**Elastin exhibits a distinctive temporal and spatial pattern of distribution in the developing chick limb in association with the establishment of the cartilaginous skeleton**

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†The authors dedicate this paper to the memory of Professor Michael Solursh, who died when this paper was in the course of publication

**SUMMARY**

In this work we have analyzed the presence of elastic components in the extracellular matrices of the developing chick leg bud. The distributions of elastin and fibrillin were studied immunohistochemically in whole-mount preparations using confocal laser microscopy. The association of these constituents of the elastic matrix with other components of the extracellular matrix was also studied, using several additional antibodies. Our results reveal the transient presence of an elastin-rich scaffold of extracellular matrix fibrillar material in association with the establishment of the cartilaginous skeleton of the leg bud. The scaffold consisted of elastin-positive fibers extending from the ectodermal surface of the limb to the central cartilage-forming regions and between adjacent cartilages. Fibrillin immunolabeling was negative in this fibrillar scaffold while other components of the extracellular matrix including: tenascin, laminin and collagens type I, type III and type VI; appeared codistributed with elastin in some regions of the scaffold. Progressive changes in the spatial pattern of distribution of the elastin-positive scaffold were detected in explant cultures in which one expects a modification in the mechanical stresses of the tissues related to growth.

A scaffold of elastin comparable to that found in vivo was also observed in high-density micromass cultures of isolated limb mesodermal cells. In this case the elastic fibers are observed filling the spaces located between the cartilaginous nodules. The fibers become reoriented and attach to the ectodermal basal surface when an ectodermal fragment is located at the top of the growing micromass. Our results suggest that the formation of the cartilaginous skeleton of the limb involves the segregation of the undifferentiated limb mesenchyme into chondrogenic and elastogenic cell lineages. Further, a role for the elastic fiber scaffold in coordinating the size and the spatial location of the cartilaginous skeletal elements within the limb bud is also suggested from our observations.

Key words: limb development, chick embryo, chondrogenesis, extracellular matrix, elastin, fibrillin, collagen, morphogenesis

**INTRODUCTION**

Mechanical forces have often been proposed as molding factors in morphogenesis. In motile organs, such as the developing heart, possible morphogenetic mechanical stress is a consequence of the organ’s function. In other cases morphogenetic mechanical forces are more obscure and were only deduced on the basis of changes in the shape of the cellular and tissue components of the embryonic organs. In these cases the cells appear to modify the surrounding extracellular matrix by mechanical traction causing a spatial realignment in the matrix, which in turn modifies the behavior of the cells; such interactions may provide positive feedback, which may be involved in complex shaping processes (Harris et al., 1981; Stopak and Harris, 1982).

Different types of molding mechanical effects have been demonstrated to be responsible for events during limb morphogenesis. Extrinsic mechanical stress originating from muscle contraction plays a significant role in joint cavitation (Mitrovic, 1982) and prevents degeneration of the long autopodial tendons (Kieny and Chevallier, 1979; Brand et al., 1985), but both joints and long tendons can be formed initially in the absence of muscle activity (Mitrovic, 1982; Shellswell and Wolpert, 1977; Kieny and Chevallier, 1979). Fine locally generated mechanical stress has also been proposed to be responsible for limb morphogenetic events (Rooney et al., 1984). The mechano-chemical theory proposed by Oster et al. (1985) and Oster and Murray (1989) takes into account the generation of tension by condensing mesenchymal cells and its subsequent transmission to the extracellular matrix as a
primary factor accounting for the morphogenesis of the limb skeleton. Experimental support for this hypothesis is scarce, but Stopak and Harris (1982) have clearly shown a molding effect of the growing cells on the surrounding extracellular matrix under in vitro conditions. Further, these authors (Stopak et al., 1985) have also found that labeled collagen injected into the limb bud is rearranged by traction of the limb mesenchymal cells. Whether this traction is also exerted in the matrix elaborated within the limb cannot be deduced from these experiments but the demonstration of extracellular matrices with tense properties (see Foster, 1982; Mecham and Heuser, 1991; Pasquali-Ronchetti, et al., 1993; Rosenbloom et al., 1993) would be of great interest and would help to confirm the role of tension in normal development.

