Fibronectin mRNA Splice Variant in Articular Cartilage Lacks Bases Encoding the V, III-15, and I-10 Protein Segments*

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Fibronectin (FN)1 is an extracellular matrix glycoprotein present in body tissues and fluids. Functionally, it is important in such diverse activities as cell adhesion, cell migration, cellular differentiation, blood clotting, opsonization, wound healing, and neoplastic transformation (Hynes, 1990). Fibronectin protein structure consists predominantly of three types of homologous repeating units (designated I, II, and III). It is encoded by a single gene, but significant protein heterogeneity results from alternative splicing of the pre-mRNA at three sites, termed extra type III domain A (ED-A), extra type III domain B (ED-B), and the variable (V) region (Schwarzbaer, 1991). The V region is sometimes also referred to as the connecting segment between the 14th and 15th type III homologous repeats (IIICS). Exons encoding ED-A and ED-B are spliced in or out in their entirety. In the V region of rat FN transcripts, however, a single 5'-splice donor site is present and results in two more V region splice variants (Vibe-Pedersen et al., 1984; Kornblith et al., 1985; Odermatt et al., 1985; Schwarzbaer et al., 1987).

Fibronectin is an important matrix constituent in cartilage, and its content is markedly elevated in articular cartilage lesions within osteoarthritic joints (Wurster and Lust, 1982; Burton-Wurster et al., 1986). Although the precise functional role of FN in normal and diseased cartilage is unknown, several unique structural features have been described. Relatively high levels of the (ED-B)1 isoform have been found in both canine and human cartilage FN (Burton-Wurster et al., 1989; Zhang et al., 1995a; Zhang et al., 1995b; Rencic et al., 1995). A small subset of cartilage FN appears to be post-translationally modified with the addition of a chondroitin or dermatan sulfate glycosaminoglycan (Burton-Wurster and Lust, 1993). We have also observed that canine cartilage FN has a subunit with an apparent molecular mass —15 kDa less than the smallest subunit of plasma FN. This subunit fails to react with two monoclonal antibodies that recognize epitopes in the III-15 segment, although at least some of these smaller subunits were still found within FN dimers (Burton-Wurster and Lust, 1989). These protein data are consistent with those of a subunit that retains the two sulfhydryl groups in the carboxyl terminus necessary for dimerization but has an internal deletion of the III-15 segment. In this study, we have examined the alternative splicing patterns of FN mRNA in articular cartilage. The results demonstrate a previously unreported splice variant that extends beyond any of the known 3'-acceptor sites in the V region and deletes nucleotides that would normally encode the 15th type III homology repeat (III-15) and the 10th type I homology repeat (I-10). This new splicing pattern is present in a majority of FN transcripts within articular cartilage and accounts for the small subunit of FN protein previously described.

EXPERIMENTAL PROCEDURES

Experimental Samples—Equine tissues were collected from a 15-year-old intact male horse immediately after euthanasia under a protocol approved by the Institutional Animal Care and Use Committee of Cornell University. The horse was euthanized because of neurological deficits caused by a congenital malformation of a cervical vertebra but had no other evidence of illness. Articular cartilage was collected from this same horse, a 3-week-old male foal, an adult male Labrador Retriever dog, and an adult male Flemish Giant/Chinchilla cross-bred rabbit. Articular cartilage samples from within each animal were pooled from multiple joints, including shoulder, elbow, hip, and stifles. All tissue samples were snap frozen in liquid nitrogen and stored at −70 °C until the time of RNA isolation.
Chondrocytes in Culture—Chondrocytes were isolated from equine articular cartilage (3-week-old foal) as described by Nixon et al. (1992). In brief, 1 g of diced cartilage fragments was incubated at 37 °C with 10 ml of 0.075% (w/v) collagenase type CL51 from Clostridium histolyticum (Worthington) until cartilage pieces were no longer visible. The cell/enzyme mixture was filtered to remove debris, and the chondrocytes were trapped in a monolayer at a density of 3 × 10^6 cells/cm² into Dulbecco’s modified Eagle’s medium (Life Technologies, Inc., catalog no. 11965) supplemented with α-ketoglutaric acid (30 μM), penicillin G (20 units/ml), streptomycin (20 μg/ml), and 10% fetal bovine serum. Chondrocytes were cultured for up to 46 days and were subcultured at weekly intervals.

