Role of CD44 in Epithelial Wound Repair

MIGRATION OF RAT HEPATIC STELLATE CELLS UTILIZES HYALURONIC ACID AND CD44v6

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The hyaluronic acid receptor, CD44, exists as multiple splice variants that appear to have a role in migration of tumor cells. The role of this receptor and its variants in normal wound repair is poorly understood. A central feature of wound repair in the liver is activation and migration of perisinusoidal stellate cells. We have examined CD44 expression by stellate cells from normal or injured rat liver, finding that it increases with injury and involves a distinct set of CD44 splice variants. Among the latter, variants containing the v6 exon (CD44v6) are strikingly increased. Analysis of migration of primary cells on transwell filter inserts reveals that only cells isolated from injured liver are migratory. Also, they move more rapidly on hyaluronic acid than on collagen I or collagen IV. A polyclonal antibody to recombinant CD44v6 blocks migration by 50%, whereas antibody to CD44v4 has no effect. The inhibition is specific for cells migrating on hyaluronic acid and is reversed by synthetic peptide representing the N terminus of the v6 protein. In conclusion, activated stellate cells use CD44v6 and hyaluronic acid for migration. Given the evidence that migration is required for progression of injury with scar formation, blockers of CD44v6 expression or function are candidates for preventing the deleterious effects of chronic fibrosis.

A prominent aspect of the liver’s response to injury involves perisinusoidal cells termed stellate cells. With injury, stellate cells undergo activation, displaying a smooth muscle/myofibroblast phenotype characterized by fibrogenesis, migration, and contractility (1). Activated cells aggregate around the injury, where they elaborate collagens and other extracellular matrix proteins that serve as a scaffold on which repair proceeds. When injury is recurrent and chronic, production of extracellular matrix outstrips its removal. Over time, the repair matrix coalesces into dense bands that are populated by activated stellate cells. This process results in the pathological appearance of fibrosis, which can progress to cirrhosis with disruption of lobular architecture and altered blood flow.

Cell migration is fundamental to wound repair, and studies have shown that stellate cells migrate to the area of injury within the liver (2). Several aspects of stellate cell migration have been examined, including chemotactic factors (3–7) and intracellular signaling pathways (6, 8, 9). However, little is known concerning the cell-matrix interactions mediating migration. There is accumulating evidence that a milieu rich in hyaluronic acid (HA; hyaluronan) is particularly conducive to migration. CD44 is well characterized as a major receptor for HA (10). Whereas it is a single gene of 50 kilobases, it is expressed as a large number of variant forms due to a region of 10 exons that undergo alternative splicing in the nascent mRNA. The N-terminal extracellular portion of the protein, representing exons 1–5, is constant. The C-terminal constant region comprises exons 17, which encodes the transmembrane portion, and exons 18–20, representing a short intracellular region. Exons 6–15 are subject to individual alternative splicing. Each may be included or excluded, giving rise to a multitude of isoforms in which the juxtamembranous region of the protein varies. The differing roles of CD44 in tissue injury and repair may reflect exonic variation. These range from local binding and transcytosis of inflammatory cells (11) and induction of chemokine and cytokine responses (10) to smooth muscle migration and contraction (12). Few data directly link individual splice variants specifically to migration, and the role of CD44 in liver injury is unknown.

Substantial effort has been directed at defining the biological roles of specific CD44 splice isoforms. Expression of CD44v6 is prominent in models of tumor metastasis (13, 14). In a model of vascular injury, it was shown that CD44v6 is up-regulated in smooth muscle cells at the luminal edge of the expanding neointima (15). However, there is a paucity of data on isoform-specific changes and their function in epithelial injury in vivo. In experimental studies of liver injury in vivo, an increase in total CD44 was noted with toxin-induced cirrhosis (16); in acute injury induced by partial hepatectomy, there was an increase specifically in CD44v6, although the cellular source of this variant was not determined (17). In the present studies, we have examined the diversity of CD44 expression in stellate cells from normal and injured liver, respectively. The results show that CD44v6 plays a major role in mediating the migration of activated stellate cells in liver injury.

