Changes in the patterns of collagens and fibronectin during limb-bud chondrogenesis

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SUMMARY

The distribution and sequence of appearance of fibronectin and of type-I and type-II collagen in the developing cartilage models of embryonic chick hind-limb buds was studied by immunofluorescence, using specific antibodies directed against these proteins. Fibronectin and type-I collagen are evenly distributed throughout the intercellular space of the mesenchyme prior to condensation of core mesenchyme of the limb anlage and formation of the cartilage blastema. With the onset of the condensation process fibronectin and type-I collagen appear to increase in the cartilage blastema compared to the surrounding loose mesenchyme, reaching a maximal density at the time of cartilage differentiation. The latter process is marked by the appearance of type-II collagen in the cartilage blastema. As cartilage differentiation progresses, type-I collagen is gradually replaced by type-II collagen; fibronectin disappears and is completely absent from mature cartilage.

The transient appearance of type-I collagen and fibronectin suggests a temporal role in cell-matrix or cell-cell interactions in chondrogenesis, since it had been shown that (a) type-I collagen substrates stimulate cell proliferation and cartilage differentiation in limb-bud mesenchyme cell cultures; (b) fibronectin mediates attachment of cells to collagen substrates; and (c) fibronectin is directly involved in cellular interactions in chondrocyte cultures.

INTRODUCTION

The mesenchyme of a vertebrate limb bud develops primarily into four types of tissues: cartilage, bone, muscle, and connective tissue. Cartilage differentiation begins with a cellular condensation process in the core of the proximal half of the limb (Fell & Canti, 1934; Jurand, 1965). While in the undifferentiated mesenchyme, cells are loosely packed and connected by sparse cellular processes. The number of intercellular associations and the cell density increase during the condensation phase (Searls, 1973; Thorogood & Hinchliffe, 1975) to form the so-called cartilage blastema, representing the bone anlage. Classically, expression of the cartilage phenotype in the blastema is indicated by the secretion of a metachromatic extracellular matrix.

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The transient condensation during the phase which precedes chondrogenesis has drawn the attention of many investigators in the past (Saunders, 1948; Ede & Agerbak, 1968; Thorogood & Hinchliffe, 1975; for review see Ede, Hinchliffe & Balls, 1977). It is in this stage that cell–cell or cell–matrix interactions which may be involved in the process of chondrogenic differentiation are likely to occur. Our previous studies have shown that the condensation of chick limb-bud mesenchyme is associated with the deposition of type-I collagen in the extracellular matrix (von der Mark, von der Mark & Gay, 1976a). This finding was in agreement with biochemical studies on collagen-type transitions during cartilage and bone formation in the chick limb bud (Linsenmayer, Toole & Trelstad, 1973). It was reported that the pre-cartilaginous limb-bud mesenchyme at stage 23–24 contained type-I collagen. Onset of type-II collagen synthesis in the limb core at stage 25–26, concomitant with the appearance of metachromatic staining of the extracellular matrix, indicated the emergence of differentiated cartilage. The type-I collagen is no longer present in mature cartilage (Linsenmayer et al. 1973; von der Mark et al. 1976a; von der Mark, 1979).

It is still unclear if the transitory appearance of the type-I collagen in the cartilage blastema has functional significance. The possibility exists that it may be involved in developmental processes. Cell culture experiments on dissociated chick limb mesodermal cells suggest that exogenous collagen substrates increase the rate of chondrogenic differentiation (von der Mark, 1978).

From studies by Linder, Vahteri, Rouslahti & Wartiovaara (1975), Stenman & Vahteri (1978) and Wartiovaara, Leivo & Vahteri (1979) it became apparent that another matrix molecule, fibronectin, is present in undifferentiated mesenchyme, but not in mature cartilage. Fibronectin, which is a major cell surface glycoprotein, mediates cell interactions with collagen (Klebe, 1974; Klebe et al. 1977). It has an affinity to native and denatured collagens, and binds preferentially to cyanogen bromide fragments in the carboxyterminal region of the collagen molecule (Kleinman, McGoodwin & Klebe, 1976; Dessau, Adelman, Timpl & Martin, 1978a). The collagenase cleavage site is present in this region of the molecule (Kleinman et al. 1978).