Elastic fibers are major components of the ECM in adult vertebrate organs and tissues subjected to mechanical tension, such as skin, lungs, blood vessels and ligaments (see Pasquali-Ronchetti et al., 1993). At the ultrastructural level elastic fibers consist of amorphous tracts surrounded by small fibrils, 10-12 nm in diameter, usually termed ‘elastin-associate microfibrils’ (Cleary and Gibson, 1983). Elastin is the major component of the amorphous material and consists of a highly insoluble protein with a characteristic rubber-like elasticity (Gray et al., 1973; Sage and Gray, 1979). Several glycoproteins have been identified in association with the microfibrillar component of the elastic fibers, including; fibrillin (Sakai et al., 1986), MAGP (microfibril-associated glycoprotein; Gibson et al., 1990), and emilin (elastic microfibril interface-located protein; Bressan et al., 1993). In general, little is known about the function of most of the elastin-associated glycoproteins, and all of them have been shown to have a wider distribution than that of elastin. The identification of alterations in elastin-associated glycoproteins in genetic diseases affecting the elastic properties of the tissues, such as the Marfan syndrome (Dietz et al., 1991; Kainulainen et al., 1992; Ramirez et al., 1993), suggests an important role for at least some of these components in the elastic functions. The study of the distribution of elastin and associated glycoproteins in new locations during embryonic development may provide important insights into, and help to clarify the significance of, the molecular complexity of the elastic matrices. However, information on the distribution of the elastic matrix components in embryonic tissues is scarce (Rosenquist et al., 1988; Foster et al., 1989; Gallagher et al., 1993; Holzenberger et al., 1993) and elastin is often thought to be a component produced in later stages of the fetal period (Clearly et al., 1967; Parks et al., 1988).

In this work we have analyzed the presence of elastic components in the extracellular matrices of the developing chick leg bud in vivo and in vitro. Elastin and fibrillin were detected immunohistochemically with monoclonal antibodies in whole-mount preparations using confocal laser microscopy. The association of these proteins with other components of the extracellular matrix was also studied with a variety of monoclonal antibodies to components of the extracellular matrix. Our results reveal the transient presence of an elastin-rich scaffold of extracellular matrix fibrillar material in association with the establishment of the cartilaginous skeleton of the leg bud. The most prominent components of this matrix scaffold are elastin fibers, extending from the ectodermal basal surface to the central chondrogenic region of the limb bud and between adjacent developing cartilages. A comparable scaffold is also formed in micromass cultures of isolated limb mesenchymal cells between the developing chondrogenic nodules. The scaffold underwent progressive changes in its spatial distribution in explant cultures in which one expects a change in the mechanical stresses of the tissues that is related to growth. A role for the elastic fiber scaffold in coordinating the size and the spatial location of the cartilaginous skeleton within the limb bud is suggested from our observations.

MATERIALS AND METHODS

Materials

White Leghorn chick embryos ranging from stage 22 to 35 (Hamburger and Hamilton, 1951) were used in the present study.

Immunohistochemistry

The leg buds were dissected free and fixed at 4°C for up to 8 hours in methanol plus 20% dimethyl sulfoxide (DMSO). The specimens were then washed in Tris-buffered saline (TBS) and digested for 30 (1-3 hours) minutes in 1600 units/ml of testicular hyaluronidase (Sigma). After a prolonged rinse in TBS they were immersed for 30 minutes in 10% goat serum in 10 mM glycine and transferred to PBS containing the primary antibody for 6 hours (undiluted hybridoma supernatant or purified antibodies diluted in PBS at a concentration of 10µg/ml). The specimens were washed again for 3-5 hours in TBS, incubated overnight in fluorescein-conjugated goat anti-mouse IgG (Cappel) diluted at 1:300, washed thoroughly in TBS and attached with Cell-Tack (Becton Dickinson Labware, Bedford, MA) to excavated slides. After dehydration in methanol the cavity of the slide was filled with a 2:1 (v/v) mixture of benzyl benzoate:benzyl alcohol and sealed with a coverglass attached to the slide by means of nail-polish. In some cases, after labeling with the second antibody the limbs were carefully dissected under the microscope and attached to the slides as described above. Controls omitting the first antibody were also made.

Elastic components of the ECM were detected by monoclonal antibodies to elastin and to fibrillin (see below). Extracellular matrix components associated with the elastic matrix were studied with antibodies obtained from the Developmental Studies Hybridoma Bank, including monoclonal antibodies to: tenascin (M1-B4, from D. M. Fambrough), laminin (31, from D. M. Fambrough), collagen type II (II-IIIb3, from T. F. Linsenmayer), collagen type III (3b2, from R. Mayne), and collagen type VI (39, from D. M. Fambrough). Monoclonal antibodies to collagens type I (Linsenmayer et al., 1979) type IV (Fitch et al., 1982) and type X (Schmid and Linsenmayer, 1985) were obtained from Dr T. F. Linsenmayer.

The specimens were examined in a Bio-Rad MRC-600 (Bio-Rad Laboratories, Richmond, CA) scanning confocal microscope equipped with a krypton-argon laser.