Cartilaginous tissue, however, presents several technical problems for RNA isolation. The tissue is hypoeutic and largely composed of extracellular matrix proteins. This results in low RNA yields of cartilage when compared with normal parenchymal tissues. In addition, chondrocytes are contained within the matrix and protected from routine extraction procedures. Finally, the direct application of RNA isolation methods that rely on differential alcohol and salt precipitations will co-precipitate large amounts of matrix proteoglycans with the RNA. To overcome these difficulties, we have combined modifications of a method originally described by Adams et al. (1992) with a commercial protocol that utilizes silica gel-based spin columns (RNeasy, QIAGEN Inc., Chatsworth, CA). Specifically, articular cartilage samples were pulverized to fine granules in liquid nitrogen using a Spek Freezer Mill (Spek Industries, Metuchen, N.J.). While still frozen, 0.5 g of the cartilage powder was transferred to a 50-ml disposable polypropylene tube containing 4 ml of guanidinium thiocyanate, 0.1 M Tris-HCl, 25 mM EDTA, and 2.5 μl of 2-mercaptoethanol, final pH 7.5. The sample was then homogenized at room temperature for 30 s and centrifuged at 1,500 x g for 10 min. The resulting supernatant was transferred to a new 50 ml disposable polypropylene tube. Homogenization and centrifugation steps were repeated on the matrix pellet, and the supernatants were pooled. 650 μl of 2 M potassium acetate (pH 4.0) was added to the pooled supernatant, mixed with a Vortex mixer, and chilled on ice for 15 min. 8.0 ml of 3 M sodium acetate, pH 6.0, was then added, mixed with a vortex mixer, and chilled on ice for an additional 15 min. The majority of solubilized matrix proteins were then removed by sequential (usually 3) extractions (1:1, v/v) with phenol:chloroform:isoamyl alcohol (24:24:1) until a white precipitate at the organic/aqueous interface was no longer evident.

The targeted region of FN cDNA was then amplified for 35 PCR cycles by RT-PCR of equine cartilage RNA using CTGGTGCTGCTGCT- CACGCTGCTGCTGCTGCTGCT- GACCTTTTACCCCTTTGTCAC as primers. These samples were initially analyzed by Northern blot hybridization using three FN cDNA probes: an 841-base pair transcript isolated from the amplified RT-PCR product of primers 6s and 6a, shown in Table I, starting with liver RNA), and the carboxyl terminus (amplified RT-PCR product of primers 7s and 7a, shown in Table I) were indeed cloned into pGEM-3Zf(-) (Promega). Madison, WI. A type II procollagen CDNA fragment extending from exon 1 to 7 was generated by RT-PCR of equine cartilage RNA using CTTGTTGCTGCTGCT- GACCTTTTACCCCTTTGTCAC as primers. The elongation factor Tu cDNA was a gift from Dr. Roy Levine (Levine et al., 1993). Radioactively labeled probes were prepared from gel-purified CDNA insert pools using [γ-32P]dCTP and random hexanucleotide primers (Prime-a-Gene, Promega) and purified with Sephadex G-50 spin columns (Boehringer Mannheim). Prehybridization, hybridization, and wash conditions followed protocols recommended by the manufacturer of the nylon hybridization membrane.

RESULTS

Cartilage Splicing Patterns of FN mRNA—Total RNA was isolated from equine articular cartilage, liver, second passage monolayer chondrocyte cultures, and peripheral blood lymphocytes. These samples were initially analyzed by Northern blot hybridization using three FN cDNA probes: an 841-base pair fragment flanking the stop codon, full-length ED-B, and full-length ED-A (Fig. 1). The first probe, which is outside of all known alternatively spliced regions in FN, detected four distinct mRNA bands in cartilage with sizes estimated at 7.3, 7.6, 8.1, and 8.4 kb. In contrast, only two FN-specific bands were detected in the liver and cultured chondrocyte samples. The 7.3-kb band was the major FN transcript in cartilage but appeared to be completely absent in both the liver and chondrocyte RNA samples. Hybridization with ED-B cDNA detected only the 7.6- and 8.4-kb bands in cartilage and a single band in chondrocyte RNA. There was no evidence of ED-B positive hybridization in the liver. The ED-A probe recognized a single band in the monolayer chondrocytes but did not demonstrate any specific hybridization in the liver or cartilage RNA sam-