Assuming that fibronectin also participates in cell–collagen interactions occurring in the cartilage blastema of the limb bud prior to expression of the cartilage phenotype, a maximum of fibronectin deposition in the developing cartilage would be expected at the time of maximal intercellular deposition of type-I collagen. In order to obtain further information on the role of matrix macromolecules, we have studied the temporal and spatial sequence of fibronectin, type-I and type-II collagen deposition in the early stages of the developing chick hind-limb bud.
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MATERIAL AND METHODS

Histological techniques

White Leghorn chick embryos were incubated at 37 °C under moisture, and staged according to Hamburger & Hamilton (1951). Limb buds at various stages were removed, fixed in 4 % formaldehyde in PBS (phosphate-buffered saline) for 30 min and transferred to 3 % sucrose in PBS, embedded in ‘Compound’ Tissue Tek (Miles, Naperville, Ill., USA) and quick frozen at dry-ice temperature. Frozen sections, 8 μm thick, were cut with a SLEE-Cryotome (Mainz, FRG) and incubated for 30 min at room temperature with antibodies to collagens or fibronectin, washed with PBS and incubated for 30 min at room temperature with fluorescein-conjugated rabbit anti-guinea pig γ globulin or goat anti-rabbit γ globulin (Behringwerke, Marburg, FRG). Double-staining experiments for simultaneous localization of type-II collagen and fibronectin were performed using rabbit antibodies to fibronectin which were counter-stained with rhodamine-labelled goat anti-rabbit γ globulin, and guinea-pig antibodies to type-II collagen which were counter-stained with fluorescein-conjugated rabbit anti-guinea-pig γ globulin.

Antibodies

Antisera to chick type-I and type-II collagen were raised in rabbits and guinea pigs, purified by immunoadsorption and tested for specificity as described earlier (von der Mark et al. 1976a). No cross-reaction of antibodies against type-I collagen was observed with type-II and type-III collagen. Nor was there cross-reaction of antibodies against type-II collagen with type-I and type-III collagen (H. Herrmann, W. Dessau, L. Fessler & K. von der Mark, in preparation). Rabbit antibodies to human plasma fibronectin (CIG) were kindly provided by Dr R. Timpl. Cross-reaction between anti-human fibronectin antibodies and chick fibronectin has been established previously (Dessau et al. 1978b).

RESULTS AND DISCUSSION

By immunofluorescence labelling we were able to identify type-I collagen and fibronectin in the developing chick limb bud as early as stage 19. Both proteins are localized in the interstitial space of the mesenchyme as well as in the ectodermal–mesodermal interface. Until stage 22–23, the extracellular type-I collagen meshwork is evenly distributed throughout the mesenchyme (Fig. 1B, C). Antibodies to fibronectin also reveal an even staining pattern, almost identical to that observed with antibodies to type-I collagen (Fig. 1A). The presence of fibronectin in the mesenchymal tissue of stages 19–23 shows that fibronectin is an early matrix protein and suggests that it may be involved in the differentiation processes.
Fig. 1. Immunofluorescent localization of fibronectin (A) and type-I collagen (B, C) in the mesenchyme of a stage-23 embryonic chick hind-limb bud. Frozen sections were stained with rabbit antibodies to fibronectin (37 µg/ml) (A) or guinea-pig antibodies to chick type-I collagen (136 µg/ml) (B, C). (C) Higher magnification of (B); bright fluorescence at the ectodermal/mesodermal interface indicates the site of the presumptive dermis. (A) and (B), × 200; (C) × 300.
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When cells in the limb core start to condense at stage 23–24, a slight increase in the intensity of type-I-collagen staining compared to the peripheral mesenchyme can be observed. At late stage 24, type-II collagen appears in the blastema in addition to type-I collagen, indicating the onset of cartilage differentiation (Fig. 2A, B). The pattern of fibronectin followed that of type-I collagen. A parallel section stained for chondroitin sulphate proteoglycans with Alcian blue in the presence of 0-3 m-MgCl₂ shows a weak reaction in the same area that type-II-collagen stain was found (not shown). In a previous study type-II collagen was first found at stage 26 in the cartilage blastema (von der Mark et al. 1976a, b). Due to improvement of the immunofluorescence technique we are now able to identify type-II collagen in addition to type-I collagen in the blastema as early as stage 24.

