Fibronectin gene expression during limb cartilage differentiation

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Summary
A critical event in limb cartilage differentiation is a transient cellular condensation process in which prechondrogenic mesenchymal cells become closely juxtaposed and interact with one another prior to initiating cartilage matrix deposition. Fibronectin (FN) has been suggested to be involved in regulating the onset of condensation and chondrogenesis by actively promoting prechondrogenic aggregate formation during the process. We have performed a systematic quantitative study of the expression of the FN gene during the progression of chondrogenesis in vitro and in vivo. In high-density micromass cultures of limb mesenchymal cells, FN mRNA levels increase about 5-fold coincident with the crucial condensation process, and remain relatively high during the initial deposition of cartilage matrix by the cells. Thereafter, FN mRNA levels progressively decline to relatively low levels as the cultures form a virtually uniform mass of cartilage. The changes in FN mRNA levels in vitro are paralleled closely by changes in the relative rate of FN synthesis as determined by pulse-labeling and immunoprecipitation analysis. The relative rate of FN synthesis increases 4- to 5-fold at condensation and the onset of chondrogenesis, after which it progressively declines to low levels as cartilage matrix accumulates. High levels of FN gene expression also occur at the onset of chondrogenesis in vivo. In the proximal central core regions of the limb bud in which condensation and cartilage matrix deposition are being initiated, FN mRNA levels and the relative rates of FN synthesis become progressively about 4-fold higher than in the distal subridge region, which consists of undifferentiated mesenchymal cells that have not yet initiated condensation. The striking increase in FN gene expression that occurs during condensation and the onset of chondrogenesis is consistent with the suggestion that this molecule may play a role in the crucial cell-cell or cell-matrix interactions involved in regulating the onset of cartilage differentiation.

Key words: fibronectin, limb, cartilage differentiation.

Introduction
The onset of cartilage differentiation in the developing vertebrate limb is characterized by a transient cellular condensation process in which prechondrogenic mesenchymal cells become closely juxtaposed prior to initiating cartilage matrix deposition. During this condensation process, critical cell-cell and/or cell-matrix interactions take place which are necessary to trigger the chondrogenic differentiation of the cells (see Kosher, 1983; Solursh, 1983 for reviews). Condensation is accompanied by the initiation of the expression of genes for the protein core of cartilage-specific sulfated proteoglycan (Kosher et al. 1986a; Mallein-Gerin et al. 1988) and cartilage-specific type IX collagen (Kulyk & Kosher, 1989), and a dramatic increase in the expression of the gene for cartilage-characteristic type II collagen (Kosher et al. 1986b; Nah et al. 1988). Several regulatory factors have been shown to play important roles in controlling chondrogenesis and cartilage-specific gene expression during condensation including cAMP (Kosher et al. 1979b; Kosher & Savage, 1980; Solursh et al. 1981; Kosher et al. 1986a), prostaglandins (Kosher & Walker, 1983; Biddulph et al. 1984; Chepeknik et al. 1984; Kosher & Gay, 1985; Gay & Kosher, 1985), and a cytoskeletal-mediated change in the shape of the cells from a flattened morphology to a rounded configuration (Zanetti & Solursh, 1984).

The onset of the critical condensation phase of chondrogenesis may be initiated, at least in part, by a progressive decline in the accumulation of extracellular hyaluronate (Kosher et al. 1981; Knudson & Toole, 1985; Kulyk & Kosher, 1987). Another matrix macromolecule that may play an important role in regulating the onset of condensation and chondrogenesis is the adhesive glycoprotein, fibronectin (FN). It has been suggested, for example, that FN actively promotes the formation of cellular aggregates during the condensation process (Newman & Frisch, 1979; Tomasek et al. 1982). This suggestion is based primarily on immunofluorescent studies which have shown that FN is present in relatively high amounts along the surfaces of the closely
aposed cells during condensation in the proximal central core of the developing limb bud (Dessau et al. 1980; Silver et al. 1981; Kosher et al. 1982; Tomasek et al. 1982). However, FN is also uniformly distributed throughout prechondrogenic mesenchyme prior to the onset of condensation (Dessau et al. 1980; Kosher et al. 1982; Tomasek et al. 1982). Therefore, it is not clear whether the apparent high concentration of FN in the condensing central core of the limb detectable by immunofluorescence represents an absolute increase in FN synthesis and deposition, or is merely a passive consequence of the higher cell density in the condensed regions. Moreover, there are conflicting reports as to whether or not FN persists or has a structural role in differentiated cartilage matrix (Dessau et al. 1980; Melnick et al. 1981; Kosher et al. 1982; Glant et al. 1985).

