Novel cartilage-specific splice variants of fibronectin

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Summary

Objective: To determine the nature of alternatively spliced isoforms of fibronectin expressed in healthy bovine articular cartilage and cartilage derived from human osteoporotic and osteoarthritic joints.

Design: Our study focused on a single alternatively spliced region of the fibronectin gene, the variable region. Bovine cartilage samples were obtained within 12 h of slaughter and human cartilage samples were obtained within 8 h of the time of joint replacement surgery. RNA was extracted and alternatively spliced isoforms of fibronectin were amplified using RT-PCR.

Results: Two novel alternatively spliced forms of fibronectin designated (V+I-10)− and (V+III-15)− were identified in bovine articular cartilage. Fibronectin is composed of multiple repeats of three types of homologous units and these two novel isoforms specifically splice out single individual repeating units. Expression of all these isoforms was dependent upon the presence of an extracellular matrix. The (V+C)− isoform was present in all samples and the (V+I-10)− isoform was distributed between both osteoporotic and osteoarthritic cartilage. However, the (V+III-15)− isoform was shown to be associated with osteoarthritic cartilage with statistical significance (P<0.015). In addition a third novel splice variant was identified and designated as III-15X. Translation of the III-15X isoform results in a truncated form of fibronectin lacking a significant portion of the C-terminus. Further RT-PCR analysis of several other tissue types suggests that these variants are cartilage specific.

Conclusion: Our results demonstrate the existence of three new cartilage specific isoforms of fibronectin and indicate that expression of one or more may be associated with osteoarthritis. © 2002 OsteoArthritis Research Society International. Published by Elsevier Science Ltd. doi:10.1053/joca.2002.0792, available online at http://www.idealibrary.com on...
cartilage-specific. In addition a third splice variant which encodes a truncated form of FN was identified in osteoarthritic cartilage. This data raises the possibility that these isoforms have specific roles in maintaining structural integrity or regulating 'outside-in' signaling in chondrocytes.

Materials and Methods

EXPERIMENTAL SAMPLES

Bovine articular cartilage was obtained from the metacarpal-phalangeal joints of cows (aged 18–24 months) within 12 h of slaughter. Human hip femoral head cartilage (osteoporosis patients aged 56–71 years) and tibial plateau knee cartilage (osteoarthritis (OA) patients aged 54–76 years) was obtained within 8 h of the time of joint replacement. Tissue was collected under the approval of the local ethical committee.

CHONDROCYTE CULTURE

Chondrocytes were isolated by collagenase digestion as follows: 0.5 g of diced cartilage (3×3×1 mm) was incubated in 5 ml Dulbecco's Modified Eagles Medium (DMEM, Life Technologies) containing 10 mg/ml bacterial collagenase (Sigma), 25 mM HEPES, pH 7.5 for 2 h at 37°C with constant mixing before being filtered through a 70 μm cell strainer. Chondrocytes were then recovered from the media by centrifugation and washed once in PBS before plating at a density of 1.5×10^3 cells per cm^2. Cartilage explants were maintained in DMEM with 100 U/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma) with media changes every 2 days. Isolated chondrocytes and the human cell lines, HepG2 (hepatoma ATCC No. HB-8065) and SW1353 (chondrosarcoma ATCC No. HTB-94) were maintained as monolayers in DMEM with 10% (v/v) FCS (Gibco BRL), 100 U/ml penicillin and 100 μg/ml streptomycin and passed after reaching confluence.

RNA ISOLATION

Isolation of total RNA was carried out using RNAzol B reagent (Biogenesis Inc. U.S.A.). Bovine and human articular cartilage were diced (3×3×1 mm) and 0.5 g wet-weight immediately placed in 0.5 ml of RNAzol B. Monolayer cultures were extracted by direct application of RNAzol B (2 ml per 75 cm²). Samples were then processed as described by the manufacturer. The quality of the RNA isolated from the OA derived chondrocytes was variable as assessed by agarose gel electrophoresis. The osteoporosis derived chondrocytes consistently yielded high quality RNA. The disparity is likely to be a consequence of the diseased state of the osteoarthritic cartilage.

