Fibronectin Gene Transcription Is Enhanced in Abnormal Wound Healing

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Abnormal wound healing in susceptible individuals can result in the formation of keloids that have an elevated content of extracellular matrix material compared to normal scars. Keloid-derived fibroblasts exhibit as much as a fourfold increase in the rate of fibronectin biosynthesis compared to fibroblasts from normal dermis and normal scars. Altered biosynthesis is due to an increase in the steady-state level of fibronectin mRNA, and in this investigation we have identified the level of fibronectin gene expression that is responsible for this increase. The rate of fibronectin gene transcription was found to be increased as much as threefold in keloid fibroblasts when compared to normal fibroblasts. Other possible changes that could account for the elevated level of fibronectin mRNA in keloids, such as increased copy number of the fibronectin gene or decreased turnover of fibronectin mRNA were also examined. The possibility of altered gene dosage was eliminated because chromosome content, G-banding patterns, and fibronectin gene content of keloid fibroblasts were all found to be normal. Analysis of fibronectin mRNA degradation revealed a half-life of approximately 13 h, and the residual fibronectin mRNA was observed to remain full length during this time period in both keloid and normal fibroblasts. Thus, altered degradation of fibronectin mRNA is unlikely to contribute to overproduction of fibronectin in keloids. Increased translational competence of fibronectin mRNA in keloids was also eliminated as a contributing factor because fibronectin mRNA remaining after one half-life were equally available for translation in both cell types. Although stimulation of transcription may not entirely account for the increase in fibronectin biosynthesis in keloids, this mechanism is best able to account for the majority of the change. *J Invest Dermatol 99:579–586, 1992*

Wound healing in susceptible individuals leads to the development of dermal fibrotic lesions, or keloids that are composed of atypical fibroblasts and an increased abundance of certain extracellular matrix components [1–3]. Keloid fibroblasts exhibit abnormal collagen metabolism [4–6] and increased fibronectin biosynthesis [7] when compared with normal fibroblasts. Although keloids have not been observed to metastasize, therapy is not straightforward because the lesions generally reappear and can be more severe following the trauma of surgical removal [2,3]. Keloids occur most commonly in individuals of darker pigmentation [2], and are found most frequently on the earlobe, chest, or back. In certain Black and Oriental populations, the incidence of this condition has been observed to be as high as 6% [8–10]. In addition, there is clearly a genetic predisposition to form keloids, the molecular basis of which remains unclear [9,11].

Fibronectins are a family of extracellular matrix and plasma glycoproteins that can promote cell migration, attachment, and spreading; thus, these molecules are important for complex biologic processes such as embryonic development, wound healing, and neoplastic transformation (reviewed in [12,13]). The basic unit of the protein is a disulfide-bonded dimer of two similar, but not identical, polypeptide chains. The dimer is secreted and can be further organized into fibrils in the extracellular matrix or can remain soluble in the circulation. Fibronectin plays several important roles in wound healing, functioning to promote clot formation, development of granulation tissue, and re-epithelialization [14,15]. The first stage in wound healing, inflammation, involves rapid deposition and polymerization of fibrin [16]. Plasma fibronectin binds and can be covalently crosslinked with fibrin to form a fibrous clot structure that promotes migration and attachment of leukocytes and fibroblasts [16–18]. A 120-kd fibronectin fragment is also chemotactic for monocytes, implying further involvement in macrophage recruitment [17]. Fibroblasts subsequently migrate into the wound area and produce additional fibronectin, which is assembled into a well-ordered, disulfide cross-linked, provisional matrix [19,20]. By means of multiple, distinct binding domains, fibronectin is able to specifically interact with other matrix proteins and macromolecules, in particular, collagen type I and proteoglycans [12,13]. This property is important in the assembly of other extracellular matrix components to form granulation tissue, a temporary structure that has many similarities to embryonic skin [21]. As healing progresses, collagen content increases, the fibrils are organized into fiber bundles, and the provisional matrix of fibronectin diminishes [19,20].

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Abbreviations:

DMEM: Dulbecco's modified Eagle's medium
DMES: DMEM with 10% fetal calf serum
DMSO: dimethylsulfoxide
DRB: 5,6-dichlorobenzimideazole riboside
DTT: dithiothreitol
EDTA: ethylenediaminetetraacetic acid
NP-40: nonidet P-40
PBS: phosphate-buffered saline
SDS: sodium dodecyl sulphate
TA: triamcinolone acetonide

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Granulation tissue is ultimately replaced by neo-dermis, which has a relatively high content of collagen type I and low content of fibronectin [20,21]. Re-epithelialization is accomplished as epidermal cells migrate over the provisional matrix provided by granulation tissue and re-establish the basement membrane. Fibronectin is important for this migration because expression of the fibronectin receptor is restricted to the migrating keratinocytes [14,22]. Fibronectin may further facilitate re-epithelialization because terminal differentiation of keratinocytes in vitro has been observed to be prevented by fibronectin [23].

