Differential Expression of Alternatively Spliced Fibronectin in Normal and Wounded Rat Corneal Stroma Versus Epithelium

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Purpose. The polymerase chain reaction was used to examine fibronectin (FN) expression during corneal scrape wounding with specific attention to the presence, absence, or gross changes of alternatively spliced FN as differentially expressed in the corneal stroma versus the epithelium in normal and wounded tissue.

Methods. Specific FN cDNA sequences were synthesized from rat cornea with total RNA and were amplified using various sets of synthetic oligonucleotide primers.

Results. The authors observed the presence and sustained the expression of total FN, EIIIA, EIIIB, and V-region FN mRNA in normal and injured corneal stroma for up to 3 weeks after scrape wounding. In contrast, complementary overlying epithelial samples were virtually devoid of FN message.

Conclusions. These data suggest that functionally different, alternatively spliced FN isoforms may be involved both in the maintenance of the normal cornea and in wound healing, and that their synthesis occurs in situ principally by the stroma rather than by the epithelium.


Fibronectin (FN) is a multifunctional, high-molecular-weight dimeric glycoprotein that is synthesized by most cells, circulates in the plasma, and is found on cell surfaces and in the extracellular matrix.1 It is thought to promote cellular attachment, migration, chemotaxis, and phagocytosis, thereby serving an important function in embryogenesis and tissue repair.2-4

In the normal rat and human cornea, FN is distributed throughout the epithelial basement membrane zone, the stroma, and Descemet's membrane, whereas it is conspicuously absent from the epithelium.5 In a variety of corneal wound models, FN appears within 12 hours as a dense linear matrix on the bare wound surface and beneath the migrating epithelium, with accompanying stromal deposition.6,7 Once the wound has reepithelialized, the amount of FN in these areas gradually decreases. Although these observations suggest a fundamental role for FN in corneal wound healing, FN deposition onto the wounded corneal surface has been convincingly shown not to be essential for successful epithelial wound closure in simple abrasions8-10 or in superficial keratectomies.11 Furthermore, despite early encouraging anecdotal reports12,13 of FN efficacy in enhancing corneal epithelial defect closure in humans, the exogenous administration of FN has failed to improve significantly the healing of persistent epithelial defects in randomized placebo-controlled clinical trials.14,15

The structure of FN has been studied extensively. The glycoprotein is composed of three types of repeating homology units designated type I, type II, and type III16 (Fig. 1). Molecular cloning has shown that a single primary messenger RNA (mRNA) transcript of FN may undergo alternative splicing in three regions, EIIIA (ED-A), EIIIB (ED-B), and V (III-CS), generating up to 12 different FN isoforms in the rat and 20 different isoforms in the human.1,17 EIIIA and EIIIB are single type III domains encoded by single
exons either included or excluded during splicing. Alternative splicing of the V region generates three possible variants in the rat and five in the human, all of which arise from exon subdivision of the coding sequences. The plasma form of FN includes the V region 50% of the time, whereas the EIIIA and EIIIB domains are spliced out. In contrast, FN synthesis in situ, or “cellular-associated FN,” typically retains these domains.

Initial immunohistochemical techniques employing anti-FN antibodies could not distinguish between the cellular and plasmatic isoforms of FN. Monoclonal antibody and molecular biologic technology have enabled the identification of alternatively spliced isoforms of FN. Using the in situ hybridization technique, mRNA containing the EIIIA and EIIIB domains has been found after cutaneous wounding. These FN isoforms are expressed in a defined pattern recapitulating that seen in embryogenesis.

Conversely, all three FN isoforms appear to be spliced out at a greater frequency in aging tissues and cells as demonstrated by the reverse transcriptase polymerase chain reaction (PCR). Endogenous FN has been reported to participate in corneal wound healing, and the presence of cellular FN has been demonstrated immunohistochemically in the cornea after anterior keratotomy. The functional significance of cellular FN and the factors influencing alternative splicing in corneal wound healing remain obscure. In addition, although keratocytes, corneal epithelial cells, and endothelial cells have been shown to synthesize FN in culture, the cell types responsible for FN production during corneal wound healing have not been identified. It has been suggested that specific FN isoforms generated by alternative splicing of FN mRNA may be more functionally important to the migration and proliferation of corneal epithelial cells during wound healing than is plasma FN.

