The accumulation of basement membrane components during the onset of chondrogenesis and myogenesis in the chick wing bud

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Summary

The study describes the distribution of several basement membrane molecules in the embryonic chick wing bud from stages 23 to 26, during the onset of myogenesis and chondrogenesis, and then later at stage 28. Laminin is localized as early as stage 23, prior to the onset of myogenesis, in regions corresponding to the position of the future dorsal and ventral myogenic areas. Other matrix components, including fibronectin, do not differentially accumulate in these same regions. Fibronectin, basement membrane heparan sulphate proteoglycan and type IV collagen are more widespread in their distribution than laminin, and are even present between mesenchymal cells. These results suggest a role for laminin in the initial differentiation of the muscle masses and emphasize that components of basement membrane can also be associated with mesenchymal cells.

Key words: laminin, type IV collagen, myogenesis, limb bud.

Introduction

The relationship between a number of extracellular matrix molecules (ECM) and muscle differentiation has been described in some detail during late stages of muscle formation (see Mayne & Sanderson, 1985, for review). Kieny & Mauger (1984) have provided such a study for the chick embryo starting at 7-5 days of incubation. Muscle fibres are sheathed by a basement membrane containing type IV collagen, laminin and heparan sulphate proteoglycan. Collagens I, III and V and fibronectin approach the basement membrane.

At stages prior to and during myogenesis, in situ studies have been less extensive. More details are available for chondrogenesis. Dessau et al. (1980) reported that fibronectin and type I collagen appear uniform in the limb mesenchyme at stage 23 but increase slightly in the prechondrogenic core at stage 24. Starting at stage 24 the presumptive dorsal and ventral myogenic regions show reduced immunofluorescence for fibronectin (Chiquet et al. 1981; Kosher et al. 1982; Tomasek et al. 1982) and a decrease in hyaluronic acid (Singley & Solursh, 1981). Both of these matrix molecules inhibit myogenesis in vitro (Podleski et al. 1979; Kujawa & Tepperman, 1983). As far as the collagens are concerned, Shellswell et al. (1980) found that, even as late as stage 27 when myogenesis is well under way, there is a paucity of immunologically detectable collagens I, III and V in the muscle-forming regions.

The in situ distributions of basement membrane components such as laminin, type IV collagen and basement membrane heparan sulphate proteoglycan in the limb bud prior to and during the onset of myogenesis and chondrogenesis have not yet been reported. The temporal and spatial appearance of these molecules might be important in the regulation of the onset of myogenesis, in particular. In vitro studies have shown that laminin promotes myoblast proliferation (von der Mark & Kuhl, 1985), migration (Ocalan et al. 1988) and differentiation (Foster et al. 1987). Occupation of integrin by extracellular matrix appears to be a prerequisite for myogenesis, since it is reversibly inhibited in the presence of CSAT, an antibody to integrin (Menko & Boettinger, 1987). Thus, it is of some interest to relate the appearance of laminin, in particular, to the onset of myogenesis.

Materials and methods

Frozen 10 μm cross-sections of wing buds from white Leghorn chick embryos from stages 22 to 28 (Hamburger & Hamilton, 1951), embedded in OCT compound, were cut
on a cryostat and treated with acetone at $-20^\circ\text{C}$ for 2 min before being stored at $-20^\circ\text{C}$. Storage time at $-20^\circ\text{C}$ for a few days to several weeks did not alter the results. Sections stained as described earlier (Solursh et al. 1982) were rehydrated in phosphate-buffered saline (PBS), incubated with primary antibody, washed three times in PBS and incubated with fluorescein-conjugated rabbit anti-mouse IgG followed by fluorescein-conjugated goat anti-rabbit IgG for monoclonal antibodies and fluorescein-tagged goat anti-rabbit IgG for polyclonal antibodies. All fluorescein-conjugated antibodies (Cappel) were diluted 1:300. Hybridoma supernatant (undiluted) or ascites (diluted 1:100) were mostly obtained through the Developmental Studies Hybridoma Bank, maintained by a contract from NICHD (NO1-HD-6-2915). These include antibodies to muscle myosin (MF-20, Bader et al. 1982), fibronectin (Gardner & Fambrough, 1983), laminin and heparan sulphate proteoglycan (Bayne et al. 1984). Monoclonal antibodies to type IV collagen were kindly provided by Dr T. F. Linsenmayer (Fitch et al. 1982). A mixture of supernatants from three different hybridomas was used in this later case. They recognize three different, characterized epitopes, as described earlier (Mayne et al. 1984), and together serve as a polyclonal antibody. A polyclonal antibody (1/100 dilution) to laminin (Collaborative Research) was used for comparison. Results were similar with monoclonal and polyclonal antibodies to laminin. Immunofluorescence micrographs were taken on a Leitz microscope with Tri-X Kodak film. In some cases for each antigen, sections were pretreated with testicular hyaluronidase [1 mg ml$^{-1}$ HSE (Worthington) in PBS] for 1 h at room temperature. Results were identical to those obtained without hyaluronidase pretreatment.