To aid in clarifying the possible role of FN in chondrogenesis, we have performed a systematic quantitative study of the expression of the FN gene during the progression of limb cartilage differentiation in vitro and in vivo. We have found that a striking transient increase in FN gene expression occurs during condensation and the onset of chondrogenesis. Our observations are consistent with the suggestion that this molecule may play a role in the crucial cell-cell or cell-matrix interactions involved in regulating the onset of cartilage differentiation.

Materials and methods
Preparation of tissue and cell culture
Wing buds were removed from stage 25 (Hamburger & Hamilton, 1951) embryos of White Leghorn chicks, and distal wing bud tips (subridge regions) were cut away from the limb buds as previously described, the size of the excised subridge regions being 0.3–0.4 mm from the distal apex of the tissue to the proximal cut edge (Kosher et al. 1979a). Ectoderm was removed from the tissue, and micromass cultures were prepared from the distal subridge mesenchymal cells as previously described (Gay & Kosher, 1984).

In addition, stage 25 wing buds were dissected with fine knives into the various discrete regions previously described in detail in which cells are in different phases of differentiation (see Fig. 3 and Kosher et al. 1981; Kulyk & Kosher, 1987). Specifically, the undifferentiated subridge region (see above) was dissected into distal (segment 1, Fig. 3) and proximal (segment 2, Fig. 3) regions, the proximodistal length (P–D) of each being about 0.2 mm. The remaining proximal portion of the limb bud was dissected into four segments (segments 3–6, Fig. 3) along the P–D axis, the P–D length of each being about 0.3 mm. The proximal segments (3–6) were further dissected into central core regions (3C–6C, Fig. 3) in which condensation and cartilage differentiation are occurring and peripheral regions (3P–6P, Fig. 3) in which nonchondrogenic differentiation is occurring. Photographs of the various regions into which stage 25 wing buds were dissected can be found in Fig. 2 of Kosher et al. (1981).

Hybridization probes
A subclone of the chicken FN genomic DNA clone FC40 (Hirano et al. 1983) was generously supplied by Dr Louis Gerstenfeld (Laboratory for the Study of Skeletal Disorders, The Children’s Hospital, Boston, MA). This genomic subclone consists of 2.8 kb of the 5’ end of the chicken FN gene inserted into the Sp65 vector (Gerstenfeld et al. 1985). Although, in some experiments, hybridizations were performed using the whole plasmid, most hybridizations were done with a 1.5 kb fragment, which was released by SmaI digestion of the plasmid and which hybridized to FN mRNA on Northern blots. A control probe consisted of the remainder (about 1 kb) of the insert plus the Sp65 vector sequences, and did not hybridize to FN mRNA. Probes were labeled with 32P-dCTP (3000 Ci mm⁻¹; Amersham) by the standard nick translation procedure of Maniatis et al. (1982) or by random oligonucleotide primed DNA labeling (Feinberg & Vogelstein, 1983, 1984). The oligo(dT)20 (Pharmacia) used in determination of total polyadenylated RNA was 5’ endlabeled with gamma-32P-ATP and T4 polynucleotide kinase (BRL) as described by Harley (1987).