We have previously reported that keloid fibroblasts accumulate significantly more cytoplasmic and cell-surface-associated fibronectin than normal fibroblasts as a result of alterations in fibronectin metabolism [7]. Although fibronectin is similarly processed, compartmentalized, and degraded by normal and keloid fibroblasts, fibronectin biosynthesis was found to be accelerated as much as fourfold in keloid-derived cells due to a corresponding increase in the amount of accumulated fibronectin mRNA [7]. Biosynthesis of the 140-kd fibronectin receptor was also found to be increased in keloid fibroblasts, suggesting some level of coordinate regulation for fibronectin and fibronectin receptor [7]. Because keloid fibroblasts exhibit extracellular matrix overproduction in vitro [4,5,7], it is unlikely that local or systemic factors are solely responsible for keloids. The fibroblasts themselves are abnormal for extracellular matrix production, and the increased content of fibronectin in keloids is due to overproduction of the molecule by fibroblasts recruited for wound healing, rather than increased deposition from the circulation [7]. However, the level at which fibronectin gene expression has been affected in keloid fibroblasts and the mechanism by which this alteration results in elevated content of fibronectin mRNA is unknown. The following possibilities could account for fibronectin overproduction and the increased cellular level of fibronectin mRNA. First, gene amplification could result in a greater than diploid content of the fibronectin gene in keloid fibroblasts, which in turn could be responsible for the elevated levels of fibronectin mRNA. Second, enhanced fibronectin gene transcription could also cause increased content of fibronectin mRNA in keloid fibroblasts. Third, decreased turnover of fibronectin mRNA, or certain species of fibronectin mRNA, could also account for the increased abundance of fibronectin mRNA in keloid fibroblasts. In order to distinguish among these possibilities we have examined and compared fibronectin gene dosage, rates of transcription initiation, mRNA turnover, and translational competence in primary cultures of keloid and normal fibroblasts. We also discuss regulatory pathways that could be involved in abnormal fibronectin gene expression in the formation of keloid lesions.

MATERIALS AND METHODS

Tissue Sources and Cell Culture The preparation and culture of early-passage fibroblast cultures from keloid and normal tissue specimens (Wound Healing Center, Medical College of Virginia, Richmond, Virginia) has already been described [7]. Dulbecco's modified Eagle's medium (4.5 g/l glucose) (DMEM) supplemented with 10% fetal calf serum (Gemini Bioproducts, Calabasas, CA), 500 U/ml penicillin, and 100 mg/ml streptomycin (DMES) was used. The four samples of keloid fibroblasts used in this investigation (patients are Black women, age range 14–57) have been previously studied and found to overproduce fibronectin compared to normal fibroblasts and contain steady-state levels of fibronectin mRNA that are correspondingly elevated [7]. Three samples of normal fibroblasts used in this investigation (two patients are Black women, ages 44 and 50; one patient is a white female, age 40) have also been previously studied for fibronectin biosynthesis and steady-state levels of fibronectin mRNA and were found to be in the normal range [7]. For all of these studies, sets of normal and keloid fibroblasts were equivalently cultured and expanded in order to keep the culture age as similar as possible at the time of analysis. During the course of these experiments, cultures showed no evidence of crisis or senescence. In some experiments HT1080C, a clonal human cell line derived from a fibrosarcoma [24], was used for comparison or control. This cell line has been studied for fibronectin content and regulation of fibronectin gene expression [25,26].

For steroid regulation assays the synthetic corticosteroid triamcinolone acetonide, TA, was added 36–48 h prior to assay at a final concentration of 5×10^{-7} M. TA was solubilized at 10^{-2} M in 100% ethanol and diluted for use as a stock solution of 10^{-5} M in phosphate-buffered saline (PBS). In some mRNA turnover experiments, cells were exposed for increasing periods of time to 5,6-dichlorobenzimideazole riboside (DRB; Sigma), an inhibitor of the activity of RNA polymerase II [27,28]. DRB was dissolved in dimethylsulfoxide (DMSO) at a concentration of 150 mg/ml and then diluted to a final concentration of 60 μg/ml in DMES. Because the carrier DMSO is present at a final concentration of 0.04%, as a control in these experiments, cells were treated with 0.04% DMSO alone.

Chromosome Analysis Keloid cells were exposed to 0.1 μg/ml colcemide (Gibco) at 37°C for 90 min. The cells were trypsinized, pelleted, incubated at 37°C for 15 min in a hypotonic solution (75 mM KCl), and then fixed in fresh, cold 3:1 methanol-glacial acetic acid. Slides were incubated at 100°C for 15 min, cooled to room temperature, and treated with 0.05% trypsin. Trypsinized slides were stained with Giemsa stain (Gurr's R66) and examined by bright-field microscopy using a Zeiss Photomicroscope with a 63× planapochromat oil immersion objective. Representative karyotypes were photographed using Kodak 2415 film.