![Fig. 1. Northern blot analysis of cartilage-specific fibronectin RNA splicing patterns. Total RNA was purified from equine liver (adult), chondrocytes (adult), cultured chondrocytes (monolayer, passage 2), and peripheral blood lymphocytes. The RNA was resolved electrophoretically in triplicate, transferred to nylon membranes, and hybridized individually with [32P]labeled fibronectin cDNA probes. The first probe recognized all splice variants of fibronectin (All FN). The second probe was specific for transcripts containing extra domain B (ED-B FN). The third probe was specific for transcripts containing extra domain A (ED-A FN).](image-url)
The presence of (ED-B) band was consistent with retention of the ED-B exon. The size of the larger band of the predicted size in each RNA sample. In the ED-B region of alternative splicing in FN and detected only as single respectively. Primer pairs 1, 2, 4, and 7 were outside of all known fully designed to flank ED-B, ED-A, and the V region, respectively. Primer pairs 3, 5, and 6 were purposefully designed to flank ED-B, ED-A, and the V region, respectively. \((\text{ED-A})^+\) symbolizes retention and deletion of extra domain A, respectively. \((\text{V}^+)^+\) and \((\text{V}^-)^+\) symbolize retention and deletion of the V region, respectively. \((\text{V}^-)^-\) symbolizes a new RNA splice variant in which nucleotides encoding the III-15 and I-10 domains are deleted together with the complete V region. The diagrammatic representation of fibronectin protein in this figure was modified from Hynes (1990).

**TABLE I**

| Name | Position | Sequence |
|------|----------|----------|
| 1s   | 219–234  | GTGGCAACTTGCTCCC |
| 1a   | 2066–2047| CACTGGTACTGACACCATG |
| 2s   | 2047–2066| CATGGGTGCAAGATACCAGTG |
| 2a   | 3329–3309| TCAAGAAGTACCTGAGGTTA |
| 3s   | 3309–3329| TACGGCTGTTGCTGAAGTA |
| 3a   | 4571–4553| CCAGGCGAGAGTTGTAA |
| 4s   | 4553–4571| TAAACAAACTCCTCCTGCTG |
| 4a   | 5372–5352| GCAATTTCCTGTGATCTCGGA |
| 5s   | 5364–5382| AGAAGTTAGAACAGGTGAG |
| 5a   | 5963–5941| AGAGCATAGACACTCACTTCATA |
| 6s   | 5941–5963| TATGAAAGTGGTGCTTATCCT |
| 6a   | 7508–7492| TCTGCACCGAATGCTC |
| 7s   | 7492–7508| GGAGAACAGTGCGACAGA |
| 7a   | 8332–8311| ATTCCTCTTAATCGACGATA |

**Fig. 2. Analysis of fibronectin mRNA structure by RT-PCR scanning.** Total RNA samples prepared from equine articular cartilage (C), cultured chondrocytes (Ch), and liver (L) were analyzed by RT-PCR using fibronectin-specific oligonucleotide primers (Table I). Amplified fragments were resolved in agarose gels and visualized by ethidium bromide staining. \(B^+\) and \(B^-\) symbolize retention and deletion of extra domain B, respectively. \(A^+\) and \(A^-\) symbolize retention and deletion of extra domain A, respectively. \(V^+\) and \(V^-\) symbolize retention and deletion of the V region, respectively. \((V^-)^+\) symbolizes a new RNA splice variant in which nucleotides encoding the III-15 and I-10 domains are deleted together with the complete V region. The diagrammatic representation of fibronectin protein in this figure was modified from Hynes (1990). bp, base pairs.