In the present study, we used PCR to examine the expression of FN mRNA isoforms in a rat corneal epithelial scrape wound model. Synthetic, region-specific oligonucleotide primers were used to amplify specific cDNA sequences to determine the presence, absence, or gross changes in alternatively spliced FN as differentially expressed in corneal epithelium versus stroma in both normal and wounded tissue.

**MATERIALS AND METHODS**

**Animal Model**

Female CD rats weighing between 220 g and 250 g (Charles River Laboratory, Wilmington, MA) were fed and housed in conformance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rats were anesthetized with an intraperitoneal injection of 0.2 ml of a 1:1 mixture of xylazine (5 mg/kg) and ketamine HCL (25 mg/kg).

Scrape wounding was performed under a Zeiss (Oberkochen, Germany) operating microscope. A 4-mm trephine was used to demarcate the central corneal epithelium of each eye, and this area was gently debrided with a Grieshaber microsurgery knife (cat. no. 681.01; Schaffhausen, Switzerland). One rat from each of three separate groups was killed by intraperitoneal injection of 0.2 ml of a 1:1 mixture of xylazine (5 mg/kg) and ketamine HCL (25 mg/kg).

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jured animals were manipulated in the same fashion as the wounded rats, including anesthesia.

RNA Extraction
Total RNA was extracted from the epithelial and stromal samples by the guanidine HCl–phenol protocol of Chomczynsky and Sacchi. Briefly, the separately pooled stromal and epithelial samples from each time point were solubilized in 0.75 ml and 0.3 ml of RNAzol B solution (Cinna/Biotex Lab, Houston, TX), respectively, with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). The generator tip of the homogenizer was cleaned in 80% ethanol and autoclaved between samples. RNA was extracted with phenol and chloroform, precipitated with isopropanol, washed with 80% ethanol, and resuspended in distilled water. Total RNA was also extracted from rat liver and from confluent rat embryo cell culture monolayers (rat 2, ATCC, Rockville, MD) grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. These specimens served as positive controls for the alternatively spliced FN isoforms.

cDNA Synthesis
FN RNA was reverse transcribed to generate cDNAs using oligo(dT) as primers. Separate samples containing 3 μg and 4 μg of total RNA per time point were denatured in distilled water for 10 minutes at 70°C and incubated at 37°C for 1 hour with the following reagents (total volume of 20 μl): 5 mM each dideoxy nucleotide triphosphate (dNTP; Pharmacia, Piscataway, NJ), 50 mM Tris-HCl pH 8.3, 20 units of RNAsin (Promega, Madison, WI), 0.5 mM oligo(dT), and 12 units (for 3 μg RNA) or 16 units (for 4 μg RNA) of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim, Indianapolis, IN). The reverse transcriptase reaction was brought to 200 μl with distilled water at the end of the incubation. A working mixture of cDNA was prepared containing equal volumes of product generated from the initial 3-μg and 4-μg RNA samples of each time point, and it was stored at −70°C until use.

Primer Selection
The primer sequences used to detect alternative splicing of FN RNA have been described. To amplify a region spanning all FN mRNA types, primers were chosen from the sequence of type 3 repeat exon 17, and they were chosen from type 1 repeat exon 10 to amplify a 272-base pair (bp) DNA fragment reflecting total FN mRNA expression (Table 1). Briefly, primers were selected on the basis of desired size and location (between 200 and 800 bp in exons flanking alternatively spliced exons) and lack of homology to other known sequences. Primers for PCR analysis were synthesized with a Gene Assembler (Pharmacia LKB, Piscataway, NJ) and purified using an NAP-10 column chromatography (Pharmacia).