Detection of DNA Encoding Fibronectin Cultured cells were removed by trypsinization, pelleted, washed in 10 mM Tris, pH 8.0, 1 mM EDTA, and 100 mM NaCl (STE), and then lysed by addition of SDS to 0.5%. Lysates were incubated at 55°C overnight with 100 μg/ml proteinase K, and repeatedly phenol extracted and ethanol precipitated. Samples were resuspended in 10 mM Tris, pH 7.5, 1 mM EDTA (TE), and incubated at 37°C for 3 h with RNase A (100 μg/ml). SDS was added to 0.5%, and samples were digested for 2 h at 55°C with proteinase K, extracted with phenol, ethanol precipitated, and dialysed against TE [29,30]. DNA was quantitated spectrophotometrically and duplicate samples (20 μg each) were denatured by incubation in 300 mM NaOH for 1 h at 70°C and neutralized by addition of ammonium acetate (pH 7.0) to 1 M. DNA was suction filtered onto nitrocellulose using a slot-blot apparatus [31], air dried, and baked at 80°C in a vacuum oven for 1 h. A ^{32}P-labeled riboprobe [29] was prepared by transcription of a cDNA clone encoding the EIII-B segment of fibronectin [32]. A fibronectin cDNA was used as probe because genomic clones might contain repetitive elements that would complicate this analysis. Slot blots were prehybridized for 2 h at 42°C using 200 μg/ml denatured salmon sperm DNA in 50% formamide, 5 × SSPE (900 mM NaCl, 50 mM $NaPO_4$, 5 mM EDTA, pH 7.4), and 5 × Denhardt's solution, 0.1% SDS. Hybridization was overnight at 42°C using 10^7 cpm of the fibronectin probe. Blots were washed at 65°C in 0.1 × SSPE, 0.1% SDS, treated for 1 h at 37°C with 0.1 × SSPE containing RNase A (20 μg/ml), air dried, and exposed to Kodak X-Omat film at −80°C.

Rates of Fibronectin Transcription Initiation Cells were trypsinized and replated to be confluent the day before isolation of nuclei. Cells were then lysed using cold lysis buffer (10 mM Tris, pH 8.0, 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.25 M sucrose, 0.1% (v/v) NP-40, 0.5 mM DTT), scraped from the plates, and nuclei pelleted at 1000 × *g* at 4°C. Nuclei were washed and resuspended in 100 μl cold wash buffer (10 mM Tris, pH 8.0, 100 mM KCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM DTT, 18% glycerol) containing 1 mM ATP, CTP, GTP, 20 μM UTP, 400 μCi [α-^{32}P]UTP (>600 Ci/mmol, New England Nuclear), and RNasin (40–80 units). Runoff transcription reactions were incubated at 27°C for 50 min. Nuclei were treated with 5 units of RNase-free DNase (Promega Biotec) at 37°C for 30 min, adjusted to contain 10 mM Tris, pH 7.7, 5 mM EDTA, 1% SDS, and 100 μg proteinase K and incubated at 45°C for 30 min. Nucleic acids were

extracted using phenol:chloroform:isoamyl alcohol (24:24:1), ethanol precipitated, and resuspended in 10 mM Tris, pH 7.5, 1 mM EDTA. Unincorporated nucleotides were removed using Biogel P60 columns. Peak fractions were pooled and ethanol precipitated. UTP incorporation was determined by acid precipitability, and samples were prepared for hybridization by resuspending equal cpm ^{32}P-labeled RNA in 5 × SSPE, 1 × Denhardt's, heating at 65°C for 5 min followed by addition of formamide to 50% and SDS to 0.3%.

The following cDNA containing plasmids were used as probes: 1) for fibronectin, pHF1, pHF154 (kindly provided by A. Kornblihtt) [33] and pHFN300 (containing a 5′-end 1.0-kb Xbal-Sall fragment); 2) for background, pAT153 [30]. Linearized plasmids were denatured by treatment with 0.4 M NaOH for 2 min at 95°C. Single-stranded DNA was chilled on ice, NaCl and $NaO_2C_2H_3$ added to final concentrations of 1 M and 0.2 M, respectively, and the DNA suction filtered onto nitrocellulose [31]. Filters were washed using 1 M NaCl, air dried, and then baked at 80°C in a vacuum oven for 1 h. Baked filters were briefly soaked in 10 mM Tris, pH 7.5, 0.3 M NaCl, 2 mM EDTA and smaller filters excised using a paper punch. The small filters were prehybridized for 1 h at 42°C using 50% formamide, 5 × SSPE, 1 × Denhardt's, 0.3% SDS, 100 μg/ml denatured salmon sperm DNA. Hybridization reactions of 200 μl were layered with paraffin oil and incubated at 42°C for at least 72 h. Duplicate or triplicate reactions were set up using 2–16 × 10^6 input cpm. Filters were washed for 15 min in each of the following: three times at 42°C with 2 × SSPE, 0.1% SDS; three times at 65°C using 0.1 × SSPE, 0.1% SDS; and once at 37°C with 0.1 × SSPE. Non-specific hybridization was removed by treating the filters for 1 h at 37°C with 0.1 × SSPE containing RNase A (20 μg/ml) and RNase T_1 (1500 units/ml). Filters were in scintillation vials with 250 μl 40 mM NaOH warmed to 70°C for 15 min, scintillation fluid was then added and vials were kept in the dark overnight prior to counting.