EXPERIMENTAL PROCEDURES

Materials—TRI Reagent was obtained from Molecular Research Center Inc. (Cincinnati, OH), collagenase was from Crescent Chemical Co. (Islayandia, NY), Pronase was from Roche Applied Science, Accudenz® was from Accurate Chemical and Scientific Corp. (Westbury, NY), and ASF104 medium was from Ajinomoto (Tokyo, Japan).

Animals, Liver Injury Model, and Cell Isolation—Sprague-Dawley rats (male retired breeders, 700 g body weight) were anesthetized with Quencher-1.

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1 The abbreviations used are: HA, hyaluronic acid; HSC, hepatic stellate cell(s); 6-FAM, 6-carboxyfluorescein; BHQ1, Black Hole Quencher-1.
with isoflurane and subjected to surgery according to a protocol approved by the Institutional Committee on Animal Care. Total bile duct ligation was carried out as described previously (18). On the fourth postoperative day, the liver was dispersed by enzymatic perfusion in situ. The method was a modification of one previously published from this laboratory. In brief, 100 USP units of heparin were injected into abdominal inferior vena cava, and cannulas were placed in the portal vein and inferior vena cava. The liver was perfused via the portal vein with 0.40 peptide hydrolysis unit/ml collagenase and 0.08% Pronase in Ham’s Dulbecco’s modified Eagle’s medium at a rate of 10 ml/min for 20 min at 37 °C. The digested liver was removed and minced with scissors and then incubated in 0.05% Pronase, 0.50 peptide hydrolysis unit/ml collagenase, and 20 μg/ml DNase I (Roche Applied Science) for 25 min at 37 °C with rotary shaking at 200 rpm. The digest was passed through sterile gauze to eliminate large debris, and the cells were pelleted at room temperature (2000 rpm for 7 min.). The pellet was resuspended in Ham’s Dulbecco’s modified Eagle’s medium and layered on a discontinuous gradient of Accudenz®; the upper and lower layers were specific gravity 17.8 and 10.4, respectively. After centrifugation in a swinging bucket rotor at 20,000 rpm for 30 min (20 °C), HSC were recovered from the interface between the two layers. Yields averaged ~2,000,000 HSC from an individual rat; purity was >95%.

Three types of stellate cell preparations were studied: (a) normal cells isolated from unoperated or sham-operated animals; the cells either were analyzed immediately or were placed in short term primary culture (up to 96 h); (b) in vivo activated cells isolated from animals subjected to bile duct ligation as described above; and (c) culture-activated cells representing normal stellate cells that were allowed to undergo spontaneous activation in primary culture (5–9 days in serum-containing medium).

Primary Culture and Migration Assay—For migration assays on transwell inserts, freshly isolated HSC were cultured initially in ASF104 medium or Dulbecco’s modified Eagle’s medium supplemented with 20% fetal bovine serum. The seeding density was ~5,000 cells/insert, with 200 μl of medium in the insert and 500 μl in the well. After 24–48 h of culture, the medium was switched to serum-free ASF104 for 12 h, which was taken as the zero time point of migration. After a migration period of 12, 24, and 36 h, inserts were fixed and stained. Ten fields (×400) with individual cells only (clusters were ignored) were randomly picked per insert. By focusing in the appropriate plane, the top or bottom of the filter was visualized for counting the number of cells on each surface. On average, a field contained 100–200 cells, and 10 fields were counted. The migration index was defined as the percentage of cells in a field on the bottom side of the filter: (number of cells on the bottom)/number of cells on the top + bottom) × 100.

Amplification and Cloning of CD44 Splice Variants—RNA extraction and purification was carried out with TRI reagent, according to the protocol provided by the supplier. The integrity of the purified RNA was verified by visualization of 28 S and 18 S bands on 1% agarose gel electrophoresis. Reverse transcription was carried out with oligo(dT) primer. The CD44 v1–v10 was amplified by PCR using primers bracketing the exons. Reverse primer was CTTCTTTATTGGGAGCACCC-TGGCC; forward primer was CTACCTGTAGACTCATGGAATCGGATT. Conditions were as follows: annealing temperature 94 °C and 30 cycles of 94 °C for 45 s; 63 °C for 45 s; 72 °C for 1 min. Amplification of CD44 with no variable exons yielded a band of 93 bp.