PCR Amplification
PCR was performed with the cDNA working mixture in a 25-μl reaction volume containing 20 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 250 mM dNTP, 2 mg/ml bovine serum albumin, specific 5' and 3' primers, and 0.5 units of Taq DNA polymerase (Boehringer). The optimal concentration for each primer set is as follows: 5 pmol glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 10 pmol total FN, and 20 pmol for each of the EIIIA, EIIIB, and V regions. Cycle numbers varied with the sample tested: 25 to 30 cycles for stromal samples and 30 to 35 cycles for the epithelium. Amplification was performed in a thermocycler model 9600 (Perkin Elmer Cetus, Norwalk, CT) programmed for denaturation at 94°C for 10 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 2 minutes. Polymerase chain reaction products were electrophoretically fractionated on a 1.5% agarose gel containing ethidium bromide, visualized by ultraviolet light, and photodocumented using polaroid photography. The observed PCR products corresponded to their expected molecular weights.

The volume of cDNA used in each reaction was determined by an initial optimization step with GAPDH. The expression of GAPDH mRNA, a "housekeeping" enzyme, is not expected to vary significantly among different cell types. Previous work in our laboratory has shown that GAPDH expression remains constant after corneal scrape wounding, at all time points tested, when analyzed by Northern blot hybridization (data not shown). Hence, it was used to monitor the efficiency of cDNA synthesis, thereby normalizing the amount of cDNA amplified by PCR in each sample. After dilution of the newly synthesized cDNA, a volume of 2 to 12 μl of each sample was amplified by PCR with GAPDH primers. The final amount of cDNA used for each individual sample was chosen such that the GAPDH PCR products, as judged by relative band intensity, were approximately the same for all samples within a group. In this way, similar amounts of cDNA were used to study FN expression. In addition, the cDNA amount chosen produced a PCR product that fell within the linear range on a standard curve to avoid the saturation effects that can result from the presence of excess cDNA template. Standard curves were constructed for each of the five primer sets used and for any given cycle number with cDNA concentrations spanning a 100-fold dilution of the original volume chosen.

Negative- and positive-control RNAs for plasma FN (rat liver) and cellular FN (rat embryo fibroblast) were used with each experiment to confirm the presence of a specific PCR amplification signal. PCR was...
repeated with the same individual sample at least three times for each of the three experimental groups.

**Southern Blot Analysis**

Southern blot analysis was performed using a nonradioactive kit (Boehringer Mannheim, Indianapolis, IN) following standard procedures. Briefly, electrophoresed 1.5% gels were blotted onto nylon membranes (ICN Biomedicals, Irvine, CA) and hybridized to each probe. Membranes were then washed separately in 2 X SSC and 0.5 X SSC, blocked for 30 minutes at 37°C, and incubated with antibody 1:5000 at 37°C for 30 minutes. After a final wash, 1 ml of Lumi-Phos 530 (Boehringer) was added and the nylon membranes were exposed to x-ray film using an intensifying screen.

**Sequence Analysis**

Direct sequencing of the expected PCR products was performed, confirming the identity of the amplified sequences according to protocols. In essence, amplified cDNA samples were purified through sepharose CL-68 (Pharmacia) and combined with 7 pmol of primers end-labeled with 32P. Mixtures of primers and template were heat denatured, and sequencing reactions were carried out with T7 DNA Polymerase (United States Biomedical, Cleveland, OH). The product was analyzed by electrophoresis on 6% denaturing polyacrilamide gels; autoradiography was carried out for 12 to 16 hours without an intensifying screen.