**Fig. 3. Species comparison of articular cartilage and liver fibronectin RNA splice variants.** Total RNA samples prepared from equine, canine, and rabbit articular cartilage and liver were analyzed by RT-PCR using primer pair 6 (Table I). Amplified fragments were electrophoretically resolved in an agarose gel and visualized by ethidium bromide staining. \(V^+\) and \(V^-\) symbolize retention and deletion of the V region, respectively. \((V^-)^-\) symbolizes a new RNA splice variant in which nucleotides encoding the III-15 and I-10 domains are deleted together with the complete V region. bp, base pairs.

The 7.3-kb band from articular cartilage was substantially smaller than the expected sizes of FN mRNAs based on existing sequence data and the published patterns of alternative splicing. To determine its origin and to compare more specifically FN mRNA structure between articular cartilage, cultured chondrocytes, and liver, we scanned the entire FN coding region by RT-PCR (Fig. 2). A series of FN-specific oligonucleotide primers was synthesized (Table I) and used to amplify each region independently. Primer pairs 1, 2, 4, and 6 were purposefully designed to flank ED-B, ED-A, and the V region, respectively. Primer pairs 1, 2, 4, and 7 were outside of all known regions of alternative splicing in FN and detected only a single band of the predicted size in each RNA sample. In the ED-B region, two bands were generated using RNA obtained from both cartilage and cultured chondrocytes. The size of the larger band was consistent with retention of the ED-B exon. The presence of \((\text{ED-B})^+\) mRNA in cartilage and cultured chondrocytes, but not in liver (Fig. 2), was in agreement with the Northern blot results (Fig. 1). RT-PCR with primers flanking the ED-A region amplified two bands using RNA obtained from the cultured chondrocytes. These two bands differed by 270 base pairs, which is consistent with retention of the ED-A exon in a portion of the chondrocyte FN transcripts. There was no evidence of \((\text{ED-A})^+\) transcripts in cartilage or liver. A novel result was obtained in the V region. RT-PCR of RNA isolated directly from articular cartilage produced a major band approximately 400 base pairs smaller than the lower band amplified from RNA obtained from liver and cultured chondrocytes. Although the two major bands amplified from liver and cultured chondrocytes were of the size expected for \(V^+\) and \(V^-\) mRNA, the band amplified from cartilage suggested a deletion of additional nucleotides. The presence of this smaller FN transcript in articular cartilage was also confirmed in samples collected from two additional species, canine and rabbit (Fig. 3). To determine the exact nucleotide sequence identity of the major (but smaller) cartilage band and to make comparisons with the two liver bands amplified using primer pair 6, these three cDNA fragments were independently cloned and sequenced. As predicted by their size, the two bands in liver result from inclusion, \(V^+\), and exclusion, \(V^-\), of the 360-base V region. The major band in cartilage, however, results from a previously unreported RNA splicing pattern that extends from the normal 5' -splice donor site of the V region to the 3' acceptor site upstream of the exon encoding the type I homologous segment 11 (I-11). This splicing pattern maintains the same translational codon reading frame but completely eliminates from the mRNA nucleotides that normally encode segments V, III-15, and I-10 in FN protein. The complete nucleotide sequence for the full-length cDNA fragment amplified with primer pair 6 from canine chondrocytes and equine liver is given in Fig. 4A. Base differences with FN sequences published for rat and human are indicated. The amino acid sequence
FIG. 4. Nucleotide sequence of canine and equine fibronectin cDNA in the region of a novel splice variant expressed in articular cartilage. The PCR-amplified 797-base pair cDNA fragments designated (V1C)2 from equine (Fig. 2) and canine (Fig. 3) articular cartilage and the PCR-amplified 1567-base pair cDNA fragments from equine liver and canine chondrocytes were purified, cloned, and sequenced as described under "Experimental Procedures." These data are available through GenBank® accession numbers U52105, U52106, U52107, and U52108. A, the 1567 canine sequence is given in its entirety. Divergence from the equine sequence and from the published rat (Schwarzbauer et al., 1987) and human (Kornblihtt et al., 1985) fibronectin sequences are indicated. The locations of the borders between protein segments are also indicated. Alternative splice sites within the V region are starred. Canine sequence data were determined from four independent clones analyzed in both orientations. Equine sequence data were determined from three independent clones analyzed in both orientations.