Stability of Fibronectin mRNA

Pulse-Chase with [^{3}H]-Uridine Cells were pulse-labeled for 5 h using [5,6-^{3}H]uridine (43.1 Ci/mmol, New England Nuclear) at a final concentration of 130 μCi/ml and label chased by washing with warm PBS followed by incubation in DMEM supplemented with 5 mM each cold uridine and cytidine [34] for 6 or 14 h. Total RNA was isolated by cell lysis in 1.5 ml 4 M guanidinium thiocyanate, 10% sarkosyl followed by pelleting through a 3 ml cushion of 5.7 M CsCl, 100 mM EDTA [35]. Isotope incorporation was determined by acid precipitability, and samples were prepared for hybridization by resuspending equal cpm ^{3}H-labeled RNA in 5 × SSPE, 1 × Denhardt's, heating at 65°C for 5 min followed by addition of formamide to 50% and SDS to 0.3%.

Filter preparation was the same as for transcription analysis. Hybridization volumes were 150 μl and approximately 7 × 10^6 input cpm was used for each reaction. Increasing input cpms confirmed that cDNAs present on the filters were in excess of the labeled RNA. Multiple hybridization reactions were prepared for each sample and hybridization was for 72 h at 42°C. Filter washing, RNase treatment, and determination of fibronectin cpm were as described above.

Inhibition of RNA Polymerase II by DRB Cultures were exposed to 60 μg/ml DRB for 0, 6, and 12 h. Uninhibited cells exposed to 0.04% DMSO revealed no significant effect of DMSO on fibronectin mRNA levels (data not shown). Total RNA was isolated as above, pellets were resuspended in TE, ethanol precipitated, and quantitated spectrophotometrically. Ten micrograms samples of total RNA were resolved on 1% agarose gels containing 2.2 M formaldehyde and RNA blotted to nitrocellulose as described [7]. The positions of 18S and 28S rRNA were determined by staining duplicate samples with 33 μg/ml acridine orange. ^{32}P-labeled probes were prepared by nick translation [7,31] of cDNA clones, pHF154 [33], which encodes approximately 2 kb of human fibronectin, and pB (provided by J. Sylvester, University of Pennsylvania), which encodes human 18S rRNA. Nitrocellulose blots were prehybridized as described [7] and hybridization was overnight at 42°C using 10^6 cpm/ml of the fibronectin probe. Blots were washed at 65°C in 0.1 × SSPE, 0.1% SDS, dried, and exposed to Kodak X-Omat film at −80°C. Quantitation of signal intensity by densitometry was performed using the BioScan Optimas program. Background was considered to be a non-hybridizing portion of the blot.

Translational Competence of Fibronectin mRNA Availability for translation was determined by quantitating rates of fibronectin biosynthesis following inhibition of transcription with DRB. Cultures containing the same number of cells were exposed to DRB or DMSO for 0 or 12 h. Cells were labeled for 1 h using 0.5 ml of methionine-free DMEM supplemented with L-glutamine and 200 μCi/ml L-[^{35}S]methionine (New England Nuclear, 1100 Ci/mmol) and cell lysates prepared as described previously [7]. Incorporation of radioactivity into newly synthesized protein was determined by precipitation with 7% trichloroacetic acid. Fibronectin was immunoprecipitated using affinity-purified, rabbit anti-human fibronectin antibody (Accurate Antibodies) overnight at 4°C. The immune complexes were precipitated at room temperature for 2 h using carrier rabbit IgG (RAGG, Antibodies, Inc., Davis, CA) and affinity-purified goat anti-rabbit IgG (GARGG, Antibodies Inc.) as second antibody. Immunoprecipitates were pelleted and solubilized in 2% SDS, and radioactivity determined by scintillation counting. SDS-PAGE analysis of replicate samples confirmed that the label was in the fibronectin band, and reprecipitation of supernatants confirmed that the primary antibody was in excess.

RESULTS

Initial study of fibronectin biosynthesis confirmed that the rates of fibronectin production in the keloid samples selected for this analysis were increased two- to fourfold over the normal samples chosen [7]. The time in culture for sets of normal and keloid fibroblasts used in these studies was kept as similar as possible and cultures showed no evidence of senescence.

Chromosome Analysis Fibronectin gene content could be altered by the increased dosage, decreased dosage, or rearrangement of chromosome 2 that carries the human fibronectin gene. Chromosome rearrangement could also result in abnormal control of fibronectin gene expression. To examine the likelihood of these possibilities, chromosome content of three keloid strains was studied by G-band analysis. Analysis of a total of 15–20 metaphase spreads for each of the keloid strains revealed no abnormalities in chromosome number or structure. In particular, there were two normal copies of chromosome 2. All three of these female-derived fibroblast strains revealed a normal 46XX karyotype. Figure 1 shows a representative G-banded karyotype of one of the keloid strains. Scleroderma is another condition of connective tissue overproduction, specifically involving collagen [36,37] and fibronectin [38]. This disease is also thought to have a genetic component and an increased incidence of chromosome breakage has been reported [39]. In this study, fragmented and/or broken chromosomes were not observed, and striking variations in the normally occurring chromosome structural polymorphisms of heterochromatin and satellites [40] were also not present.