Determination of steady-state FN mRNA levels
FN mRNA levels were determined at various times during the progression of micromass culture and in various limb regions using a slight modification of the dot-blot hybridization procedure of Cheley & Anderson (1984), as previously described (Kosher et al. 1986a). Briefly, cells and limb tissue were solubilized by sonication in 7 M guanidine–HCl in 0.1 M-potassium acetate buffer, pH5, and RNA selectively precipitated with ethanol (Cheley & Anderson, 1984). RNA pellets were dissolved, heated, and serially diluted samples spotted onto nitrocellulose, as described (Cheley & Anderson, 1984). The stringent hybridization and washing protocols previously described were utilized (Kosher et al. 1986a,b), and levels of hybridizable FN RNA sequences were quantified by densitometry as described (Kosher et al. 1986a,b) using a BioRad Model 620 video densitometer. Several film exposures were scanned to be sure the densities of the dots were in the linear response range of the preflashed film (Kodak X-OMat XAR-5). FN mRNA levels were normalized to the total poly(A)⁺ mRNA content of samples by hybridizing portions of the same RNA samples used for determination of FN mRNA with 32P-labeled oligo(dT)20 as described by Harley (1987).

Estimation of the relative rate of FN synthesis by pulse-labeling and immunoprecipitation analysis
Micromass cultures and the various limb regions were washed with methionine-free F12 medium containing 10% fetal bovine serum (FBS), and then pulse-labeled for 20 min at 37°C in methionine-free F12 medium containing 10% FBS and 1–2 M Ci mm⁻¹ of [35S]methionine (1326 Ci mm⁻¹; Amersham). The cells and tissues were washed with ice-cold Hanks’ balanced salt solution, and sonicated in extraction buffer containing 1 M urea, 10 mM Tris–HCl (pH 7.6), 1% sodium deoxycholate, 1% Triton X-100, 1 mM-dithiothreitol (DTT), and protease inhibitors (0.1 mM-6-aminocaproic acid, 1 mM-phenylmethylsulfonyl fluoride (PMSF), and 10 mM-sodium EDTA). The sonicates were generally stored at −20°C prior to immunoprecipitation analysis.

Samples of the sonicates (200 μl) were lysed for 15 min on ice by the addition of 80 μl of 10% sodium deoxycholate, 80 μl of 10% Nonidet P-40, 40 μl of water, and 100 μl of a buffer containing 10 mM-Tris–HCl (pH 7.6), 1% deoxycholate, 1% Triton X-100, and protease inhibitors (0.1 mM-6-aminocaproic acid, 1 mM-PMSF, and 10 mM-EDTA). The lysates were centrifuged at 12000 revs min⁻¹ for 15 min, and the supernatants (500 μl) were supplied with 100 μl of immunoprecipitation buffer containing 100 mM-Tris–HCl (pH 7.4), 150 mM-NaCl, 0.05% sodium deoxycholate, 0.1% SDS, 1 mg ml⁻¹...
bovine serum albumin, and protease inhibitors (1 mM-PPSF, 5 mM-benzamidine-HCl, and 5 mM-EDTA). Two portions (25 μl) of the samples were removed for determination of total incorporation of [35S]methionine into TCA-precipitable protein, and the remainder of the samples supplied with 30 μl of anti-fibronectin antiserum (rabbit anti-human FN antiserum; Collaborative Research). The samples were incubated with continuous agitation at 4°C overnight, after which they were supplied with 50 μl of protein A-agarose (Boehringer Mannheim). Following a 4-5 h incubation with continuous agitation at 4°C, the immunoprecipitates were collected by centrifugation for 5 min at 4500 revs min⁻¹, and were then washed 5 times with immunoprecipitation buffer. The immunoprecipitates were then solubilized by boiling for 5 min in electrophoresis sample buffer containing 0-625 M-Tris-HCl (pH 6-8), 4% SDS, 10% glycerol, 20 mM-DTT, 5% beta-mercaptoethanol, and 0-025% bromophenol blue, and SDS-polyacrylamide slab gel electrophoresis was carried out by the method of Laemmli (1970) using a 5% resolving gel and 3.5% stacking gel. Following electrophoresis, gels were impregnated with anti-fibronectin antiserum (rabbit anti-human FN antiserum; Collaborative Research). The samples were incubated with continuous agitation at 4°C, the immunoprecipitates were collected by centrifugation for 5 min at 4500 revs min⁻¹, and were then washed 5 times with immunoprecipitation buffer. 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Hours 24 48 72 96

Fig. 2. (A) Fluorograph of an SDS-polyacrylamide gel demonstrating the amount of [35S]methionine-labeled immunoprecipitable FN accumulated by distal subridge mesenchymal cells during 20 min pulse-labeling periods at various times during the course of their chondrogenic differentiation in micromass culture. (B) The relative rate of FN synthesis at each time point was determined by dividing the amount of [35S]methionine-labeled immunoprecipitable FN accumulated during the 20 min pulse (as determined by densitometry of the labeled FN bands on fluorographs) by the total amount of 35S-labeled TCA-precipitable protein accumulated during the pulse. The amount of labeled FN/total labeled protein at each time point is presented as an amount relative to that at 24 h of culture, which was arbitrarily set to 1000. Values are the means of 2 determinations ±range.