**RESULTS**

Polymerase chain reaction was used to examine FN expression during corneal scrape wounding with specific attention to the presence, absence, or gross changes in alternatively spliced FN message as differentially expressed by the corneal stroma and epithelium in normal and wounded tissue. Primers were selected from exons flanking introns for total FN and from exons spanning each of the alternatively spliced FN exons to obviate the inadvertent amplification of genomic DNA. The primer sequences and the respective sizes of their expected amplified products are presented in Table 1. Briefly, amplification with primers flanking introns for total FN result in an expected product of 272 bp, whereas primers specific for GAPDH yield 195 bp. Primer pairs spanning the EIIIA region result in a 526-bp product if this exon is spliced in (EIIIA+) and a 256-bp product if it is spliced out (EIIIA-). Similarly, the 367- and 240-bp PCR products correspond to the EIIIB+ and EIIIB- isoforms, respectively. Finally, three different products of 619, 344, and 259 bp are expected with PCR amplification using V region primers. By virtue of alternative splicing and exon subdivision, the 619-bp product contains the complete V region, the 544-bp product retains part of the V region, and the 259-bp fragment lacks it completely. Amplification of the correct sequences and the identification of the amplified product specified by each primer pair for the different alternatively spliced FN isoforms were confirmed both indirectly, because the size of the PCR products equals the expected size calculated from the distance separating the two primers in the FN mRNA sequence, and directly by sequence analysis of each of these products.

All corneal epithelial and stromal samples from each of the three groups of animals underwent initial PCR optimization for 30 cycles with primers specific for the housekeeping gene GAPDH. The isointensity of the expected 195-bp PCR product for GAPDH at all time points in both the corneal epithelium and stroma (Figs. 2A, 2B) confirms that similar amounts of cDNA were amplified in each of the different samples.

**Normal and Wounded Corneal Epithelium**

The complete absence of EIIIA, V region, and total fibronectin mRNA expression in both normal and
FIGURE 2. GAPDH optimization of (A) corneal epithelial and (B) stromal cDNAs. Lane M, size markers (f < 174, Hae III digest); lane 1, normal cornea; lanes 2 to 7, wounded cornea: 15 minutes, 4, 24, 48 hours, 1 and 3 weeks, respectively. Similar GAPDH mRNA expression was shown in different samples for both the stroma and epithelium as judged by band isointensity. The same volumes of cDNA used in those experiments were chosen for subsequent amplification with each of the alternatively spliced FN primer sets. The amplified PCR product is consistent with the 195-bp expected size demonstrated by fragment sequencing.

wounded epithelial tissue in all time points is readily apparent (Figs. 3A, 3B, 3D; lanes 1 to 7). Although EIIIB− (367 bp) FN appeared faintly in normal and wounded epithelium, there was no expression of alternatively spliced EIIIB+ (640 bp) FN at any time point (Fig. 3C; lanes 1 to 7). Rat fibroblast and liver samples served as positive controls yielding the expected PCR products for total FN and for each isoform tested, yielding the expected PCR products (Figs. 3A, 3B, 3C, 3D; lanes 8, 9). Polymerase chain reaction was carried out at 30 cycles for each experiment involving the corneal stroma.

Three groups of animals were used for three independent experiments, and PCR was repeated three times for each sample. Although the time points in group 1 (5, 15, 30, 45 minutes; 1, 2, 4, 8, 16, 24, and 48 hours) were expanded in groups 2 and 3 (15 minutes; 4, 24, 48 hours; 1 and 3 weeks) to include later time points, the results from the latter two groups are identical, whereas those of the former follow the same trend, namely, the presence and sustained expression of total FN, EIIIA, EIIIB, and V region FN mRNA in both normal and wounded corneal stroma at all time points, and the lack thereof in complementary epithelial samples (data not shown).