Fibronectin Gene Copy Number Although the karyotype of these keloid strains appeared normal, small rearrangements or amplification of the gene encoding fibronectin might not be detectable by chromosome analysis. In order to compare fibronectin gene copy number in keloid and normal fibroblasts, equal amounts of genomic DNA isolated from the keloid and normal fibroblast strains were examined for fibronectin coding sequences by slot-blot analysis. Figure 2 shows the results of a representative experiment comparing the relative content of fibronectin coding sequences in genomic DNA in one set of keloid and normal fibroblasts. No apparent difference in the signal intensity of DNA encoding fibronectin was observed in this set or others, suggesting that the fibronectin gene

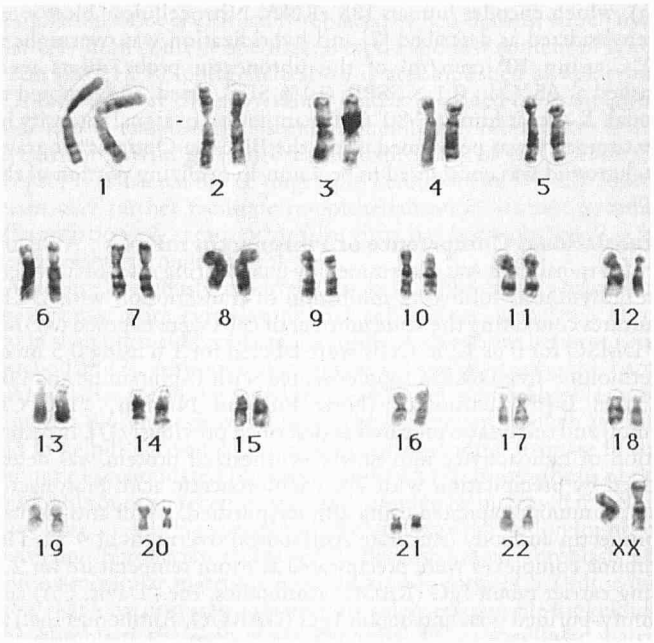

Figure 1. G-banded karyotype of fibroblasts from a keloid patient.

copy number in keloid cells is not sufficiently elevated to account for the increased steady-state amount of fibronectin mRNA.

Rates of Fibronectin Transcription Initiation Because amplification of the fibronectin gene was unlikely to cause the elevated fibronectin mRNA content of keloid cells, transcription initiation was examined. Transcription rates were measured by nuclear run-off RNA synthesis [41] and compared for keloid and normal fibroblasts. A human fibrosarcoma cell line, HT1080C [24,26], was also included for comparison, because we find that the fibronectin mRNA level in these transformed cells is about 10–20 times lower than the level in normal fibroblasts [7], and decreased fibronectin content following neoplastic transformation can be the result of diminished transcription [42]. Approximately equal amounts of acid precipitable input cpm were used for all samples and two- to threefold increased input cpm were also used to confirm the presence of excess probe on filters. HT1080C cells revealed a very low rate of fibronectin mRNA production (Table I), which is at the limit of detection using this approach. In contrast, the keloid and normal fibroblasts revealed readily detectable and significantly higher rates of fibronectin mRNA synthesis. Specifically, in two separate determinations, the rate of fibronectin gene transcription was two- to threefold higher in keloid fibroblasts than in two different normal fibroblast strains (Table I). This increase in the rate of fibronectin gene transcription corresponds reasonably well with the fourfold increase in the rate of fibronectin biosynthesis exhibited by this keloid strain compared to normal.

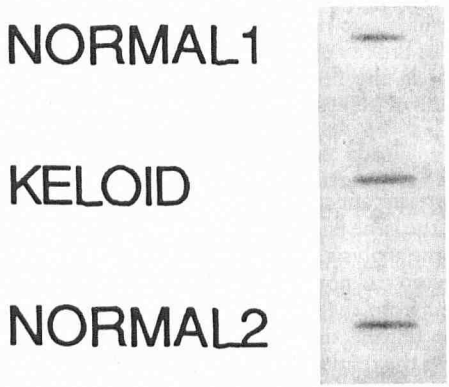

Figure 2. Relative fibronectin gene content of normal and keloid fibroblasts. Twenty-microgram samples of DNA were denatured, filtered onto nitrocellulose, and probed for fibronectin coding sequences.

Fibronectin mRNA Turnover Steady-state levels of mRNA depend upon rates of mRNA synthesis, processing, and turnover; thus, in addition to transcription, other mechanisms might be involved in fibronectin overproduction. Although the increased rate of fibronectin gene transcription can account almost entirely for the elevated fibronectin mRNA content of keloid fibroblasts, we wanted to confirm this finding by examining other potential control mechanisms in the normal and keloid pairs used for transcription analysis. Also, because the number of cells required for analysis of mRNA turnover is considerably less than for transcription, it was possible to examine an additional normal and three additional keloid samples.

