the abilities of fibronectin to interact with the surfaces of mesenchymal cells and their extracellular matrix components, in conjunction with the temporal and spatial distribution of anchor filaments, also suggest the possibility that these fibronectin-containing filaments may function in organizing the dermis during feather morphogenesis.

MATERIALS AND METHODS

Fertile chicken eggs were obtained from a commercial White Leghorn stock. Eggs were incubated in a Favorite incubator at 37°C and 70% relative humidity. Embryos were staged according to Hamburger and Hamilton ('51). Anterior backskin and underlying tissue were excised from embryos of Stages 28-36 (6-10 days of incubation) for indirect immunofluorescence. Anterior backskin was dissected free of underlying tissue for electron microscopy.

Goat anti-chick cellular FN antibody was a generous gift of Dr. Kenneth Yamada (Yamada, '78).

Indirect immunofluorescence

Tissues were processed according to the technique of Sainte-Marie ('62). Following fixation in cold 95% ethanol and dehydration in

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100% ethanol at 4°C, tissues were cleared in cold toluene and embedded in paraffin at 56-57°C. Sections were deparaffinized in cold toluene and rehydrated in cold phosphate-buffered saline (PBS). They were incubated in anti-FN antiserum for 30 min at 37°C, rinsed three times in PBS, and incubated in rabbit anti-goat fluorescein-conjugated antibody (Miles) for 30 min at 37°C. Controls included use of preimmune serum, incubation with only the fluorescein-conjugated antibody, and omission of both antibody incubation steps. Sections were viewed with a Reichert UV microscope with a mercury vapor light source and an FITC-interference filter combination.

Immunoelectron microscopy

Tissues were excised in Tyrode's balanced salts solution and incubated at 37°C for 40 min in goat anti-chick antiserum at a 1:30 dilution in Tyrode's solution. They were then rinsed five times in Hanks' balanced salts solution and incubated with ferritin-conjugated rabbit anti-goat antibody (1.0% IgG in sterile solution of PBS, Miles) at a 1:10 dilution in Tyrode's solution for 40 min at 37°C, and rinsed again in Hanks' solution. Controls were done using either preimmune serum or Hanks' balanced salts solution. The tissues were then fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer and 0.1 M sucrose for 1 hr, and postfixed in 2% $0s0_4$ in 0.1 M cacodylate buffer for 1 hr. Following dehydration in a graded alcohol series, tissues were embedded in Spurr's resin and sectioned at 60-80 nm. Sections were stained with lead citrate for 2 min, or with bismuth subnitrate for ferritin enhancement (Ainsworth and Karnovsky, '71), for 45-60 min. Sections were viewed on a JEOL-JEM 100B transmission electron microscope.

RESULTS

Indirect immunofluorescence demonstrated that fibronectin is distributed throughout the basement membrane and dermis of anterior backskin at all stages studied. No fluorescence was seen in the overlying epidermis. All controls were negative. Most significantly, we found that anchor filaments are intensely fluorescent with monospecific anti-FN antibody. Invariably, these FN-positive filaments extend into the dermis from the basement membrane underlying basal epidermal cells which themselves appear stretched in the direction of the dermis.

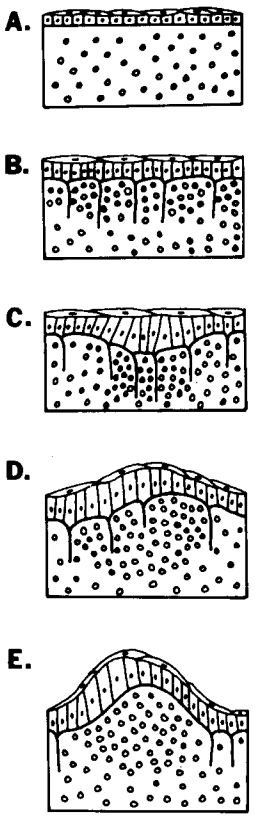
The stages of feather development have been previously described (Wessells, '65). A schematic representation of the stages of feather development depicting the distribution of FN-positive anchor filaments is shown in Text-figure 1. FN-positive anchor filaments are first seen in preplacode backskin during stages of dense dermis formation where they extend for lengths of up to 77 μm into the dermis. They are abundant beneath the epidermal placodes of the feather germs at the dermal condensation stages (Fig. 1), although some anchor filaments are also present between the feather placodes. Anchor filaments are no longer present in the elevated feather germ, but they still may be seen in adjacent tissue (Fig. 2).