Antibodies to elastic matrix components

The monoclonal antibody for elastin (mAb 10B8) was produced by immunizing Balb/c mice (Jackson Laboratories) with preparations of chick tropoelastin from 17-day-old lathyric chick embryo aorta, extracted and purified according to established methods (Rich and Foster, 1982). The immunization schedule, fusion protocol and cloning of hybridomas were performed as described previously (Sakai et al., 1982). Hybridomas were screened by immunofluorescence using sections of chick aorta. mAb 10B8 was selected and shown to be specific for elastin by immunoblotting of purified chick tropoelastin and by immunoprecipitation of [3H]valine-chick tropoelastin from extracts of chick aorta organ cultures (see Fig. 1). We found that the epitope recognized by mAb 10B8 is conserved in bovine and human tissues, since it reacts with tissues from all three species. A
commercially available monoclonal antibody specific for bovine elastin (mAb BA-4) (Sigma), which crossreacts with human but not with chicken elastin, was used as a control.

The specificity of the fibrillin monoclonal antibody (mAb 201) has been described (Sakai et al., 1986). While the immunizing antigen was human, mAb 201 recognizes chick fibrillin (Gallagher et al., 1993).

For these experiments, mAb 10B8 and mAb 201 were purified from cell culture medium using a Protein G/Sepharose column (Pharmacia). The antibody was eluted from the column with 0.1 M glycine/HCl (pH 2.5), neutralized, and dialyzed against phosphate buffered saline (PBS). The antibodies were typically stored at concentrations of 1-2 mg/ml.

**Immunoblotting**

Immunoblotting of elastin obtained from cultures of human aortic smooth muscle cells (Clonetics) was performed as described previously (Sakai et al., 1986).

For immunoprecipitation, aortae from 17-day-old chicks were dissected and incubated overnight in Dulbecco’s modified Eagle’s medium (made without valine) with 50 µCi/ml [3H]valine (Amersham). The aortae were then extracted in 50 mM Tris-HCl, 0.5 M NaCl, pH 7.5, in the presence of protease inhibitors (3 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). The extract was clarified by centrifugation and the supernatant was applied to a 10B8 antibody affinity column, constructed by coupling antibody to CNBr-activated Sepharose (Pharmacia) according to the manufacturer’s instructions. The column was washed extensively with PBS containing 0.05% Tween-20. Bound protein was eluted with 0.1 M glycine/HCl (pH 2.5) and visualized by SDS-PAGE and fluorography.

**Organ cultures**

Organ cultures were made from explants from leg autopodia between stages 28 and 31. The whole autopodium and tissue fragments containing the following elements: (i) only an isolated digit; (ii) a digit plus the adjoining interdigital tissue; (iii) two digits with the intervening interdigital tissue; and (iv) only a tissue wedge obtained from the third interdigit; were transferred to 35 mm dishes and cultured for 24-48 hours in Ham’s F-12 medium supplemented with 10% fetal calf serum and antibiotics. In some cases the explants were allowed to attach to the dish surface and in other cases they were maintained floating in the medium. At the desired stage, the explants were washed in saline and processed for immunohistochemistry as described above.

**Micromass cultures**

High-density micromass cultures were prepared according to the Ahrens procedure (Ahrens et al., 1977). Limb mesodermal cells were disaggregated from whole limb buds at stage 22-23 after a short digestion with 0.1% trypsin and 0.1% collagenase in calcium- and magnesium-free saline. The cells were then resuspended at a concentration of 2×10^5/µl in Ham’s F-12 medium (Gibco) supplemented with 10% fetal calf serum and antibiotics. Drops (10 µl) of cell suspension were plated out in 35 mm tissue culture dishes. After 1 day of culture a small fragment of ectoderm, isolated by trypsin-pancreatin treatment from limb buds of stage 23, was located at the top of some cultures and allowed to grow for 3 days (Solursh et al., 1981).

Extracellular matrix distribution in the cultures was studied immunohistochemically in micromasses with or without ectoderm, following the procedure described above.

**RESULTS**

**Monoclonal antibody to elastin**

Monoclonal antibody 10B8 reacted in ELISA with the chick tropoelastin used for immunization; this antibody also yielded a lamellar immunofluorescent staining pattern in cross-sections of chick aorta (data not shown). mAb 10B8 immunoprecipitates chick tropoelastin (Fig. 1) and immunoblots human elastin (data not shown). The commercially available mAb BA-4 immunoblotted the same band identified by mAb 10B8 in preparations of human elastin. However, unlike mAb BA-4, mAb 10B8 binds to chicken elastin and can be used for tissue localization studies without requiring unmasking of tissue section with trypsin.

**The elastic matrix of the developing limb bud**

Two successive and distinct patterns of elastin distribution were found in the developing limb bud. Initially, elastin was observed forming a complex scaffold within the undifferentiated limb mesenchyme. The scaffold was established in a proximo-distal sequence in the limb bud concomitantly with the proximo-distal pattern of chondrification of the limb skeletal elements. During the next stages elastin was observed in the developing limb structures with expected elastic properties such as joints and tendons.