B
predicted by the new mRNA splice junction in articular cartilage is shown in Fig. 4B. We designate this new splicing pattern for FN transcripts (V+I-10).

To confirm independently of a PCR-based assay that the 7.3- and 7.6-kb FN transcripts in articular cartilage lack nucleotides encoding protein segments III-15 and I-10, additional Northern blot analyses were conducted (Fig. 5). Separate cDNA probes specific for the III-15 and I-10 segments both hybridized to the 8.1- and 8.4-kb bands but failed to recognize the smaller cartilage transcripts as distinct bands. In contrast, the banding patterns in RNA isolated from liver and 21-day (second passage) articular cartilage transcripts is only barely detectable. The kinetics of this change was examined by comparing chondrocytes after 1, 3, 7, 10, 14, 21, and 46 days in culture. The RT-PCR band profile of FN transcripts (V+C) is shown in Fig. 4B. We designate this new splicing pattern similar to that of liver. Although the III-15 and I-10 segments are deleted together with the complete V region, respectively. (V+C) symbolizes a new RNA splice variant in which nucleotides encoding the III-15 and I-10 segments are deleted together with the complete V region. No bands were amplified from articular cartilage RNA when the reverse transcriptase was omitted (−RT, lane 14). No contaminant bands were observed when RNA was omitted (−RNA, lane 15).

**DISCUSSION**

In this report, we demonstrate a new FN splicing pattern in articular cartilage that extends beyond the normal V region. This new FN splice variant is not present in the liver and is lost over time when chondrocytes are removed from their extracellular matrix and placed in monolayer cultures. We have termed this splice variant (V+C) "C" denotes the cartilage-sensitive region that includes 411 nucleotides that would normally be translated into the III-15 and I-10 segments. The absence of nucleotides encoding III-15 and I-10 segments is explained by our previous observation that two monoclonal antibodies specific for epitopes within this segment
fail to recognize the small cartilage FN protein subunits in Western blots (Burton-Wurster and Lust, 1989).

Chondrocytes cultured in monolayer for up to 46 days demonstrate a progressive decrease in steady-state levels of (V+C) FN broadly parallel to the loss of type II procollagen expression. Since an RT-PCR assay was used to follow the changes in FN splicing, however, these data do not determine the exact quantitative relationships. Alternative splicing of ED-A has been previously associated with chondrocyte de-differentiation. (ED-A) FN is not normally expressed in cartilage but appears when chondrocytes are cultured and is further increased in passaged monolayer cells (Burton-Wurster et al., 1988; Burton-Wurster and Lust, 1989; Bennett et al., 1991). The expression of (ED-A) FN can be modulated by the addition of dibutyryl cAMP (Leipold et al., 1992) or transforming growth factor β1 (Zhang et al., 1995a). In contrast, (ED-B) FN is expressed at high levels by chondrocytes within articular cartilage (15–35%), remains relatively high in primary chondrocyte cultures (18%), and is sensitive to the addition of transforming growth factor β1, but is decreased by the addition of dibutyryl cAMP to the culture medium (Zhang et al., 1995a). Loss of the cartilage-specific (V+C) splicing pattern of FN along with the appearance of the (ED-A) isoform may prove to be an early and even more sensitive marker of chondrocyte de-differentiation in culture than is the loss of type II procollagen and aggrecan core protein expression.

Since the liver is the primary source of plasma FN, the two splice variants generated by RT/PCR from both equine and canine liver RNA are consistent with a distribution in which approximately half of the protein subunits lack the V region and half include all or a part of the V region. This is similar to what has been reported for mouse, human, rat, and cow but unlike that for the chicken in which no FN transcripts have the V region totally excluded (Schwarzbauer et al., 1985; Kornbluth et al., 1985; Norton and Hynes, 1987; Schwarzbauer, 1991). For human and rat FN, the V region and the first half of the III-15 segment are encoded by one exon (Tamkun et al., 1984; Vibe-Pedersen et al., 1986). There is, however, an internal acceptor site immediately preceding the bases encoding III-15 that permits the entire V region to be spliced out. This region is conserved in the dog and horse, as is the alternative splice acceptor site that results in a deletion of the first 25 amino acids of the V region. In humans but not in the rat, an alternative splice donor site (GTGAG, beginning at base 881, Fig. 4A) permits deletion of the final 31 amino acids in the V region and accounts for the total of five different FN splice variants in the V region of humans versus only three in the rat.