RT-PCR, CLONING AND SEQUENCE ANALYSIS

For RT-PCR, first strand cDNA was synthesized from total RNA using Superscript II reverse transcriptase (Life Technologies). 1 μg of RNA was reverse transcribed in a final volume of 20 μl using random hexamer oligonucleotides as primers. Human multiple tissue cDNAs were obtained commercially (Clontech, U.S.A.). Oligonucleotide primer positions in the V region are indicated in Fig. 1. The nucleotide sequences were as follows: VDF: 5′-TATG AAGTGAGTGTCTATGCTCT-3′; VDR: 5′-TTCTGCCACTG TTCTCC-3′; FNIII-14F: 5′-GGGTCTCCTCCCAGAGA AGT-3′; FNI-10R: 5′-CGAGTCATCCGTAGGTTGGT-3′; FNIII-15F: 5′-TCCGTGGCAGCCACGTGTAAG-3′; FNI-11R: 5′-GGCTCATCGTGCCATT TT-3′ and type II collagen T2F: 5′-GGAGATCCTCCCGGCTGAGGGAACACCA-3′ and T2R: 5′-GAATGGGCGTCAGGTCAGGTCAGC-3′. GAPDH primers were obtained from Stratagene. All transcripts were amplified from 1 μl of first strand cDNA reaction using Expand DNA polymerase (Boehringer). PCR reactions contained 25 pmol of each primer, 0.2 mM dNTPs and 0.5 U Expand DNA polymerase in a final volume of 25 μl, and were amplified for 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 60 s, followed by a final extension period of 72°C for 10 min. Reaction products were resolved in agarose gels alongside 1 kb DNA ladder (GibcoBRL), stained with ethidium bromide (10 μg/ml) and visualized by UV transillumination. For cloning, amplified cDNA fragments were gel purified, using the QIAquick procedure (QIAGEN) and ligated into pCR2.1-TOPO (Invitrogen) following the manufacturer’s instructions. Recombinant plasmids were transformed into TOP10 E. coli (Invitrogen), grown in L-Broth containing ampicillin (100 μg/ml) and purified by anion-exchange chromatography following the manufacturer’s instructions (QIAGEN). DNA sequence was
The significance of the distribution of FN splice variants was determined using a two-tailed Fisher's exact test.

Results
SPLICING PATTERN OF FN IN BOVINE ARTICULAR CARTILAGE

To investigate alternative splice forms of FN (shown schematically in Fig. 1), RT-PCR was performed using VDF/VDR primer pairs with cDNA prepared from bovine metacarpal–phalangeal articular cartilage and the human cell lines, SW1353 (chondrosarcoma) and HepG2 (hepatoma). Based upon previously described splicing events this analysis was predicted to produce up to four V+ products, a V− product and additionally, in cartilage, the cartilage-specific (V+C)− product. Figure 2 shows that these products were successfully amplified with the expected distribution. In addition, in the cartilage sample there were two additional products intermediate in size between the V− and (V+C)− transcripts. These were cloned, sequenced and found to correspond to alternatively spliced transcripts which, if translated, would specifically delete the amino acid sequences of the variable region and either the fifteenth type III domain (III-15) or the tenth type I domain (I-10). We have designated these isoforms (V+III-15)− and (V+I-10)− respectively. The nucleotide sequences have been submitted to the EMBL/Genbank databases under accession numbers AJ320525 & AJ320526.

SPLICING PATTERN OF FN IN HUMAN ARTICULAR CARTILAGE

To determine whether the novel splice forms were also present in human tissue, RNA was derived from human hip femoral head cartilage obtained at the time of joint replacement surgery. RT-PCR analysis indicated splicing in the V domain [Fig. 3(A)]. The splice forms identified included the (V+I-10)− and (V+III-15)− isoforms as well as the previously described V+, V−, and (V+C)− isoforms. The (V+I-10)− and (V+III-15)− PCR products were cloned and sequenced to confirm their identity. The nucleotide sequences have been submitted to the EMBL/Genbank databases under accession numbers AJ320528 & AJ320529.