Discussion

A critical event in triggering the differentiation of limb mesenchymal cells into chondrocytes is a transient cellular condensation process in which prechondrogenic mesenchymal cells become closely apposed and interact with one another prior to initiating cartilage matrix deposition. It has been suggested that FN is involved in regulating the onset of condensation and chondrogenesis by actively promoting prechondrogenic aggregate formation during the process (Newman & Frisch, 1979; Tomasek et al. 1982). The present study demonstrates that a striking transient increase in FN gene expression

Fig. 3. (A) The various regions into which stage 25 wing buds were dissected. The chondrogenic central core tissue (regions 3c–6c) in the proximal limb regions was surgically separated from the nonchondrogenic peripheral tissue (regions 3p–6p) (see Kosher et al. 1981 for details and photographs). (B) Steady-state levels of FN mRNA in the various regions of stage 25 wing buds. The levels of FN mRNA and total poly(A)+ RNA in each region were determined by densitometry as described in Materials and methods. The amount of FN mRNA/total poly(A)+ RNA in each region is presented as an amount relative to that in region 6c, which was arbitrarily set to 1000. Values are the means of 2 determinations ±range.
does indeed occur during condensation and the onset of chondrogenesis in vitro. High levels of FN gene expression also occur in the proximal central core regions of the stage 25 limb bud in which condensation and cartilage matrix deposition are being initiated. Thus the relatively high amount of FN previously detected by immunofluorescence along the surfaces of the closely apposed cells during condensation (Dessau et al. 1980; Silver et al. 1981; Kosher et al. 1982; Tomasek et al. 1982) appears to reflect an actual increase in FN synthesis and deposition, and is not simply a passive consequence of the higher cell density in prechondrogenic aggregates. These observations are consistent with the suggestion that FN may play a role in the crucial cell–cell or cell–matrix interactions involved in regulating the onset of chondrogenesis.

There are several ways in which FN might be involved in influencing condensation and cartilage differentiation. As alluded to above, it may simply function as an adhesive protein that promotes cell aggregation, thus allowing the close cellular juxtaposition and interactions required to trigger differentiation. Furthermore, it has recently been demonstrated that prechondrogenic limb mesenchymal cells are selectively translocated to FN-rich regions of type I collagen gel matrices (Newman et al. 1985), and it has been suggested that such a FN-dependent ‘matrix-driven translocation’ may be involved in the formation of prechondrogenic aggregates during limb development (Newman et al. 1986). The FN-dependent matrix-driven translocation of limb mesenchymal cells is dependent on heparin-like molecules on the surface of the cells and the amino-terminal domain of the FN molecule (Newman et al. 1987), and preliminary studies indicate that a monoclonal antibody against the amino-terminal heparin-binding domain of FN inhibits the formation of prechondrogenic aggregates in vitro (S. A. Newman, personal communication). In addition to facilitating condensation by functioning as an adhesive protein and/or by promoting matrix-driven translocation, FN might be involved more directly in mediating the critical interactions occurring during the process, perhaps by virtue of its ability to interact with other cell surface or matrix macromolecules. Immunofluorescent studies have shown that, in addition to FN, type I collagen (Dessau et al. 1980; Silver et al. 1981) and a large mesenchymal-characteristic chondroitin sulfate proteoglycan molecule (Kimata et al. 1986) are present in apparently high amounts in the condensing central core of the limb bud. Since these three macromolecules directly bind to one another (Yamagata et al. 1986), an interaction between them may be involved in the condensation phase of chondrogenesis.