The first sign of elastin scaffold formation was detected at stage 25-26. At this stage the limb bud appears elongated and shows a clear autopodial plate without distinguishable digital elements. In whole mounts stained for cartilage the earliest anlage of the stylopodial (femur) and zeugopodial (tibia and fibula) cartilages are observed. A dorsal view of the limb under the confocal microscope, immunostained for elastin, reveals a
precise distribution of elastin fibers in the anterior half of the distal stylopodium and through the zeugopodium (Fig. 2A). The autopodial plate at this stage lacks elastin immunolabeling. Elastin fibers run perpendicularly to the longitudinal axis of the limb from the anterior margin towards the central zone of the bud, where they terminate. In transverse sections of the limb the pattern of elastin fibers can be clearly appreciated (Fig. 2B). Fibers are always restricted to the anterior half of
Elastin fibers were detected by stage 27-28 but the basic scheme of a fibrillar system running from the ectoderm surface towards the central cartilaginous core of the limb is conserved. The fibrillar system observed in previous stages is no longer restricted to the anterior half of the limb but elastin fibers originate from most of the ectodermal surface and penetrate deeply, reaching the surface of the developing cartilages (Fig. 2C). In the next stages the pattern of elastin distribution in the zeugopod and stylopod becomes obscured by the formation of muscle bellies, which causes an extensive redistribution of the extracellular matrix components. In the following stages elastin is detected at the zones of muscle insertion and at the level of origin of the tendons (not shown).

From stage 28 the distribution of the elastic scaffold extends to the developing autopodium where a sharp increase in elastin immunoreactivity is observed in association with the formation of the metatarsal cartilages and digital rays. As can be seen in Fig. 2D, numerous fibers are now detected radiating from the condensing digital rays towards the ectodermal basal surface and towards the developing interdigital spaces. In the interdigital spaces the fibers coming from each digit are rather confluent and tend to be directed towards the distal margin of the interdigit where they are lost in association with the distal subridge mesenchyme. No anterior regionalization of the elastin fibers was detected in this region, since elastin fibers are associated with all the developing digital rays.

From stage 29 two distinct components of the elastin fibers can be distinguished in the autopodium (Fig. 2E). One component originates from the digital rays and radiates out towards the ectoderm and interdigital space (Fig. 2E-G). The other component occupies each interdigital space (Fig. 2E,G) and appears as a bundle extraordinarily rich in elastin fibers condensed in the space limited by the distal end of the metatarsal cartilages and diverging in a fan-like fashion at the distal portion of the interdigit where the fibers are lost either in the prechondrogenic tips of the digits or in the ectodermal basal surface (Fig. 2H). From stage 33 the interdigital fibrillar components become progressively lost in association with interdigital tissue regression, and by stage 35 the elastin-positive material appears as a network of thick fibrillar bundles, which are concentrated at the distal margin of the regressing interdigit. The radiating elastin-containing fibers associated with the digital rays become progressively substituted in a proximo-distal fashion due to the rearrangement of the extracellular matrix associated with the development of the tendons. As can be seen in Fig. 3A, the developing tendons are positive for elastin labeling and in the course of their trajectory along the surface of the developing phalanges they send numerous filamentous projection, which appear to anchor the tendons to the perichondrial surface, giving a feather-like appearance to the tendons when observed at high magnification.

As we have mentioned above, in addition to the fibrillar scaffold associated with the developing skeleton, from stage 29 elastin also appears associated with the developing muscles in the zones of insertion or tendon origin, and also in the developing joints and in the long tendons of the autopodium, as described above, but a detailed analysis of this elastin-positive matrix is beyond the scope of this study (unpublished data).

In contrast to elastin, fibrillin exhibited a more restricted pattern of distribution (Fig. 3B,C). The early fibrillar pattern of the limb bud at stage 25-26 and the elastin associated with the digital rays and the interdigital tissue were all negative for fibrillin. The first appearance of fibrillin was detected in the autopodium at stage 28-29 in association with the formation of the earliest anlage of the developing tendons. At this stage fibrillin produced a fibrillar labeling pattern under the ectodermal surface of the developing ankle that was concentrated in the proximal part of the developing digital rays. From stage 32 fibrillin labeling was restricted to the perichondrium, joints and tendons. At the level of the joints fibrillin was codistributed with elastin but elastin labeling was more intense. Fibrillin labeling was also noted in the perichondrium (Fig. 3B), which is also slightly positive for elastin. In the developing tendons fibrillin was detected in the main tendinous blastemas (Fig. 3C) but not in the thin fibrillar component anchoring the tendons to the developing phalanges.