The splicing profile of the V-region of FN in other human tissues was determined using RT-PCR [Fig. 3(B)]. The (V+III-15)−, (V+I-10)− and (V+C)− splice variants were not detected in heart, placenta, liver or lung.

SPLICING PATTERN IN HUMAN OSTEOPOROTIC AND OSTEOARTHRITIC CARTILAGE

To determine whether FN isoform expression was altered in disease we compared the expression profiles using RNA derived from seven human osteoporotic hip femoral head cartilage samples and ten human osteoarthritic knee tibial plateau cartilage samples. In order to...
enable greater resolution of PCR reaction products primer pairs were designed to focus on individual splice variants. For all primer pairs the agarose gel results for three of the osteoporotic and four of the osteoarthritic samples are shown in Figs 4 and 5 and the complete data set is detailed in Table I.

The primer pair III-14F/I-10R enabled us to assess specific expression of the (V+III-15)− isoform as it could not amplify the (V+I-10)− isoform. The (V+III-15)− isoform was detected in one of the seven osteoporotic femoral head cartilage samples (sample 1, Table I) and eight of the 10 osteoarthritic tibial plateau cartilage samples (Fig. 4 & Table I). Using a two-tailed Fishers exact test expression of the (V+III-15)− isoform in osteoarthritic cartilage was shown to be statistically significant (P=0.015). The longer V+ isoforms were successfully amplified in the osteoporotic samples but were not detected in the osteoarthritic samples. This is probably a consequence of the quality of the RNA obtained from the tissue reducing the synthesis of longer cDNA products.

The primer pair III-15F/I-11R enabled us to look at specific expression of the (V+I-10)− isoform. As shown in Fig. 5 and Table I this isoform was detected in all seven of the osteoarthritic cartilage samples but was found only in three of the osteoarthritic samples. Using a two-tailed Fisher’s exact test expression of the (V+I-10)− isoform in osteoarthritic cartilage was shown to be statistically significant (P=0.01). An additional PCR product was detected in four of the osteoarthritic samples (Fig. 5). This product was cloned, sequenced and shown to correspond to a FN isoform resulting from a splice event which removed 139 nucleotides from within the III-15 domain. Translation of this DNA sequence demonstrates that the result of the splicing event is the introduction of a frame shift resulting in the addition of four new amino acids in the III-15 domain before premature termination of the coding sequence. It is possible that additional 5′ splicing events associated with this isoform might correct for this frame shift but this would probably result in significant alteration of the upstream amino acid sequence. We have designated this splice variant III-15X and deposited it in the EMBL/Genbank databases under accession number AJ320527.

EXPRESSION OF NOVEL FN ISOFORMS IS MATRIX DEPENDENT

To examine whether these additional splice variants were expressed in a matrix dependent manner, as has been described for the (V+C)− isoform9, a single bovine metacarpal–phalangeal joint was used to establish cartilage explant cultures, and to isolate and seed chondrocytes in monolayer culture. RNA was isolated from these culture systems as well as directly from the original tissue. RT-PCR analysis was carried out as described in Materials and methods. Figure 6 indicates that the splicing profile after 14 days of explant culture was identical to that of cartilage extracted directly after removal from the joint. However, the chondrocyte monolayer culture showed a time dependent loss of the (V+C)− transcript and the novel (V+III-15)− and (V+I-10)− isoforms. This loss was associated with the decreased expression of type II collagen.