The comparable sequence in the dog and horse is ATGAG, which is identical to that in the rat. Loss of the invariant GT dinucleotide suggests that the dog and horse, like the rat, will have only three splice variants within the V region and not five.

In addition to the smaller and cartilage-specific (V+C) isoform, we also detected by Northern blot hybridization and RT-PCR, FN transcripts in cartilage that have a size consistent with V region splicing patterns comparable to that observed in the liver. Burton-Wurster and Lust (1989) previously estimated that cartilage FN was 80% V+. This was based on the generation of a high percentage of a 30-kDa heparin binding fragment after thermolysin digestion, reflecting the presumed insertion of the thermolysin-sensitive V region between the type III-14 and III-15 segments (Pande et al., 1987). The accuracy of this assay, however, is invalidated by the deletion of the III-15 domain in the (V+C) isoform. Therefore, based on the transcriptional results reported here, the earlier quantitative estimate of V+ appears to be much too high. Renic et al. (1995) reported that FN splicing in seven human articular cartilage samples exhibited a pattern in which the V region was deleted. They would not, however, have been able to identify the (V+C) splice variant since the antisense primer they chose for PCR amplification was within the I-10 region.

From the Northern analysis data in Fig. 1, it is clear that the cartilage-specific (V+C) splice variant may include or exclude the ED-B segment. Direct quantitation of 32P-labeled decay events on the hybridization membrane by phosphomager analysis (Fuji Biologic, Stamford, CT) suggests the following approximate distribution of FN transcripts in adult equine articular cartilage: 63% (B-BV), 21% (B+BV+V), 11% (B+B+V), and 5% (B+B+BV+V). In total, the 26.74 ratio of B:BV transcripts is entirely consistent with published observations (Zhang et al., 1995a, Renic et al., 1995). It is also interesting to note that the percentage of (ED-B) + transcripts is 25% in (V+C) splice variants, which is roughly comparable to the 31% in the V+/V+ splice variants. Based on the observed patterns of alternative RNA splicing, the predicted major protein isoforms of FN expressed in adult equine articular cartilage are summarized in Fig. 8. PCR data with primer pair 6 also support, however, the presence of other minor V region splice variants in cartilage (Figs. 2, 3, and 7). These minor variants were not independently resolved by our Northern blot analyses. The possibility of subdivisions within
the C region (nucleotides encoding III-15 and I-10) must also be considered and is not resolved by these data.

Functions for some of the splice variants in the V region have been investigated and include the identification of sites that are important for cell adhesion and FN dimer secretion and the ability to affect covalent cross-linking in fibrin clots (Schwarzbauer, 1991). Functions for the ED-B and ED-A regions remain speculative but may be related to the assembly of FN dimers into pre-existing matrices. Consistent with that idea, Zhang et al. (1995b) have shown that newly synthesized (ED-B) F N is preferentially retained in the cartilage matrix compared with that of (ED-B) F N.

The tissue-specific pattern of (V+C) − expression and its loss by chondrocytes in monolayer culture suggest that this FN isoform may play an important role in articular cartilage matrix organization. It is not yet known if the (V+C) − splice variant is also a predominant isoform in other cartilaginous tissues such as meniscal, nasal, costal, and tracheal cartilage. Tracheal cartilage in particular will be of interest since Zhang et al. (1995a) found that it is the only cartilaginous tissue that does not express the (ED-B) F N isoform of FN. At present, we have no idea what properties the loss of the III-15 and I-10 segments may confer on the mature FN protein. The III-15 domain contains a sulfhydryl group that may be involved with activity within articular cartilage. Although these and other possibilities need to be independently investigated, the tissue-specific pattern of (V+C) − FN expression suggests that this isoform has an important function in articular cartilage.

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