Codistribution of other extracellular matrix components with elastin was observed from stage 28-29 during the formation of joints, tendons and perichondrium of the autopodial elements (Table 1). In addition, tenasin, laminin, and collagens type I, type III and type VI, were also found taking part in the formation of a complex extracellular matrix scaffold associated with the undifferentiated mesenchymal tissue. However, none of these matrix components paralleled the pattern of distribution of elastin. Fibrillar elements immunolabeled for collagen type VI and tenasin were observed to be arranged in a preferential dorso-ventral pattern (Fig. 3D,E). These fibrils run through the undifferentiated mesenchyme of the autopodium from the ectodermal basement membrane of opposing ectodermal surfaces (dorsal and ventral) in the
proximity of the developing digital rays. As can be seen in Fig. 3D-E the number of these fibrils was much more reduced than the fibrillar component detected with elastin labeling. Further, both collagen type VI and tenascin were abundant components of the ectodermal basement membrane while elastin was absent from this structure. Laminin was mostly found in the ectodermal basement membrane but in the distal region of the autopodium laminin-positive tracts running up to 100 µm deep into the subectodermal mesenchyme were also observed (Fig. 3F). Collagen type I- and type III-positive fibrils were also detected in the interdigital spaces running in a proximo-distal direction paralleling in some way the arrangement of the developing tendons (Fig. 3G-I). At the distal part of the autopodium, collagen type I was also detected taking part in the fibrillar system rich in collagen type VI and tenascin (Fig. 3H). None of these components of the extracellular matrix scaffold of the developing autopodium was detected in association with the elastin fiber component located in the anterior half of the limb at earlier stages.

**Elastin distribution in organ and cell cultures of the limb**

Explant cultures of the whole autopodium for periods up to 48 hours exhibit a pattern of elastin distribution similar to that found in vivo (Fig. 4A). Explants of autopodial fragments consisting of one or two digits also exhibited a normal pattern of elastin distribution during the first 24 hours of culture. In longer culture periods modifications in the spatial distribution
Elastin in the developing chick limb

**Fig. 3.** Whole-mount chick limbs immunolabeled for elastin (A), fibrillin (B,C), tenascin (D), collagen type VI (E), laminin (F), collagen type I (G,H), and collagen type III (I). (A) Detailed view of a developing tendon at stage 35 after elastin immunolabeling. Note the positive labeling of the main tendon blastema (T) and the presence of fibrils anchoring the tendon to the surface of the adjoining phalange. Bar, 100 µm. (B,C) Two successive optical sections of digits II and III at stage 35 after fibrillin immunolabeling. Bar, 200 µm. (B) The level of the digital cartilages showing positivity in the perichondrium and developing joints. (C) Positivity of the developing tendons. Note that fibrillin does not label the anchoring fibers of the tendons observed A. (D) Distal tip of digit III and its adjacent interdigital tissue at stage 30 after collagen type VI immunolabeling. The perichondrial surface of the developing cartilage and the ectodermal basement membrane (arrows) exhibit positive labeling. Note the presence of positive fibrils crossing the interdigital tissue. Bar, 100 µm. (E) Detailed view of the tip of digit III (D) and its adjacent interdigital tissue at stage 29 immunostained for tenascin. Positive fibrils are observed running dorsoventrally in the interdigital space. The surface of the developing cartilage is positive for tenascin and displays tenuous fibrils running towards the interdigit. Bar, 100 µm. (F) Detailed view of the distal part of the autopodium at stage 29 after laminin immunolabeling. Note that in addition to the continuous labeling of the basement membrane and in the cartilage, fibrillar tracts are also present running for a short trajectory into the subectodermal mesenchyme. Bar, 100 µm. (G) Dorsal view of the autopodium at stage 32 showing the appearance of a developing tendon (T) after immunolabeling for collagen type I. Note the abundance of collagen fibers running parallel to the main tendinous blastema. Bar, 100 µm. (H) Distal tip of digit III and adjoining interdigital tissue at stage 30 after immunolabeling for collagen type I. Note the positivity of the ectodermal basement membrane and the presence of fine tracts running longitudinally through the interdigit. Bar, 100 µm. (I) Distal part of the autopodium at stage 31 after collagen type III immunolabeling. The optical section corresponds to the level immediately under the ectodermal surface of the interdigit and shows the presence of fibrils with a preferential longitudinal arrangement. D, digit III. Bar, 200 µm.

Table 1. Distribution of ECM molecules in relation to elastin during the development of the autopodium

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<th>Joints</th>
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<td>Collagen type X</td>
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<tr>
<td>Tenascin</td>
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<td>Laminin†</td>
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*Elastin was the only element detected that formed fibrils anchoring the tendons to the developing phalanges (see Fig. 3A).
†Collagen type II was only detected in the ectodermal basement membrane and in the cartilage.
‡Collagen type IV was only detected in the wall of the developing blood vessels of the limb.
§Collagen type X at the studied stages was restricted to the hypertrophic cartilage of the diaphysis of the skeletal pieces.
¶A faint labeling of the perichondrium and joints was detected with anti-laminin antibody.

of elastin were detected in relation to the characteristics of the cultured tissue fragments.