Quantitation of mRNA—For quantitation of mRNA by an RNase protection assay, total cellular RNA was isolated as above and suspended in water at a concentration of ~1 μg/ml. Approximately 40 μg were used for assaying CD44. As an internal loading control, S-14 mRNA, which encodes a ribosomal protein, was measured in the same samples using 10 μg of total RNA; expression of S-14 by stellate cells changes minimally in liver injury (18). For probe preparation, the appropriate CD44 region was cloned into pGEM4Z. Radiolabeled cRNA was prepared using ~1 μg of cut plasmid, [32P]CTP (80 μCi) and T7 or SP6 RNA polymerase, depending on the orientation of the cloned fragment. After digestion with DNase, the labeled material was purified with a NucTrap column and counted. The final mixture contained ~10⁶

CULTURE SUBSTRATUM

HYALURONIC ACID

COLLAGEN I

PLASTIC

FIG. 1. Migration of hepatic stellate cells 4 days after injury induction. The freshly isolated cells were plated at high density on a surface coated with either HA or collagen I or on uncoated culture plastic. After attachment (24 h), a wound was created in the monolayer with the tip of a pipette. Migration of cells into the wound area was observed over the subsequent 48 h. Migration was greatest on HA.

CD44 splice variant mRNA expressed by normal, culture-activated stellate cells or in vivo activated stellate cells. Liver injury was induced by total bile duct ligation, as described under “Experimental Procedures.” In the case of normal or in vivo activated stellate cells, isolates were checked for purity and then processed immediately for RNA (without a period of culture). The region of CD44 between exon 5 and exon 16 (v1 to v10) was amplified by reverse transcription-PCR. On the left is shown an agarose gel of the amplified products from each reaction. The arrow identifies a DNA fragment of 735 bp that was particularly prominent in extracts from in vivo activated cells. The products of amplification for each cell preparation were mass-cloned into pGEM-3Z (Promega Corp.), which was grown in JM105 competent Escherichia coli and selected by standard methods. The gel shows the size distribution of individual clones.
cpm of probe and cellular RNA. The hybridization temperature was 55 °C. Samples were digested with T2 RNase.

The primer sequences and TaqMan® probes used for quantitative PCR are as follows (F, forward; R, reverse). Primers were V0-F (TCC-ACCATTGAAAGACACCC), V4-R (CACTGGGTCCGTTCCTGG), V6-R (CCCCTGCCATTCATTCTCAA), V0/11-F (CGGGATGACGCCTTTATT), and V0/11-R (CCAGCTAATTCGGATCCATGA). Probes were V0 (6-FAM-CAGAACCAGGAACGGACCCAGTGG-BHQ1), V4 (6-FAM-CAGAACCAGGAACGGACCCAGTGG-BHQ1), V6 (6-FAM-TGGGCAGATCTAATAGCACAACAGAAGAG-BHQ1), V0/V11 (6-FAM-ACCTGGCCACCAGATGGAGACT-BHQ1).

The amplification conditions were as follows: 25 °C for 10 min, 48 °C for 40 min, 95 °C for 5 min. All assays were checked in preliminary studies for efficiency and linearity. Expression levels were normalized to an internal standard (glyceraldehyde-3-phosphate dehydrogenase).

Immunological Assays—Polyclonal antibodies were raised in rabbits using recombinant rat CD44v4 (amino acids 384–423) or V6 (amino acids 463–505) as the immunogen. IgG was purified from whole antisera on a HiTrap™ N-hydroxysuccinimide-activated HP column (Amersham Biosciences). In migration assays, the antibody was introduced at 10 μg/ml. For Western blots, HSC cell lysate (50 μg) was separated on a 7.5% gel by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Hybond™-P; Amersham Biosciences). After blocking with 5% skim milk, the membrane was incubated with rabbit anti-rat CD44v6 polyclonal antibody at 1:1000 dilution. Controls included nonspecific rabbit IgG in place of the primary antibody. Detection was by enhanced chemiluminescence (ECL-plus; Amersham Biosciences).

For validating the specificity of the inhibitory effect of the anti-v6 antibody on HSC migration, synthetic peptides representing the N-termini of CD44v6 were synthesized.
terminal or C-terminal half of the v6 domain were prepared. The sequences were as follows: N-terminal peptide (CD44v6N), WADPNSTTEEAATQKEKWFE; C-terminal peptide (CD44v6C), NEWQGKNPPTPSEDSHVTEGTTA. The peptides were mixed with the polyclonal rabbit anti-v6 and then introduced into the migration assay described above.