Pulse-chase labeling of RNA was used to quantitate fibronectin mRNA degradation. Total RNA was isolated from sets of keloid and normal fibroblasts, quantitated for nucleic acid content and incorporation of ^{3}H-uridine. Because the specific activity of total RNA did not vary significantly over this time period (presumably due to rRNA, which is stable and abundant), input cpm were kept approximately equal for all samples and time points. Fibronectin mRNA remaining after increasing chase times was expressed as fibronectin cpm contained in 10^6 total cpm, and linear regression was used to determine lines that best fit the data. This analysis revealed that the content of fibronectin mRNA decreased similarly in both keloid and normal fibroblasts during 14 h of chase, and that degradation of fibronectin mRNA followed first-order kinetics. Similar results were obtained for all keloid and normal pairs tested, and the results of a representative experiment using one pair are shown in Fig 3. The time required to decrease fibronectin mRNA content by one-half under these conditions was not significantly different for the two cell types, and was approximately 13 h for normal fibroblasts and approximately 14 h for keloid fibroblasts (Fig 3, *arrows*). These results are consistant with enhanced fibronectin gene transcription as the major alteration of fibronectin gene expression in keloids.

Although pulse-chase analysis is quantitative and does not rely on complicating inhibitors, mRNA quality or length is not assessed. It was possible that degradation of fibronectin mRNA molecules is qualitatively different in keloid and normal cells. The remaining labeled fibronectin mRNA might be degraded to different extents in the two cell types, and more sensitive or resistant subsets of fibronectin mRNA not revealed. Because both intact and partially degraded fibronectin mRNA would be detected by filter hybridization, it was unclear whether the remaining "aged" fibronectin mRNA was full length and if any or all of it was available for translation in normal and keloid cells.

5,6-dichlorobenzimideazole riboside (DRB), an inhibitor of the activity of RNA polymerase II [27,28] was used to examine the length of "aged" fibronectin mRNA in normal and keloid fibroblasts, and to independently confirm fibronectin mRNA turnover times. Because DRB is an inhibitor of RNA polymerase II transcription [27,28], all mRNA present in the cell following exposure to DRB was synthesized prior to DRB treatment. Cultures of keloid and normal fibroblasts were exposed to 60 μg/ml DRB for 0, 6, and 12 h. Total RNA was isolated and quantitated, and equal amounts were resolved on formaldehyde agarose gels, transferred to nitrocellulose, and then probed for fibronectin mRNA. The same blots were later reprobed for 18S rRNA to confirm that samples contained the same amount of total RNA that resolved and transferred as expected (data not shown). Figure 4*A* shows a representative experiment with one of the keloid strains and one of the normal strains. Fibronectin mRNA migrated with a characteristic size of approximately 8 kb and, as expected, was present at an elevated level

Table I. Fibronectin Gene Transcription Is Enhanced in Keloid Fibroblasts[a]

Sample	Input[b] (cpm)	Background[c] Filter (cpm)	Fibronectin[d] Filter (cpm)	Fibronectin[e] (cpm)	Transcription[f] Relative Rates
Normal3	5×10^6	49	108	59	1.0
Keloid3	5×10^6	54	176	122	2.1
	10^7	84	326	242	2.1
Normal4	2×10^6	34	86	52	1.0
Keloid3	2×10^6	52	202	150	2.0
	6×10^6	90	452	362	2.3
HT1080C	4×10^6	40	50	10	0.1

[a] In two separate experiments, nuclei were isolated, runoff transcripts labeled and purified, and hybridization reactions set up for normal and keloid samples as described. HT1080C is a human fibrosarcoma cell line that has a much lower content of fibronectin mRNA than normal fibroblasts. Hybridization efficiency was not determined.

[b] Acid precipitable cpm used for hybridization reactions.

[c] Average cpm present on background filters containing pAT153 DNA.

[d] average cpm present on fibronectin filters. Replicate determinations of fibronectin cpm differed by less than 15% for the normal and keloid samples and 30% for HT1080C samples.

[e] Background cpm have been subtracted from fibronectin cpm. Fibronectin specific cpm should be compared within a particular experiment, because hybridization efficiency was not determined. Because it is not possible to accurately estimate cpm contributed by the entire fibronectin initial transcript (~70 kb versus 5 kb of coding sequences used as probe), the fibronectin specific cpm do not indicate the contribution of fibronectin transcripts to total transcripts.

[f] Ratios of fibronectin cpm/normal fibronectin cpm are reported, and the values for the normal samples are set to 1.0. Ratios represent relative rates of fibronectin gene transcription and thus can be compared among experiments.

in the keloid strain (Fig 4*A*). There was no obvious appearance of partial degradation products in the DRB-treated samples (Fig 4*B*), demonstrating that after 12 h of aging, residual fibronectin mRNA remained full-length in both normal and keloid fibroblasts. This observation suggests that if a particular fibronectin mRNA undergoes degradation, the process is rapid and extensive. Based on changes in the signal intensity, fibronectin mRNA content in the 12-h DRB-treated keloid sample appeared to be approximately half that of the untreated keloid sample (Fig 4*B*). Similarly, the 12-h DRB-treated normal sample contained about half the amount of fibronectin mRNA as did the untreated cells (Fig 4*B*). Although this approach is not as well suited for quantitative analysis as pulse-chase labeling of RNA because an inhibitor is used, there is good agreement with the previous estimate of a half-life of 13–14 h for fibronectin mRNA sequences in normal and keloid cells.