Results

Frozen cross-sections were prepared from wing buds at several stages of development. Particular emphasis was given to comparing stage 24, before the onset of myoblast fusion (Hilfer et al. 1973), or the presence of immunologically detectable muscle myosin (Swalla & Solursh, 1986), or chondrogenesis (Dessau et al. 1980), with stage 26, during early stages of overt myogenesis and chondrogenesis. At stage 24 (Figs 1A and 2A,B) laminin is detected in the subectodermal basement membrane. In addition, dorsal and ventral regions can be seen to exhibit a punctate, positive fluorescence for laminin. Fainter, but similar, regions

![Fig. 1. Fluorescence micrographs of cross-sections through the stage-24 wing bud, all cut at the level of the presumptive humerus. (A) Stained with anti-laminin. Besides the bright ectoderm and its basement membrane note the presence of dorsal and ventral regions indicated by a punctate fluorescence (arrows). (B) Stained with antibody to basement membrane heparan sulphate proteoglycan. Besides the subectodermal basement membrane, there is antigen throughout the limb mesenchyme. There is no particular localization in the premuscle regions, however. (C) Stained with anti-type IV collagen. Note the widespread distribution of the antigen. It appears relatively reduced in the avascular peripheral mesenchyme except for the dorsal and ventral premyogenic regions. The ectodermal basement membrane (arrow) does not appear to be extensively immunoreactive. (D) Stained with anti-fibronectin. While the antigen is widespread there is a slight relative reduction in fluorescence in subectodermal regions including the presumptive dorsal and ventral muscle regions (arrows). (Bar, 100 $\mu$m.)](image-url)
could also be observed in the proximal part of the wing bud at stage 23, but not earlier (not shown). At this stage, basement membrane heparan sulphate proteoglycan is widespread throughout the limb mesenchyme and in the subectodermal basement membrane (Figs 1B and 2C,D). There is no particular

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**Fig. 2.** Higher magnification phase-contrast (A,C,E) and fluorescence micrographs (B,D,F) from those shown in Fig. 1. (A,B) Stained with anti-laminin. The ectoderm and especially the subectodermal basal lamina stain brightly. Punctate staining is visible among the mesenchymal cells. (C,D) Stained with anti-basement membrane heparan sulphate proteoglycan. There is immunoreactive material in the subectodermal basement membrane and throughout the mesenchyme, including the peripheral avascular zone. (E,F) Stained with anti-type IV collagen antibodies. Note the punctate staining in the mesenchyme with a relative paucity of immunoreactive material in the subectodermal mesenchyme and occasional indications of staining in the subectodermal basement membrane (arrow). (Bar, 100 μm.)
Fig. 3. Fluorescence micrograph of a cross-section cut at the level of the developing radius and ulna through a stage-26 wing bud stained for muscle myosin. Note the presence of muscle myosin in the dorsal and ventral myogenic regions. Chondrogenic elements can also be detected at this time. (Bar, 100 μm.)