EXAMINATION OF GENOMIC DNA SEQUENCE OF THE HUMAN FN GENE

Examination of the described FN exon–intron organization shows that the novel (V+III-15)−, (V+I-10)− and III-15X isoforms are all generated by splicing events between
known exons. The formation of a complete III-15 domain requires appropriate splicing of two exons. In the case of the III-15X isoform the second exon is skipped leading to the potential frame-shift and premature termination codon. Sequence analysis of the two III-15X exons identifies purine rich elements (PREs) immediately upstream of both of the 5′ donor sites. PREs have recently been shown to exert a regulatory effect upon splicing of the ED-B exon in FN12.

**Table I**

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Two-tailed Fisher's exact test

The presence or absence of each FN splice variant was determined by RT-PCR. The statistical significance of the distribution was determined using a two-tailed Fisher's exact test.

**Fig. 5.** Expression of the (V+I-10)− isoform in human osteoarthritic and osteoporotic cartilage. (A) RT-PCR was carried out using the FNIII-15F and FNI-11R primers as described in Materials and methods. The results for the three osteoporotic (OP) samples and four osteoarthritic samples used in Fig. 4 are shown, as well as a water control and size markers (SM). The position of the novel III-15X isoform is indicated. (B) A schematic diagram of the isoforms indicating the position of the primers and the expected PCR product sizes.

**Fig. 6.** Expression of novel FN isoforms is dependent upon the presence of the extracellular matrix. Cartilage and chondrocytes were cultured as described. RNA extracted and RT-PCR carried out using primers specific for FN, Type II collagen and GAPDH. Lane 1—bovine articular cartilage from the metacarpal–phalangeal joint extracted on day 1, Lane 2—bovine articular cartilage extracted after explant culture for 14 days, Lane 3—primary bovine chondrocytes were isolated from the metacarpal–phalangeal joint and extracted after 7 days in monolayer culture, Lane 4—primary bovine chondrocytes isolated from the metacarpal–phalangeal joint and extracted after 14 days in monolayer culture, Lane 5—water control. Size markers (SM).
When chondrocytes were isolated and cultured in the absence of a matrix, expression of the (V+III-15)− and (V+I-10)− isoforms is dependent upon the presence of an ECM and loss of either of these domains would significantly impair the ability of fibronectin to incorporate into the cartilage ECM environment. The III-15 domain may also be required for binding to chondroitin sulfate, the major glycosaminoglycan attached to aggrecan. Consequently selective deletion of the III-15 or I-10 domains may impact upon rate and type of matrix synthesized. Studies using deminectins, truncated FN constructs, have indicated that the (V+C)− isoform is secreted as a homodimer and is not capable of heterodimerization with V+ isoforms. Also, a mutant FN lacking the I-10 to I-12 region when expressed by two to one. It remains to be determined whether the (V+III-15)− and (V+I-10)− isoforms are expressed as proteins and would be able to heterodimerize with the (V+C)− isoform or favor monomeric secretion. In addition FN is involved in ‘outside-in’ signaling events via interactions with several integrins and a number of other proteins and would be able to heterodimerize with the (V+C)− isoform or favor monomeric secretion.

Fig. 7. Human FN genomic organization. The human FN cDNA is depicted spanning from the end of the III-14 domain to the end of the I-11 domain. The position of the introns is marked in the sequence and indicated on the right are the identity of the spliced domains. The positions of several putative purine rich elements (PREs) upstream of 5′ acceptor sites are underlined.

Discussion

We have examined the mRNA splicing events in the variable region of the FN gene and have identified two novel cartilage specific isoforms of FN designated (V+III-15)− and (V+I-10)−. Both variants are expressed in juvenile bovine articular cartilage and adult human articular cartilage. This is the first description of multiple splicing events in humans involving the sequences encoding the III-15 and I-10 domains.

It has been reported previously that the major FN isoform in equine and canine cartilage is (V+C)−8. Our RT-PCR experiments confirm that this observation extends to human cartilage. The relative intensities of PCR products suggest that the novel (V+III-15)− and (V+I-10)− isoforms may be minor species, although it should be noted that RT-PCR is not a truly quantitative measure. We have examined the expression profiles of the FN splice forms in osteoporotic hip and osteoarthritic knee cartilage and adult human articular cartilage. This is the first description of multiple splicing events in humans involving the sequences encoding the III-15 and I-10 domains.