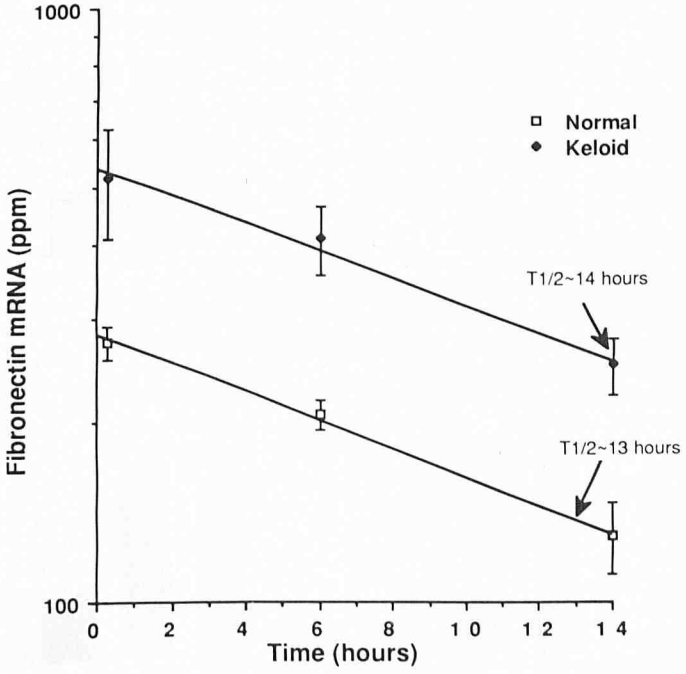

Figure 3. Relative rates of fibronectin mRNA degradation in normal and keloid fibroblasts. Cells were pulse-labeled for 5 h with ^{3}H-uridine and the label chased for 6 or 14 h. The fibronectin specific cpm are reported as parts per million (ppm) of the input RNA. 7×10^6 input RNA cpm were used for all samples and time points. 2×10^6 input RNA cpm for the time 0 samples were also used to confirm excess probe on filters. Background was approximately 10–20 cpm/10^6 cpm for the keloid fibroblasts and has been subtracted. Background was lower for the normal fibroblasts, approximately 5–10 cpm/10^6 cpm, and has also been subtracted. There is no correction for hybridization efficiency.

To confirm the validity of this approach, fibronectin mRNA levels were also examined in HT1080C cells where treatment with glucocorticoids is known to diminish degradation of fibronectin mRNA [25]. RNA polymerase II transcription was inhibited by DRB for 0, 6, and 12 h in untreated and glucocorticoid (triamcinolone acetate [TA])–treated cultures of HT1080C cells. Total RNA was isolated and analyzed for fibronectin mRNA content as described above. As expected, glucocorticoid treatment significantly increased the level of fibronectin mRNA (Fig 4*C*, compare *lanes a* and *g*). TA treatment reduced the rate of fibronectin mRNA degradation, because the fibronectin mRNA content diminished more slowly in the TA-treated cells (Fig 4*C*, compare *lanes d–f* with *g–i*). Following 6 h of inhibition with DRB, the level of fibronectin mRNA appeared unchanged in the TA-treated cells (Fig 4*C*, compare *lanes g* and *h*), whereas the fibronectin mRNA content of the untreated cells was clearly diminished (Fig 4*C*, compare *lanes d* and *e*). Thus, this approach can be used to compare relative rates of mRNA degradation and to assess mRNA length during the turnover process; however, only relative changes are meaningful because it is assumed that DRB treatment does not alter rRNA content, which is used as a constant for this determination.

Translational Competence of Fibronectin mRNA Because the kinetics and process of fibronectin mRNA turnover appeared to be similar in keloid and normal fibroblasts, it was of further interest to determine whether the remaining "aged" fibronectin mRNA were equally available for translation in the two cell types. Availability for translation was determined by quantitating rates of fibronectin biosynthesis following inhibition of transcription for 12 h (approximately one fibronectin mRNA half-life) with DRB. Metabolic labeling and specific fibronectin immunoprecipitation were used as a measure of fibronectin biosynthesis and, thus, the translational competence of the remaining fibronectin mRNA. Keloid and normal cultures were exposed to DMSO or DRB (solubilized in DMSO) for 0 or 12 h. Cells were then pulse labeled for 1 h using L-[^{35}S]-methionine, newly synthesized fibronectin specifically immunoprecipitated and quantitated. In a representative experiment using one pair of keloid and normal cells, it can be seen that the rate of fibronectin biosynthesis was considerably higher in the keloid fibroblasts than the normal fibroblasts, as expected for the uninhib-