DISCUSSION

The distribution and expression of FN in normal and wounded tissue has been studied, employing a variety of techniques and several models of both corneal and cutaneous wound healing.5-8,11,20-22,24 After epithelial scrape debridement, corneal epithelial wound healing is characterized by the early migration and proliferation of neighboring epithelial cells over the denuded area. The functional role of FN in this process is evidenced by the universal appearance of a dense linear FN–fibrinogen matrix on the bare wound surface and within the migrating epithelium within 12 hours after wounding.6 Although wound closure is usually complete within 48 hours, FN is detectable by immunohistochemical techniques for up to 2 weeks after wounding.6 It has been suggested that the early appearance of FN may be derived from the plasma via the limbal vessels and the tear film.6 However, such
FIGURE 3. Polymerase chain reaction analysis of FN mRNA expression in the normal and wounded corneal epithelium. Lane M, size markers (qX 174; Hae III digest); lane 1, normal cornea; lanes 2 to 7, wounded cornea: 15 minutes, 4, 24, 48 hours, 1 and 3 weeks, respectively; lane 8, rat 2 embryonic fibroblasts; lane 9, liver; lane 10, negative control. (A) Total FN mRNA expression: The 272-bp PCR products are present only in rat (lane 8) and liver (lane 9) positive controls. (B) EIIIA mRNA expression: EIIIA+ (526 bp) and EIIIA- (256 bp) are present in rat fibroblasts (lane 8) as expected, whereas only EIIIA- is present in liver (lane 9) positive controls. Both normal and wounded epithelial samples are devoid of EIIIA FN message (lanes 1 to 7). (C) EIIIB mRNA expression: EIIIB+ (640 bp) is present in rat fibroblasts, and EIIIB- (367 bp) appears in rat and liver controls as expected. EIIIB- is also faintly present in normal and wounded specimens (lanes 1 to 7). (D) V region mRNA expression: Rat fibroblast and liver controls are appropriately positive for V+ (617 bp and 544 bp) and V- (259 bp) FN mRNA (lanes 8 and 9). V region FN message is completely absent from normal and wounded samples (lanes 1 to 7).

deposition and the exogenous administration of plasma FN have been well documented to be inessential for successful wound closure in animal studies and fail to improve significantly the healing of persistent epithelial defects in randomized, placebo-controlled clinical trials in humans.

The availability of monoclonal antibodies and the high degree of sensitivity and specificity of molecular biologic techniques, such as in situ hybridization and PCR for alternatively spliced isoforms of FN, have allowed the facile distinction between plasma and cellularly derived FN. Hence, an assessment of the relative contribution of endogenously produced FN and the identification of alternatively spliced FN isoforms, which may be more functionally important to wound healing, are possible.

Alternative splicing of FN is developmentally regulated by the appearance of EIIIA+, EIIIB+, and V+ region isoforms in fetal and tumor cells and by their absence in aging tissues and cells. Using in situ hybridization, both EIIIA+ and EIIIB+ FN expression have been demonstrated in a temporally and spatially defined pattern after punch biopsy in a rat model of cutaneous wound healing. Although normal, unwounded rat skin expressed FN mRNA, there was no expression of alternatively spliced domains.

Regarding corneal wound healing, the presence and persistence of cellular FN in the corneal stroma for up to 3 months after anterior keratectomy has been demonstrated immunohistochemically. Previous studies in our laboratory using in situ hybridization have demonstrated that stromal keratocytes pos-
Most recently, our group has demonstrated the production of EIIIA+, EIIIB-, and V+ FN mRNA after corneal epithelial scrape wounding, anterior keratectomy, and alkali burns in a rat model when whole cornea samples were analyzed by PCR.

In the present study, we used the PCR technique to demonstrate the presence and sustained expression of total FN mRNA and alternatively spliced EIIIA, EIIIB, and V region FN message in both normal and injured corneal stromal tissue for as long as 3 weeks after scrape wounding. In contrast, the overlying complementary epithelial samples were virtually devoid of FN expression, with the exception of low level EIIIB-message, the identity of which was confirmed by direct sequence analysis. Although mRNA production frequently corresponds to synthesized protein, this relationship is not invariably and requires further experimental confirmation. The faint, yet consistently detectable, EIIIB—message in the epithelial samples may represent a forme fruste expression of this molecule that is never translated into protein. The otherwise complete absence of FN mRNA and its alternatively spliced isoforms from the normal rat corneal epithelium in our study is consistent with the previous immunohistochemical localization of FN to the stroma and Descemet's membrane but not to the epithelium. Although in vitro studies have shown that human and rabbit keratocytes, bovine and rabbit endothelial cells, and rabbit corneal epithelial cells all share the capacity for FN synthesis in cell culture, the cell types responsible for FN production during corneal wound healing...
in vivo have yet to be identified. The results of the present study suggest that in the rat, in addition to plasma-derived FN, FN is produced in situ by the corneal stroma rather than by the epithelium in both normal and wounded states, and that this synthesis entails the production of alternatively spliced isoforms.