When the explants contain only a digit and adjacent interdigital tissue, the fibrillar pattern radiating from the digit remains conserved but the former interdigital elastic system becomes condensed at the margins of the explant (Fig. 4B). Explants containing two digits and the intervening interdigital result in the formation of two digital rays, which tend to fuse at the distal end leaving an oval interdigital region between them (Fig. 4C). In these cases, while the elastin fibrillar component radiating out from the developing digits observed in vivo remained unaltered, the interdigital elastin component lost its fan-like appearance of the in vivo condition (Fig. 4D).

An extreme modification of the interdigital elastic system is obtained in cultures of explants containing only a wedge of interdigital tissue. In these cases the interdigital mesenchyme forms a prominent cartilaginous element (Fig. 4E), which exhibits a newly formed radiating complex of elastin fibrils (Fig. 4F) similar to that found in the digital rays. This elastin-positive fiber system exhibited a more precise radial arrangement when the explants were attached to the culture dish than in explants floating free in the culture medium.

Elastin fibrils were also detected in high-density micromass cultures of dissociated limb mesoderm. As previously described (Ahrens et al., 1977) the limb mesoderm under these culture conditions undergoes intense chondrogenesis, forming abundant cartilage nodules, detectable by Alcian Blue staining after 3 days of culture, surrounded by non-chondrogenic mesenchyme. Elastin was very abundant between the chondrogenic nodules. The first identification of elastin fibers in these cultures occurred at day 3, concomitant with the formation of cartilage aggregates (Fig. 5A). At day 4 of culture the chondrogenic aggregates were more prominent and elastin was very abundant between the chondrogenic nodules (Fig. 5B). At the zones of confluence of several chondrogenic aggregates elastin fibers showed a well-defined radial pattern of distribution joining the surface of adjacent cartilage nodules (Fig. 5C), resembling the pattern of elastin distribution observed in transverse sections of the interdigital spaces in vivo. Placement of a small piece of ectoderm at the top of the micromass after 1 day of culture causes a local inhibition of chondrogenesis (Solursh et al., 1981), and immunostaining for elastin shows an increased deposition of elastin in the subectodermal region that is devoid of cartilage (Fig. 5D). At high magnification and with the advantage of the confocal microscope to study optical sections of the tissue, it can be clearly observed that the elastin fibers in this region become aggregated in thick bundles of fibrils that appear anchored by one of their ends to the ectodermal basal surface (Fig. 5E-F).

Fibrillin was also detected in the micromass cultures but it showed a pattern of distribution very different from that of elastin. The first identification of fibrillin was at day 2 of culture in association with the earliest chondrogenic aggregates (Fig. 5G). From day 3 of culture fibrillin showed an intense positivity on the outer surface of the chondrogenic nodules (Fig. 5H). A diffuse labeling of the intercartilaginous joints was detected with anti-laminin antibody.
DISCUSSION

Elastin constitutes a unique extracellular matrix component with rubber-like properties conferring tensile functions that are required to meet the demands of mechanical stress in vertebrate tissue (Sage and Gray, 1979). Most studies of elastic matrices...
have been focussed on adult tissues and organs that are normally subjected to periodic mechanical deformations such as skin, pulsatile blood vessels, lung and some ligaments where elastin comprises a very high percentage of their dry weight (Sage and Gray, 1979). On the basis of the data from these studies it has been largely assumed that elastin is a component formed in late fetal periods of development and its function has been mostly correlated with gross and continuous tissue deformation (Parks et al., 1988; Pasquali-Ronchetti et al., 1993). However, recent studies with the developing chick embryo, using in situ hybridization techniques, revealed that tropo-elastin mRNA is expressed early during development (Selmin et al., 1991; Holzenberger et al., 1993), but the pattern of elastic fiber distribution in relation to morphogenetic events remains to be clarified.

Our study reveals the involvement of elastin during the morphogenesis of the limb skeleton in vivo and in vitro. In the course of this study we have also observed that elastin in precise patterns of fiber arrangement is present in many other developing embryonic tissues, including the outflow tract and atrioventricular cushion tissue of the heart, the early developing lung, the notochord and the somites (unpublished data). Further, in contrast to previous studies on elastogenesis in vitro by bovine nuchal ligament fibroblasts (Mecham et al., 1984), we have also found that chondrogenic mesodermal limb cell cultures are very elastogenic. While nuchal ligament fibroblasts decrease elastogenesis markedly in vitro in the absence of contact with a previous elastic matrix, limb mesodermal cells retain an intense elastogenic potential in vitro. All these findings point to new, previously unsuspected functions for elastic matrices during embryonic development.