Between stages 24 and 26, fibronectin and type-I and type-II collagen occur together in the developing cartilage of the long bones. Fibronectin and type-I collagen are most prominent in the cartilage blastema, but are also present in
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Peripheral mesenchyme, while type-II collagen is restricted to cartilage. At stage 25 the staining with antibodies to type-I and type-II collagen and fibronectin reveals the Y-shaped blastema, representing the anlage of femur, tibia and fibula (Fig. 3A–D). Type-I collagen and fibronectin reach maximal density at that stage.

With further maturation of cartilage, type-I collagen and fibronectin begin to disappear from the cartilage matrix and are gradually replaced by type-II collagen (Fig. 4c). Fibronectin and type-I collagen, however, persist in the perichondrium (Fig. 4A, B) in other connective tissues such as tendons and ligaments, and in vessels. The mature cartilage matrix is completely devoid of both proteins (see also Dessau et al. 1978a).

These results show that in the developing limb, type-I collagen and fibronectin occur at maximal density at a stage which coincides with onset of expression of the cartilage phenotype. Although not conclusive, this suggests that both proteins may be involved in cell–matrix or cell–cell interactions which may be required for chondrogenic differentiation. In several other systems the ability of collagen substrates to stimulate cell proliferation and differentiation has been described. Collagen types I–IV stimulate corneal differentiation (Meier & Hay, 1974; Hay, 1977), somite differentiation (Kosher & Church, 1975; Lash & Vasan, 1978, von der Mark, 1978) and fusion of myoblasts (Konigsberg & Hauschka, 1965).

The possibility exists that the collagen of the mesenchymal extracellular matrix in the prechondrogenic mesenchyme serves as a substrate which allows cell proliferation. It may thus contribute to the cell condensation process by increasing the cell density. In support of this view is the finding reported here that extracellular type-I collagen and fibronectin are co-distributed. Fibronectin may participate in cell–collagen interactions during the condensation phase. Alternatively, fibronectin may be directly involved in cell–cell interactions. The latter hypothesis is strengthened by the fact that fibronectin is completely absent from mature hyaline cartilage in which cellular interactions are prevented by an extensive extracellular matrix (Dessau et al. 1978a).

A transient appearance of type-I collagen before cartilage differentiation has also been observed in vitro. When dissociated limb-bud mesodermal cells are

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**Figure 3**

Co-distribution of fibronectin (A), type-I collagen (C) and type-II collagen (B, D) in the cartilage blastema of a stage-25 limb bud.

(A, B): Double staining with rabbit anti-fibronectin (A) and guinea-pig anti-type-II collagen on the same section (B).

(C, D): Consecutive sections stained with guinea-pig anti-type-I collagen (C) and guinea-pig anti-type-II collagen antibodies (D).

† Femur. × 200.
Fig. 4. Cross section through tibia of a stage-29 chick embryo leg. Immunofluorescence labelling of consecutive sections with anti-fibronectin (A), anti-type-I collagen (B) and anti-type-II collagen (C) shows that fibronectin and type-I collagen disappear from the mature cartilage matrix but become prominent in the surrounding connective tissue. T = tibia, F = fibula, × 150.
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cultured at densities greater than confluency, cell aggregates form after 2–3 days in culture, and subsequently develop into nodules of hyaline cartilage (Caplan, 1970; Solursh & Reiter, 1975; von der Mark & von der Mark, 1977, Ahrens, Solursh & Reiter, 1977). Those prechondrogenic aggregates show an extensive extracellular meshwork of fibronectin and type-I collagen (Sasse & von der Mark, in preparation), which becomes replaced by type-II collagen and chondroitin sulphate proteoglycan with progressive maturation of the cartilage.

Direct involvement of fibronectin in cellular interactions is also suggested from studies on matrix production by chondrocytes from hyaline cartilage (Dessau et al. 1978a). Dissociation of the cartilage matrix and release of chondrocytes reinitiates the synthesis of fibronectin which connects cells within a colony by short, intercellular strands. This intercellular fibronectin meshwork may allow the development of a chondrocyte colony in which cells can reexpress the cartilage phenotype and deposit extracellular cartilage matrix. Similar to the differentiating cartilage in the limb bud, the extracellular fibronectin disappears with progression of matrix accumulation in chondrocyte colonies.

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