RESULTS

Substratum Dependence of Stellate Cell Migration in Two Dimensions—As an initial screen for cell-matrix interactions mediating migration, we studied the movement of stellate cells in two dimensions. Cells isolated from normal liver or after bile duct ligation were plated on chamber well slides coated with specific matrix components mixed with collagen I (which fosters attachment of both quiescent and activated stellate cells). With relatively high density plating, the cells were confluent after 2–3 days of culture. A sterile pipette tip was used to clear a track in the plate, and migration of cells into the area was monitored. Stellate cells isolated from normal rat liver showed no significant migration on any surface (data not shown). Activated stellate cells migrated much more rapidly on a surface with HA than on collagen I alone (Fig. 1).

CD44 Expression by Hepatic Stellate Cells—These initial observations suggested that HA fosters migration of activated stellate cells. Therefore, we probed for expression of the HA receptor, CD44, initially by reverse transcription-PCR using primers bracketing the v1–v10 variable exon group. The substrate was mRNA from freshly isolated normal stellate cells, freshly isolated in vivo activated stellate cells, or culture-activated stellate cells (see “Experimental Procedures”). As shown (Fig. 2), gel analysis of the PCR results revealed multiple CD44 variants from all preparations (Fig. 2, left). By comparison with cells from normal liver, stellate cells from the injured liver exhibited several additional bands including one of 735 nucleotides, which was particularly prominent (Fig. 2, arrow). This band was excised from the gel, cloned, and sequenced, and it was found to represent CD44 containing v6–v10. Interestingly, it is not increased in the RNA extract of culture-activated stellate cells.

The products of amplification from normal and in vivo acti-
Vibrated stellate cells were mass cloned (Fig. 2, right) and sequenced for identifying individual splice variants. The composite results are shown in Fig. 3. Overall, the number of variants from in vivo activated cells was increased. All contained v6, and some of these had exons (v2 and v3) that were not present in the variants from normal cells. When we quantified expression of the v6 isoform using an RNase protection assay, we found it to be markedly up-regulated in activated stellate cells (Fig. 4). Of note, in culture-activated cells, the level of expression is similar to that of quiescent (normal) cells.

The profiles shown in Fig. 3 also indicate that activated stellate cells generate variants without v8 and/or v9. To assess their importance, we quantified the mRNA for v8–v10. We found that CD44v8–v10 was nearly as abundant as CD44v6 mRNA (data not shown). This confirms that the principal v6-containing variant is CD44v6–v10 rather than isoforms lacking v7, v8, and/or v9. Expression of variants containing CD44v6 was assessed also at the protein level by immunoblot using our polyclonal antibody to CD44v6. The data are consistent with the mRNA findings, in that the higher molecular weight forms are relatively increased in activated stellate cells from injured liver. Also, activated stellate cells exhibit several lower molecular weight forms, which are undetectable in extracts of quiescent stellate cells (Fig. 5).

For quantifying the isoforms with variable exons v1–v6, we used real time PCR with primers and probe as shown (Fig. 6A). We also examined the level of expression of “standard” CD44 (sCD44), which has no variable domains, using a probe that straddles the invariant exons on either side of the variant region (v0/11); sCD44 is the principal form in hematological cells (19). With injury, there is a 5–6-fold increase in expression of v4-containing and v6-containing forms as well as sCD44. Given that all of the splice variants that we identified with v4 also contain v5 and v6 (Fig. 3), it appears that these variant exons appear largely as a group. Thus, in addition to CD44v6–v10, CD44v4–v10 (with or without v2 and v3, respectively) is relatively highly expressed. Interestingly, sCD44 (with no variant exons) is a minor fraction of the total, 25–30% in resting stellate cells and 15–20% in in vivo activated cells. Total CD44 was determined using primers and probe for an invariant exon (v0, data not shown).