In the unwounded rat cornea, all FN isoform mRNAs were expressed, including EIIIA+ and EI-IIA−, EIIIB+ and EIIIB−, and the three V region isoforms. Although initial studies in our laboratory revealed variability in the expression of EIIIA+ FN mRNA when whole, uninjured corneal samples were studied by PCR, subsequent experiments by two independent investigators confirmed the consistent expression of all three FN mRNA isoforms in normal rat cornea. This suggests that all FN isoforms play a role in the maintenance of the normal cornea.

After scrape wounding, all FN mRNA isoforms were expressed at all time points, with minor variations in band intensity among samples and when compared to normal controls. Although the amount of cDNA in different samples was normalized by initial optimization with GAPDH and all amplifications were performed within the linear range to avoid the saturation effects of excess cDNA template, the present assay does not permit quantitative statements with respect to the level of FN mRNA expression among samples. However, a proportional analysis of alternatively spliced FN mRNA isoforms for any given time point, as judged by relative band intensity (i.e., the relative brightness of EIIIA+ compared to EIIIA− for normal cornea), is itself internally controlled and has been reported previously. In the present study, the relative predominance of EIIIA− to EIIIA+, the equivalence of EIIIB+ and EIIIB− with the exception of the 1-week time point, and the more pronounced V+:V− ratio in both normal and wounded corneal stroma suggest that some isoforms may be more functionally relevant than others in the maintenance of normal cornea and during wound healing. Whereas dynamic kinetic changes in the expression of these various FN mRNA isoforms may be present after scrape wounding among time points and compared to the normal controls, such analysis awaits the application of truly quantitative PCR, already underway in our laboratory.

The functional significance of the various FN isoforms and the factors influencing alternative splicing are unclear at present. TGF-β, retinoic acid, and vitamin D have been reported to increase the relative amount of mRNA for EIIIA and EIIIB. FN is involved in a number of processes including cell adhesion, migration, proliferation, chemotaxis, and collagen remodeling by virtue of its multifunctional domain structure with binding sites for fibrin, heparin, collagen, DNA, and cell attachment. The major FN cell binding domain, orRGDSsequence, has specificity for the classical FN receptor, the α5β1 integrin, which has been reported to appear in response to epithelial abrasion and keratectomy. Furthermore, the polypeptide receptor for an alternative cell binding sequence in the V region (V1) is the α4β1 integrin, which is expressed in a variety of cell types, including T cells. These observations and the results of our research suggest that functionally appropriate forms of FN may arise from alternative splicing of the primary transcript, and therefore they modulate the migration and attachment of different cellular populations in response to wounding, aging, and growth factors.

In summary, we observed the presence and sustained expression of total FN mRNA, as well as that of alternatively spliced EIIIA, EIIIB, and V region isoforms in both normal and injured corneal stroma for up to 3 weeks after scrape wounding. Stable, proportionate differences within alternatively spliced species were noted, as opposed to frank quantitative changes in the level of mRNA expression among samples. In contrast, complementary overlying epithelial samples were virtually devoid of FN message. These data suggest that functionally different, alternatively spliced FN isoforms may be involved both in the maintenance of the normal cornea and in wound healing, and that they are synthesized principally by the stroma rather than by the epithelium.

Key Words

PCR (polymerase chain reaction), fibronectin isoforms, alternative splicing, epithelial scrape wounds, wound healing

References

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