At stage 26, muscle myosin can be detected in dorsal and ventral muscle masses (Fig. 3). Chondrogenesis is also well under way (Dessau et al. 1980). At this same time, the localization of laminin to the muscle regions is more apparent than at the earlier stages (Fig. 4A). Similarly, basement membrane heparan sulphate proteoglycan is detected in the muscle-forming regions and the subectodermal base-

enrichment in the premuscle regions, in contrast to laminin. Type IV collagen (Fig. 1C) also has a widespread distribution (Fig. 2E,F), including the presumptive myogenic regions. Surprisingly, type IV collagen is only faintly detectable at the light microscopic level in the subectodermal basement membrane and in other areas in the subectodermal mesenchyme. Fibronectin is widespread at stage 24 including the subectodermal basement membrane (Fig. 1D) but is decreased relatively in the dorsal and ventral, presumptive myogenic regions, as reported earlier by Kosher et al. (1982).

Fig. 4. Fluorescence micrographs of cross-sections through a stage-26 wing bud, all cut at the level of the developing radius and ulna. (A) Stained with anti-laminin. Note that besides the bright ectoderm and the subectodermal basement membrane, there is a punctate fluorescence in the regions of the dorsal and ventral myogenic areas (arrows). (B) Stained with antibody to basement membrane heparan sulphate proteoglycan. The subectodermal basement membrane and dorsal and ventral myogenic masses are positive for the antigen. There are also some areas throughout the mesoderm, including the skeletal elements, which are positive. (C) Stained with antibodies directed against type IV collagen. There is a punctate fluorescence in the dorsal and ventral myogenic regions but other areas around the cartilage elements exhibit a brighter fluorescence. Antigen is just detectable in some regions of the subectodermal basement membrane. (D) Stained with anti-fibronectin. The dorsal and ventral myogenic regions show a relatively reduced fluorescence for fibronectin compared to other regions. (Bar, 100 μm.)
ment membrane (Figs 4B and 5C,D). There are also some regions around the skeletal elements which are positive for the antigen. Type IV collagen, another component of basement membranes, is detected in just some locations of the subectodermal basement membrane (Fig. 5E,F). As at stage 24, it is detectable

Fig. 5. Higher magnification phase-contrast (A,C,E) and fluorescence (B,D,F) micrographs from Fig. 4 of stage-26 wing buds. (A,B) Stained with anti-laminin. The ectoderm and subectodermal basement membrane are immunoreactive. Like stage 24, there is also punctate staining in the subectodermal avascular mesenchyme. (C,D) Stained with antibody to basement membrane heparan sulphate proteoglycan. There is staining in the subectodermal basement membrane and among the mesenchymal cells. (E,F) Stained with antibodies to type IV collagen. There is more staining in the subectodermal basement membrane (arrow) than at stage 24. The subectodermal mesenchyme shows less immunoreactivity than the more internal mesenchyme. (Bar, 100 μm.)
in the two muscle regions (Fig. 4C), but is more intensely fluorescent in the areas that surround the muscle masses and skeletal elements. It is also apparent that staining for fibronectin is still reduced in the muscle-forming areas relative to other parts of the limb bud (Fig. 4D).

At stage 28, laminin has a similar distribution to that at earlier stages. It is present in the subectodermal basement membrane and in the dorsal and ventral myogenic regions (Fig. 6A). By this stage, basement membrane heparan sulphate proteoglycan has become relatively more localized to the dorsal and ventral myogenic regions (Fig. 6B) and subectodermal basement membrane. However, it is still detectable in the perichondrium and soft connective tissues. Type IV collagen has a similar distribution as at stage 26 (Fig. 6C), being present in the subectodermal basement membrane, dorsal and ventral muscle masses and in soft connective tissues.