Although the present study and the others described above strongly indicate that FN plays a positive role in regulating the onset of chondrogenesis, it has been demonstrated somewhat paradoxically that under certain culture conditions exogenous FN inhibits the chondrocytic phenotype and cartilage matrix production (Pennypacker et al. 1979; West et al. 1979; Swalla & Solursh, 1984). The inhibitory effect of FN may be attributable to its ability to maintain the cells in a flattened morphology, since cytochalasin D, which disrupts the actin cytoskeleton and causes cell rounding, overcomes the inhibitory effect of FN (Zanetti & Solursh, 1986). It is noteworthy, therefore, that the extracellular glycoprotein tenascin interferes with the attachment and flattening of cells upon FN substrates, and causes cell rounding (Chiquet-Ehrlmann et al. 1988). It has recently been demonstrated that tenascin is present in the condensing central core of the developing limb bud, and that exogenous tenascin stimulates the formation of cartilage nodules by limb mesenchymal cells in vitro (Mackie et al. 1987). On the basis of these observations, it seems conceivable that the presence of tenascin might allow FN to promote precartilage aggregate formation, while preventing its cell flattening effect, thus allowing the change in cell shape to a rounded configuration that may be necessary for subsequent differentiation. Mackie et al. (1987) have suggested that tenascin may override the ‘anti-chondrogenic’ effect of FN by causing cell rounding.

We have recently demonstrated that transforming growth factor-beta (TGF-beta) is a potent promoter of the chondrogenic differentiation of embryonic chick limb mesenchymal cells, and have suggested that a possible mechanism by which TGF-beta might regulate chondrogenesis is by controlling the production of, or the levels of receptors for, extracellular matrix molecules such as FN that are involved in the crucial cell–cell or cell–matrix interactions that trigger chondrogenesis (Kulyk et al. 1989). TGF-beta stimulates the formation of FN in fibroblasts (Ignotz & Massague, 1986; Roberts et al. 1986), and controls the levels of receptors for FN in several cell types (Ignotz & Massague, 1987). It will, therefore, be of considerable interest to investigate the relationship of TGF-beta to
the striking increase in FN gene expression that occurs at the onset of limb chondrogenesis.

The results of the present study indicate that FN continues to be synthesized at relatively high levels as cartilage matrix deposition is proceeding during early chondrogenesis. This observation is consistent with previous immunofluorescent studies which have shown the presence of FN throughout differentialed cartilage rudiments during early limb development (Kosher et al. 1982; Tomasék et al. 1982). Therefore, the disappearance of FN is not required for cartilage differentiation to occur or for maintenance of the chondrocytic phenotype as others have suggested (Pennypacker et al. 1979; West et al. 1979). FN synthesis does, however, decline to quite low levels late in the differentiation process. FN may, however, persist even in mature cartilage, and has, in fact, been suggested to play a structural role by mediating the attachment of chondrocytes to type II collagen (Glant et al. 1985).

Both FN mRNA levels and the relative rate of FN synthesis increase 4- to 5-fold at the onset of chondrogenesis, and concurrent progressive declines in FN mRNA levels and FN synthesis occur as cartilage matrix accumulates. This suggests that FN gene expression during chondrogenesis is regulated primarily at the transcriptional level, although changes in processing of nuclear transcripts and/or changes in mRNA stability might also conceivably be involved (see, for example, Dean et al. 1988). The apparent transcriptional regulation of FN gene expression during cartilage differentiation contrasts with the apparent translational regulation of type I collagen gene expression during the process. Differentiated chondrocytes, which have ceased synthesizing detectable amounts of type I collagen protein, nevertheless contain as much cytoplasmic type I collagen mRNAs as do prechondrogenic limb mesenchymal cells that are synthesizing the protein (Kosher et al. 1986b; see also Focht & Adams, 1984; Kravis & Upholt, 1985; Saxe et al. 1985). It may be noteworthy, however, that at 96 h of micromass culture, which is well after overt cartilage formation, FN mRNA levels remain about 2-fold higher than they were before condensation, whereas the relative rate of FN synthesis at 96 h is lower than it was before condensation. This observation suggests some posttranscriptional regulation of FN gene expression may occur following overt differentiation (see also Adams et al. 1987).

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References


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