We have also confirmed that expression of the (V+C)− isoform is dependent upon the presence of an ECM and that this is also the case for the (V+III-15)− and (V+I-10)− isoforms8. When chondrocytes were isolated and cultured in the absence of a matrix, expression of the (V+III-15)−, (V+I-10)− and (V+C)− isoforms was lost within 7 days and this was coincident with the loss of expression of type II collagen. Thus, expression of these isoforms of FN appears to be associated with a chondrocytic phenotype and lost during the dedifferentiation observed when chondrocytes are placed in monolayer culture.

Alternative splicing of the V region was observed between all four described donor and acceptor splice sites when only the V region was subject to splicing. However, only the most 5′ and most 3′ sites were used in splicing events involving the III-15 and I-10 sequences. A recent study has shown that the splicing of the ED-B exon is regulated in part by purine-rich elements (PREs) which act as enhancer elements and are required for accurate 5′ splice site selection in the absence of a strong consensus sequence12. A separate study has also implicated TGCATG repeats in regulating ED-B splicing13. The critical sequence elements required to regulate 5′ splice site selection have not yet been identified; however, splice suppressors have been described in the FN ED-A exon which rely on secondary structure for activity14,15. In the cartilage specific splicing profiles we have described PREs are located immediately upstream of the majority of the splice sites. These sequences may be involved in regulating the choice of 5′ splice site selection through interaction with chondrocyte-specific splicing factors.

We have demonstrated that the existence of mRNA species for the (V+III-15)− and (V+I-10)− variants. If these isoforms of FN are expressed then one can speculate on their functional significance. The III-15 and I-10 segments have been implicated in regulating the rate and type of matrix synthesized. Studies using deminectins, truncated FN constructs, have indicated that the (V+C)− isoform is secreted as a homodimer and is not capable of heterodimerization with V+ isoforms. Also, a mutant FN lacking the I-10 to I-12 region when expressed in COS cells is secreted with monomers exceeding dimers by two to one. It remains to be determined whether the (V+III-15)− and (V+I-10)− isoforms are expressed as proteins and would be able to heterodimerize with the (V+C)− isoform or favor monomeric secretion.

In addition FN is involved in 'outside-in' signaling events via interactions with several integrins and a number of other proteins and would be able to heterodimerize with the (V+C)− isoform or favor monomeric secretion.
ECM molecules, for instance the RGD site in the tenth type III repeat has been shown to interact with the α5β1 and the αvβ3 integrins, while the V and III-14 domains are recognized by the α4β1 integrin. All of these integrins are expressed by chondrocytes. There are also many domains in FN which contain cryptic activities only realized by conformational change or proteolytic cleavage. Expression of the III-15X variant lacking sequences C-terminal to the III-15 domain would prevent dimerization and possibly expose a sequence within the III-14 domain that has been shown to have antiadhesive properties. Deletion of the V region in concert with either the II-15 or I-10 domains is likely to cause conformational changes in the surrounding domains and may impact upon the associated matrix interactions or signalling events. It may also alter the relative susceptibility to proteolytic cleavage. In OA elevated levels of FN and catabolic FN fragments are found in the cartilage and synovial fluid. Loss of the V, III-15 and/or I-10 domains may alter the type of fragments generated.

In conclusion, this study describes three additional splicing events that occur in the sequence encoding the V, III-15 and I-10 regions of FN. The (V+III-15)−, (V+I-10)− and III-15X isoforms appear to be cartilage-specific and their expression is dependent upon an extracellular matrix. The RT-PCR analysis, while not being truly quantitative, implies that the (V+III-15)−, (V+I-10)− and III-15X isoforms are minor splice variants in cartilage when compared with the predominant (V+C)− isoform. The physiological role of these variants in normal and diseased cartilage remains to be determined.

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References