Migration of Stellate Cells in Transwell Culture—Freshly isolated stellate cells were placed in transwells prepared with various matrix components and incubated for 24 h to allow regeneration of surface proteins that might have been damaged by the enzymatic isolation. The upper and lower surfaces of transwell filters were visualized individually by setting the plane of focus of the microscope objective. UV fluorescence reveals the retinoid-containing vesicles that are characteristic of stellate cells and was used to confirm stellate cell identity (Fig. 7). The cells from normal or injured liver attached in serum-free medium with comparable efficiency and were morphologically similar. With an additional 24 h of incubation in serum-free medium, most cells remained round. In serum-containing medium, they rapidly assumed a fibroblastic shape. Under either condition, cells with a fibroblastic profile were incapable of migration. Only rounded cells migrated (Fig. 8).

Stellate cell migration was tested on transwell filters coated with HA, collagen I, or collagen IV; uncoated filters were included for comparison. Cells isolated from normal liver showed minimal migration on any of these surfaces (Fig. 9a). In contrast, activated cells from injured liver were migratory on all substrata and notably so on HA. The migration rate on HA was

FIG. 9. Migratory activity of stellate cells from normal or injured liver on various substrata. Normal stellate cells (nHSC, a) show minimal migration on any substratum, whereas in vivo activated cells (vaHSC; b) are migratory, moving most rapidly on HA.

FIG. 10. Effect of antibody to CD44v4 or CD44v6 on migration of in vivo activated stellate cells on inserts prepared with the indicated substrata. Only anti-CD44v6 was effective, and only for cells placed on HA-coated filter inserts. The data are expressed as percentage of the migration index when nonspecific rabbit IgG was used in place of the indicated antibody.
double that on collagen I or uncoated plastic. Collagen IV supported an intermediate rate of migration (Fig. 9 double that on collagen I or uncoated plastic. Collagen IV supported an intermediate rate of migration (Fig. 9).

**Role of CD44v6 in HA-mediated Migration of Hepatic Stellate Cells**—We next asked whether the strikingly increased expression of CD44v6 was involved in migration of activated stellate cells. For this purpose, antibody to the v6 domain was introduced into the migration assay described in the legend to Fig. 9. Antibody to v4 was used for comparison. Anti-v4 reduced migration and was specific for HA; when the substratum was collagen I, collagen IV, or uncoated plastic, it had no effect. Anti-v4 had no effect on migration on any of the substrata (Fig. 10). To confirm that the effect of anti-v6 was specific to this exon, we mixed v6 domain peptide, either N-terminal or C-terminal, with the antibody before adding the mixture to the migration assay. As shown (Fig. 11), the N-terminal peptide reversed the effect of anti-v6, whereas the C-terminal peptide had no effect. This result establishes the specificity of the antibody inhibition and indicates that the migration-facilitating region of CD44v6 resides in its N-terminal half.

**DISCUSSION**

The goal of our studies was to define the cell-matrix interactions that mediate stellate cell migration. We found that the HA receptor, CD44, plays a key role. This extracellular matrix receptor system changes rapidly in liver injury in vivo, both qualitatively and quantitatively. Particularly prominent is an increase in CD44v6, which appears as a component of multiple splice variants but is dominated by v6–v10 and v4–v10. Based on antibody inhibition studies, the v4 exon appears to have no direct role in migration. The role of v7–v10 is speculative. We found that a blocking antibody to v9 reduced migration but with about half the effect of anti-CD44v6; this could indicate a role for v9 or could involve steric hindrance of v6 by antibody binding to v9.

The way in which the v6 variable exon exerts its effect is speculative. It has been reported that high affinity binding of HA by CD44 requires the presence of v4–v7 (20). Also, the presence of exons v4–v7 fosters clustering of CD44 within the membrane, an event that allows formation of a complex with a matrix proteinase (gelatinase B or matrix metalloproteinase-9) (21). By altering the pericellular matrix and cell-matrix attachment, the latter may facilitate migration. It has been shown that stellate cell migration is blocked by inhibitors of matrix metalloproteinase-2 and -9 (7). These proteinases are present in the liver, matrix metalloproteinase-2 being secreted by Kupffer cells and matrix metalloproteinase-9 by stellate cells (22).

Although expression of CD44v6 appears to be important to stellate cell migration, its role is not exclusive. Collagens are part of the wound repair environment, and our data indicate that stellate cells utilize type IV collagen as a migration substratum (Fig. 9). Migration on collagen IV is unaffected by anti-CD44v6, as expected, because this protein is not a ligand for CD44. However, it is blocked by antibody to α1/β1 integrin, which is expressed by stellate cells (23). The question of whether other HA receptors, such as RHAMM, are involved (10) has not been examined as yet.