At the ultrastructural level, anchor filaments are seen as bundles of fine filaments as previously described by Kallman et al. ('67). These bundles merge with the lamina densa of the basal lamina complex (Fig. 3). Again, the anchor filaments invariably extend from the basal lamina beneath epidermal cells which protrude into the underlying dermis. No distinct axial periodicity was evident in the filament bundles. The individual filaments which make up the bundle ranged from 11 to 19 nm in diameter. Many fibroblasts make close contact with the anchor filaments, and several fibroblasts, abutted against the bundle, were oriented with their long axes parallel to the anchor filament bundle. Immunoferritin electron microscopy confirms that FN is intimately associated with these anchor filaments, since ferritin is specifically distributed along the length of the anchor filaments, both at the periphery and within the bundle (Fig. 4). Controls showed ferritin labeling to be specific for FN.

DISCUSSION

Anchor filaments in the dermis may establish the pattern of epidermal placodes. In fact, tissue recombination experiments have demonstrated that the information for placode pattern formation resides in the dermis (Linsenmayer, '72; Novel, '73), which is most likely the site of origin for the FN-positive anchor filaments (Kischer and Keeter, '71).

The appearance of anchor filaments prior to as well as during dense dermis formation suggests that they may participate in the establishment of this dense dermis, which characterizes early feather-forming regions of the skin. Formation of this dense dermis involves cell migration from the underlying dermatome



Text-Fig. 1. Schematic representation of feather morphogenesis. A. Pre-Stage 29: A cuboidal epithelium overlies the loose mesenchyme of dorsal skin prior to feather development. B. Stage 29: A dense dermis forms beneath the columnar epithelial cells in feather-forming skin. Anchor filaments are present at intervals along the basement membrane. C. Stage 30: Epidermal placodes are present and dermal condensations subsequently form beneath them. Anchor filaments are abundant in the dermis. D. Stage 33: The feather primordium begins to elevate. E. Stage 35: The feather has elevated well above the skin surface. Anchor filaments are not present in the feather.

(Mauger, '72). FN-containing anchor filaments may function by binding presumptive dermal fibroblasts, by acting as a substratum for cell migration, and/or as a chemotactic agent for presumptive dermal fibroblasts. FN has been shown to have such biological activities. For example, cells in suspension attach preferentially to surfaces coated with FN (Grinnell and Feld, '79). Also, an increased mobility of both normal and transformed cells upon addition of fibronectin has been demonstrated (Ali and Hynes, '78), as well as an ability to promote directional migration of fibroblasts (Postlethwaite et al., '81).

It is also conceivable that anchor filaments may participate in the formation of discrete dermal condensations beneath the epidermal placodes. There is some evidence that increased mitosis is involved in formation of dermal condensations (Wessells, '65), but cell migration is probably the primary cause (Stuart et al., '72). Here also, FN may be involved in chemotaxis and fibroblast migration, as well as cell-cell and cell-substratum adhesion. Interestingly, we find that fibroblasts are oriented along the anchor filaments during stages of dermal condensation formation, suggestive that cells may use the filaments as a substratum for migration.

Anchor filaments may also participate in the establishment of the fibrous lattice. Oriented cells occur between the dermal condensations, and fibers (believed to be collagen) are oriented in a similar pattern (Stuart and Moscona, '67). It has been suggested that this fibrous lattice is responsible for organizing the dermal condensations by providing the structural framework upon which cells migrate (Stuart and Moscona, '67; Stuart et al., '72). Perhaps anchor filaments direct the organization of the fibers by virtue of FN's ability to bind collagen.

It is perhaps the disappearance of these anchor filaments that permits differential growth of the feather germ. Kischer and Keeter ('71) suggested that increased mitotic activity may be localized to areas of the skin where anchor filaments are not present.

And finally, anchor filaments may actually consist of bundles of FN filaments. Fibrils of similar diameter, identified immunologically as FN, have been found on the surfaces of cells in culture (Furcht et al., '78; Singer, '79). FN has the capacity to become cross-linked by formation of intermolecular disulfide bonds (Hynes and Destree, '77; Keski-Oja, et al., '77; Yamada et al., '77), and by being a specific substrate for transglutaminase (Keski-Oja et al., '76). Cell-surface FN is in fact often found to exist as multimers (Hynes and Destree, '77) and is a known fibrillar constituent of loose connective tissues.