In the limb bud prior to the establishment of muscle bellies, elastin exhibits a constant pattern of arrangement, forming a fibrillar scaffold from the ectoderm to the chondrogenic skeletal pieces at the early stages of formation of the limb skeleton. There are several characteristics of this scaffold that are particularly interesting as putative factors of importance in skeletal morphogenesis. The fibers of the scaffold are never arranged randomly but exhibit a constant orientation repeated at the same stage in all the embryos studied. Further, the pattern of fiber arrangement follows closely the sequence of formation of the skeletal elements and remains unaltered in organ culture conditions provided that the morphology of the growing explant maintains the characteristic shape found in situ. When the explant consists of a tissue fragment isolated from its normal neighboring elements, the elastin pattern undergoes changes that parallel the altered pattern of growth and chondrification of the explant. The appearance of the elastin scaffold both in vivo and in vitro is coordinated with the establishment of distinct cartilage elements and it exhibits a permanent association with the ectoderm basal surface. This last feature is reinforced by the ability of the ectoderm to reorientate and aggregate elastic fibers when located at the top of micromass cultures. Taking together all these features along with the well-known biomechanical properties of elastic matrices (see Mecham and Heuser, 1991; Pasquali-Ronchetti et al., 1993; Rosenbloom et al., 1993), it can be proposed that elastin scaffold formation may be linked to the occurrence of mechanical stress associated with the anisotropic growth of the limb with tissues of different physical characters. If this assumption is true, the elastin scaffold provides molecular evidence for the hypothesis involving mechanical tension in the morphogenesis of the limb skeleton (Oster et al., 1985; Rooney et al., 1984). On the other hand, the precise arrangement of the elastin scaffold joining the different skeletal pieces with the ectodermal limb cover might constitute an anchoring system accounting for the maintenance of the appropriate distance between the different limb tissues and for the coordination of growth.

A major question concerning the morphogenesis of the long bones of the limb is why, once the cartilaginous rudiments are formed, the early shape is maintained and growth is restricted to their proximo-distal axis, while increase in thickness is almost fully inhibited (Rooney et al., 1984). The perichondrium has been proposed to be responsible for stopping lateral outgrowth of the cartilage rudiments of the long bones (Rooney et al., 1984), but the formation of the perichondrium, especially at the level of the epiphyseal regions, is a relatively late event. This study reveals the occurrence of a precise landmark of elastic matrix that delimits, both in vivo and in vitro, the lateral edge of the developing cartilages from the surrounding, apparently undifferentiated mesenchyme. The sequential study of micromass cultures reveals that the formation of the cartilage aggregates is accompanied closely by the deposition of an elastic matrix in the adjoining nonchondrogenic tissue. This elastic matrix undergoes progressive differentiation showing precise patterns of fibrillary arrangement, as observed in vivo, concomitantly with the growth and differentiation of the cartilage nodules. Further, and again resembling the events observed in vivo, the location of an ectodermal fragment on the top of the culture stimulates elastogenesis in precise fibrillar patterns. All these features suggest that the differentiation of cartilage aggregates in the developing limb bud involves the segregation of the initially undifferentiated mesenchyme into two distinct cell lineages: chondrogenic and elastogenic. This concomitant segregation of the early undifferentiated mesenchyme may contribute to preventing further incorporation of cells into the chondrogenic foci. Ectoderm appears to promote the elastogenic lineage and this might account for the proven antichondrogenic effect of the ectoderm in vitro (Solursh, 1984; Zanetti and Solursh, 1986) and in some in vivo conditions (Hurle and Gañan, 1986).

The restricted distribution of elastin to the anterior half of the limb bud between stages 25 and 26 is a striking feature, which might be explained by differences in the amount of elastin associated with the formation of the anlage of the tibia in comparison to a more reduced amount associated with the formation of the fibula (see Archer et al., 1983; Hampe, 1960). However, since differences between mesodermal cells of the anterior and posterior segments of the limb have been found to be of fundamental importance for limb patterning (Bryant and Gardiner, 1992), in the absence of detailed information on possible further functions of elastin (see Hornebeck et al., 1986) in developing systems, we cannot discount the possibility of a relation between the anterior polarized distribution of elastin and the previous distinctive commitment of the limb mesoderm.