The use of a particular receptor-ligand system depends not only on receptor expression but also on the stage of injury and the composition of the “injury” matrix. HA is prominent in early injury. Stellate cells produce it in primary culture (24). In liver injury, their production of HA increases in parallel with expression of CD44 (16). HA is a large polysaccharide (ranging up to 10,000 kDa), which combines with proteoglycans such as versican to form giant water-holding complexes. This hydrodynamic property of HA-proteoglycan may be fundamental to its role in migration and metastasis, altering the pericellular space so as to loosen the anchoring attachments of cells (11). A matrix rich in HA is found at the leading edge of migrating smooth muscle cells (25). The rounded shape of actively migratory cells in our studies (as in Fig. 7c) may be due to pericellular HA, which is presented by the precoated filter and, in the case of activated cells, is also endogenous. Cells with a fibroblastic morphology increase with time in culture, develop anchoring attachments, and become nonmigratory (Fig. 7d). The observations in culture suggest two stages of stellate cell activation: an early stage of active migration, followed by the development of actin cables and focal adhesion to matrix molecules that render the cells stationary. Based on the studies with culture-activated stellate cells, the latter event is accompanied by downregulation of CD44v6 at the cell surface.

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\(^2\) S. Kikuchi and D. M. Bissell, unpublished observation.

\(^3\) S. Kikuchi, A. De Fougerolles, and D. M. Bissell, unpublished data.
Unlike previous studies, the present one has examined CD44 splice variants in vivo by interrogating cells that are isolated directly from normal or injured tissue. We captured the in vivo phenotype by minimizing the time that the cells were maintained in culture. Numerous observations have established that normal stellate cells undergo a spontaneous change of phenotype as they adapt to culture. The cells acquire several characteristics of myofibroblasts that have been characterized broadly as mimicking the response to injury (26). Our focus in these studies was on stellate cells activated in vivo, in part because differences between in vivo activated and culture-activated stellate cells have been noted already (23). The present results confirm that important differences exist and are especially striking with respect to the CD44 receptor and migration. The expression of CD44v6 by culture-activated stellate cells was very low relative to that of in vivo activated stellate cells; indeed, it resembled that of resting cells. Culture-activated cells have a fully developed myofibroblast phenotype, with stress fibers and focal adhesion to the substrate. Given this and their low expression of CD44v6, they appear to model stellate cells in the second stage of the injury response, which are within fibrous bands and stationary; they are not a model of the acute response to injury.

The initial evidence for stellate cell migration in injury was from morphological studies of human liver with alcohol-related damage. Alcohol induces zone 3 (centrilobular) injury, with accumulation of “transitional” (partially activated) and myofibroblastic cells in this zone and depletion of midlobular (zone 2) stellate cells (2). The current view is that stellate cells migrate toward the injury while becoming progressively activated. When fully activated, they elaborate an injury matrix and occupy it. With maturation, the recently synthesized collage nous fibrils form tracts, to which stellate cells anchor and contract in response to agonists such as endothelin (27). Thus, migration of stellate cells in liver injury appears to be inherent to the formation of dense scar tissue in chronic injury and has a central role in the progression of chronic injury to cirrhosis and functional compromise.

Therapeutic blockade of stellate cell migration has not been explored to date but is attractive, based on insights from this and other recent work. Several potential targets exist, both extracellular (e.g. a matrix receptor) or intracellular (e.g. a receptor kinase or signaling moiety). CD44v6 is attractive for the reason that it appears to be up-regulated dramatically and selectively in injury; thus, an inhibitor may have little effect on normal homeostatic function of this receptor. Also, if the liver (or other epithelial tissue) were the sole site of injury, tissue targeting would be unnecessary. The fact that blockade of CD44v6 reduces migration by only 50% can be viewed as a safety factor rather than a limitation. In chronic epithelial injury, repair is highly dynamic, with concomitant fibrogenesis and fibrolysis (1). The goal is not to abrogate fibrogenesis completely but rather to tilt the process toward net fibrolysis. Inhibiting migration through blockade of CD44v6 merits study as a treatment strategy.

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