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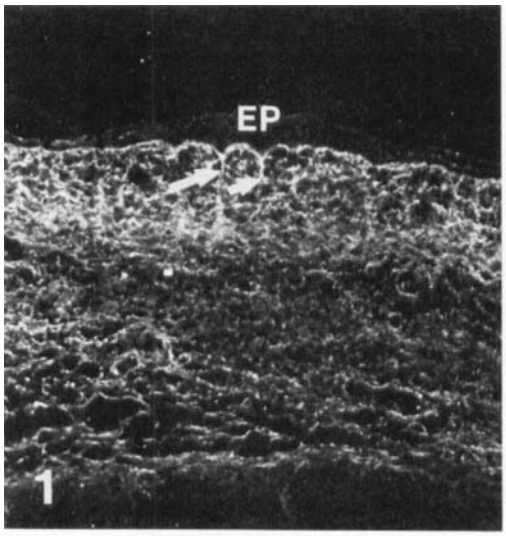
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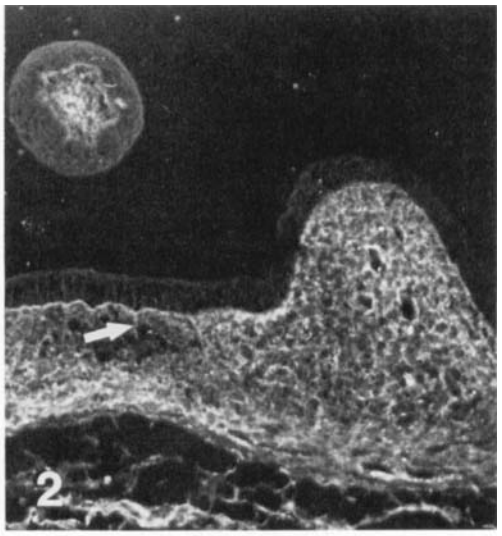
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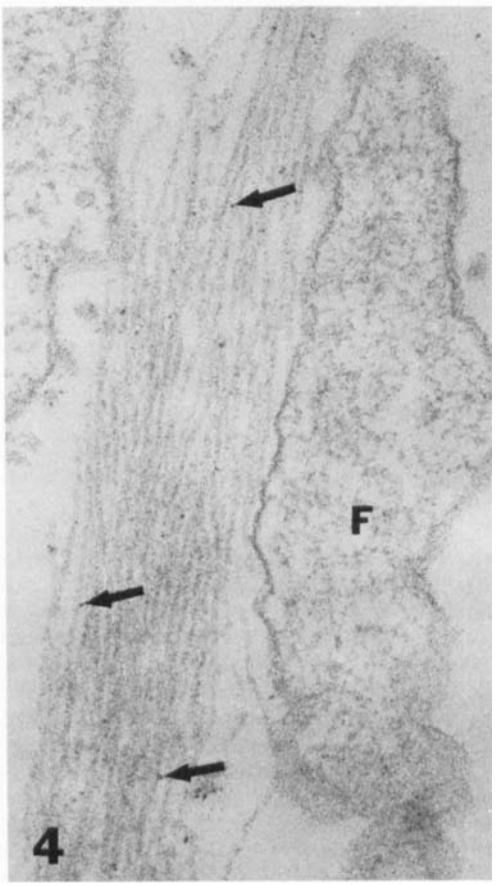
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Fig. 1. Anti-FN fluorescence photomicrograph of an epidermal placode/dermal condensation stage (Stage 32) feather germ. Anchor filaments (arrows) are evident beneath the epidermal placode (EP) (×210).

Fig. 2. Anti-FN fluorescence photomicrograph of an elevated feather germ (Stage 35). Anchor filaments are not present in the dermis of the feather germ but can be seen in the adjacent skin (arrow) (×210).

Fig. 3. Transmission electron micrograph of a Stage 32 backskin labeled with ferritin. The anchor filament (large arrow) is a bundle of fine filaments which merges with the lamina densa (LD) underlying an epidermal cell which protrudes into the dermis. Fibroblasts (F) are closely associated with the anchor filament bundle. Ferritin (small arrows) identifies FN in the anchor filament (×40,300).

Fig. 4. Transmission electron micrograph of an anchor filament bundle found among cells of a dermal condensation in Stage 31 backskin. Ferritin identifies FN in the anchor filament (arrows). A fibroblast (F) is oriented parallel to the bundle (×63, 333).