In a previous paper we have described the occurrence of an extracellular matrix scaffold within the limb bud that is arranged in a dorsoventral pattern of fibrils attached to opposite ectodermal basal surfaces (dorsal and ventral) (Hurle et al., 1989). Tenascin, fibronectin and collagen type I were found to
be the major constituents of this scaffold. In that study we identified such a scaffold with a complex system of fibrils that were observed in paraffin sections stained with silver. In this study the use of a wider set of antibodies, along with the advantage of using the confocal microscope to study the distribution of immunolabeled matrix components in whole-mount specimens, allowed us to clarify several aspects of this extracellular matrix scaffold. On one hand, the argyrophilic fiber system clearly corresponds to the elastin scaffold described here in association with chondrogenesis. On the other hand,
Fig. 5. Micromass cultures of chick limb mesoderm from stage 22 limb buds after immunolabeling for elastin (A-F) and fibrillin (G-I). (A) Illustration showing the presence of patches of fibrillar matrix positive for elastin after 3 days of culture. This matrix is located in the mesenchyme surrounding the developing chondrogenic nodules. Bar, 200 µm. (B) Four days of culture, showing the abundance of elastin-positive fibrils surrounding the chondrogenic nodules (C). Bar, 100 µm. (C) Optical section through the zone of confluence of four cartilage nodules at the same plane of the tissue showing the presence of elastin-positive fibrils with a precise radial pattern of arrangement. Bar, 100 µm. (D) Low magnification view of micromass cultured for 5 days in which a fragment of ectoderm was placed on the top surface on day 2 of culture to inhibit chondrogenesis. Note the accumulation of elastin-positive matrix in the central region of the micromass corresponding to location of the ectoderm fragment (E). Bar, 300 µm. (E-F) Two successive optical sections showing a detailed view of the ectodermal region shown in 24. In (E) focus is at the subectodermal fibrillar matrix. In (F) the focus is at the level of the ectoderm showing the termination at this level of the elastin bundles indicated by arrows in E. Bar, 100 µm. (G) Two-day-old culture showing the earliest appearance of fibrillin-positive matrix associated with the zones of chondrogenic mesenchymal condensations. Bar, 100 µm. (H) Four-day-old culture showing maximum labeling for fibrillin in the surface of the developing cartilages. Bar, 10 µm. (I) Five-day-old micromass in which an ectodermal fragment (E) was located at the top surface on day 2. Note the halo of inhibition of fibrillin deposition in relation to the ectoderm fragment. The intense fluorescent region associated with the ectoderm (+) corresponds to autofluorescence as assessed by using the rhodamine filter. Bar, 200 µm.

this study reveals that the so-called 'dorso-ventral fibrillar system' constitutes a more complex scaffold containing additional matrix components, including collagens type III and type VI, and most likely laminin. Whether the components of this matrix share common functions with the elastin scaffold cannot be ascertained from this study. Elastogenesis by nuclear ligament fibroblasts has been found to be critically dependent on the presence of other extracellular matrix components (Mecham et al., 1981, 1984). Further, assemblies of different extracellular matrix components may be of fundamental importance in extracellular matrix function, including the establishment of the patterns of adhesion to the cellular components. However, in this study none of the reported matrix components exhibited a pattern of distribution paralleling the elastin scaffold, although they may be associated with particular regions of the elastin scaffold.

The different patterns of distribution of elastin and fibrillin observed in this study constitute an unexpected feature of the elastic matrix of the developing limb bud. Fibrillin is currently considered to be a constant component of the elastic matrix and has been characterized as the fibrillar component associated with the amorphous tracts of elastin (Sakai et al., 1986). Early studies on elastogenesis showed that this fibrillar elastic component precedes the appearance of the amorphous insoluble elastin, suggesting a possible role as a precursor of the elastic matrices facilitating in some way the crosslinking of tropoelastin to form insoluble elastin tracts (Ross and Bornstein, 1969; Cleary et al., 1981). In this study fibrillin and elastin showed relatively similar patterns of distribution only in later stages of development in association with joints, peri- chondrium and tendon differentiation; but even in these cases differences in distribution were significant. Elastin, for example, is associated with the fibrillar tracts anchoring the tendon blastemas to the developing phalanges, while fibrillin was only present in the main tendon blastema. In early stages of development fibrillin was not detected in the elastin scaffold. This different pattern of distribution was particularly remarkable in micromass cultures where fibrillin and elastin showed opposite patterns of distribution in the surface of the cartilage aggregates and in the extracellular matrix of the non-chondrogenic tissues, respectively. The location of an ectodermal fragment on top of the cultures emphasized this difference, causing a local increase in elastin fiber deposition and an intense inhibition of fibrillin distribution. While we cannot discard the possibility of the presence of masked fibrillin associated with the elastin scaffold of the limb or the occurrence in the chick of different types of fibrillins lacking the epitope recognized by our antibody (see Ramirez et al., 1993), all the observations mentioned above suggest that the different patterns of fibrillin and elastin distribution are characteristic of the elastic matrix of the developing limb.

Thanks are due to Karen Jensen for help and advice with the immunohistochemical techniques, and to John Busse for photography. This work was funded in part by grants to J.M.H. (DGICYT, from the Spanish government), M.S. (NIH grant HD05505) and an award to L.Y.S. from the Snriner’s Hospital for Crippled Children.

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(Received 15 April 1994 - Accepted 24 May 1994)