Discussion

This study demonstrates the localization of laminin in the subectodermal basement membrane and in the regions of the presumptive dorsal and ventral myogenic regions prior to overt myogenesis at stage 26 in the wing bud. Type IV collagen is also present at this early stage but has a more widespread distribution, including the premyogenic areas. Unexpectedly, it is relatively less detectable in the subectodermal basement membrane or subjacent mesenchyme than in the central regions of the limb bud. On the other hand, heparan sulphate proteoglycan, another basement membrane component, is detected throughout the limb mesenchyme as well as in the subectodermal basement membrane and subjacent mesenchyme. It is possible that some epitopes could be masked, preventing the early detection of type IV collagen in the basement membrane. However, pretreatment with testicular hyaluronidase has no effect on the results. It is clear that a typical, intact basal lamina is already well developed even at the presumptive stages of the limb bud (Jurand, 1965; Berczy, 1966;
Smith et al. 1975; Kaprio, 1977). However, light microscopic localization of the basement membrane need not reflect localization at the ultrastructural level (e.g. Martins-Green & Erickson, 1987). Nevertheless, this apparent asynchrony in the accumulation of three major basement membrane components warrants further investigation, particularly at the ultrastructural and biosynthetic levels. With the onset of myogenesis, the intensity of laminin staining increases, as does that for basement membrane heparan sulphate proteoglycan and type IV collagen. As discussed earlier, some other ECM components like fibronectin and collagen types I, III and V are expressed at relatively low levels in the early myogenic regions.

This early localization of laminin to the premyogenic areas might be of significance in relation to the formation of the muscle masses. Myoblasts do synthesize laminin in vitro (Kuhl et al. 1982; Olwin & Hall, 1985). It is most interesting that a laminin substratum enhances myoblast adhesion (Kuhl et al. 1986) and promotes myoblast proliferation (von der Mark & Kuhl, 1985) and migration (Ocalan et al. 1988). Foster et al. (1987) have shown that rat skeletal myoblasts become responsive in terms of increased proliferation and differentiation to a laminin substratum at a particular stage in development. The present results are consistent with a role for laminin in regulating the proliferation, migration and formation of the early muscle masses in situ, with the development of cell responsiveness playing a major role.

The brachial nerve begins to grow into the wing bud at stage 24 (Roncalli, 1970; Bennett et al. 1980). Two branches form, which grow directly into the dorsal and ventral muscle masses. It is noteworthy that laminin substrata promote axon elongation (Baron-van Evercooren et al. 1982; Faivre-Bauman et al. 1984; Manthorpe et al. 1983; Rogers et al. 1983). Whether laminin plays a role in vivo in muscle innervation is not known. However, Lewis et al. (1981) have shown that at least the major nerve branches form in muscleless wings. It still needs to be determined whether the myogenic cells, the associated connective tissues or both are responsible for the early accumulation of laminin in the premyogenic regions. As suggested by Tomaselii et al. (1986), multiple neuronal interactions might be involved in neurite outgrowth. Additional experimental studies are clearly needed to clarify the role of laminin in myogenesis in situ.

Basement membranes are an extracellular matrix that separates epithelia from associated stroma (Timpl et al. 1982). The appearance of basement membrane components, such as laminin, basement membrane heparan sulphate proteoglycan, type IV collagen and fibronectin, has often been used to study the formation of epithelia from mesenchyme (e.g. Ekbloom, 1981; Thesleff et al. 1979; O'Shea, 1987). In this light, it is unexpected to find basement membrane heparan sulphate proteoglycan and type IV collagen associated with limb bud cells other than ectoderm, vascular endothelia and skeletal muscle.

The basal lamina components within the mesenchymal compartment are not likely to be derived entirely from epithelial sources, including vascular endothelia. Ultrastructural studies indicate that basal lamina are not present around capillaries in the limb bud until much later in development (Drushel et al. 1985). The distribution of these ECM components shows no relationship to the vascular pattern and heparan sulphate proteoglycan is even found in the peripheral limb mesenchyme, which is known to be avascular (Feinberg et al. 1983). On the other hand, type IV collagen and laminin appear relatively reduced in the subectodermal mesenchyme compared to the central core region.

The widespread distribution of basement membrane molecules, such as basement membrane heparan sulphate proteoglycan and type IV collagen, suggests that under some circumstances they are not specific to basement membranes but are components of other extracellular matrices. In support of this suggestion, Chen & Little (1985) have shown that embryonic mesenchyme cells can produce organized fibres of type IV collagen, which are not contained in basement membranes. Furthermore, the lack of a constant codistribution of some of these basement membrane molecules supports the idea that their synthesis and accumulation are not tightly coregulated.

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References


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