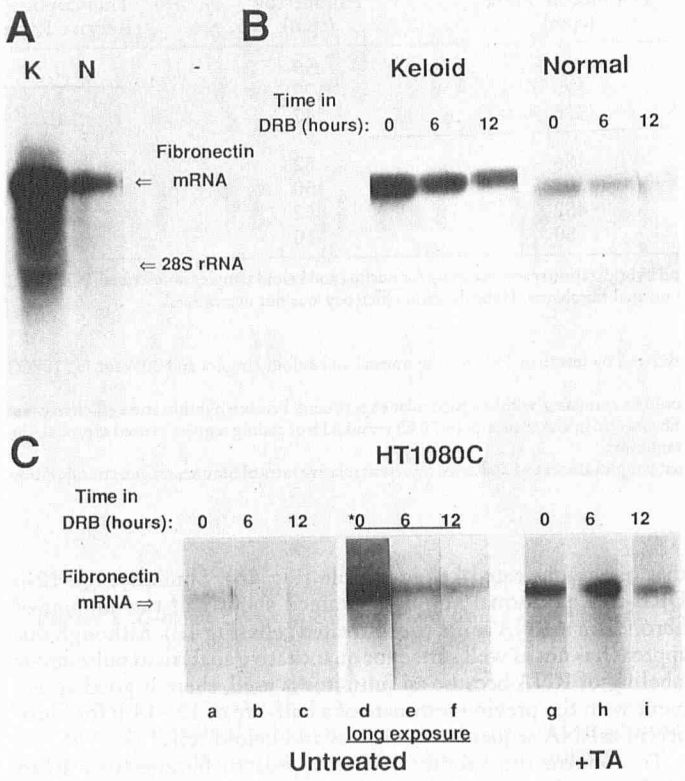

Figure 4. Inhibition of transcription by DRB. *A*) Comparison of fibronectin mRNA content of keloid and normal fibroblasts. Ten micrograms of total RNA was applied to the gel for each sample. *B*) Cells were exposed for increasing time to DRB and total RNA isolated, and quantitated. Ten-microgram samples were resolved on formaldehyde agarose gels, transferred to nitrocellulose, and probed for fibronectin mRNA. *C*) HT1080C is a human fibrosarcoma cell line that makes very little fibronectin mRNA. For comparison of relative fibronectin mRNA content *lanes a–c* (no treatment) represent the same exposure time as *lanes g–i* (TA treatment). Fibronectin mRNA has a longer half-life following treatment of the cells with glucocorticoid hormones such as triamcinolone acetonide (TA). For comparison of fibronectin mRNA turnover, a longer exposure of the RNA from the untreated cells has been included *(lanes d–f)*.

ited cells (Fig 5). Following 12 h of DRB inhibition, total protein synthesis was found to be reduced to approximately 30% of the uninhibited rate in both normal and keloid cells (Fig 5). The rate of fibronectin biosynthesis in the DRB-inhibited cells was 29% of the uninhibited rate in normal cells and 34% of the uninhibited rate in keloid cells (Fig 5). Similar results were obtained for other keloid and normal pairs, suggesting that the fibronectin mRNA remaining after one half-life was equally available for translation in normal and keloid fibroblasts. Also, under these conditions, the translational capacity of "aged" fibronectin mRNA is not significantly different from that of an average mRNA, because the decrease in fibronectin biosynthesis was very similar to that for total protein synthesis. However, "aged" fibronectin mRNA is not entirely available for translation because 12 h is approximately one half-life for fibronectin mRNA, and fibronectin biosynthesis is considerably less than 50% of the uninhibited rate for both normal and keloid cells. Thus, the half-life of translationally functional fibronectin mRNA is less than 12 h for both normal and keloid fibroblasts. This might be due to differences either in the translational machinery or the fibronectin mRNA itself. Because this was observed for both normal and keloid cells, differences in translational competence of fibronectin mRNA are unlikely to contribute to fibronectin overproduction in keloids.

DISCUSSION

Transcription initiation is most likely the molecular control point in fibronectin metabolism that has been altered in keloid fibroblasts causing a two- to fourfold stimulation of fibronectin biosynthesis in abnormal wound healing. This is likely to be a significant change because fibronectin production accounts for at least 1% of total protein in normal fibroblasts. The magnitude of the change in the rate of transcription initiation is nearly sufficient to account for the observed enhancement of fibronectin production, suggesting that additional mechanisms are unlikely to contribute to fibronectin overproduction. Other possible mechanisms, such as increased gene dosage or decreased mRNA turnover, were ruled out as providing major contributions to fibronectin overproduction in keloids. Stimulation of transcription initiation is most likely to occur by modification of either the DNA of the fibronectin gene regulatory region or the enzymes and protein factors that mediate transcription. G-band karyotype analysis revealed no abnormalities of chromosome 2, which carries the gene for fibronectin, suggesting that the gene and its adjacent regulatory region(s) have not undergone major rearrangements. Also, because keloids exhibit a coordinate increase in the basic components of the extracellular matrix, an alteration in a common metabolic pathway that regulates extracellular matrix production is a more likely explanation. Thus, we favor the idea of a change in the transcription apparatus, or a *trans* acting modification, which might involve specialized transcription factors.

Enhanced fibronectin gene transcription in keloids could be di-

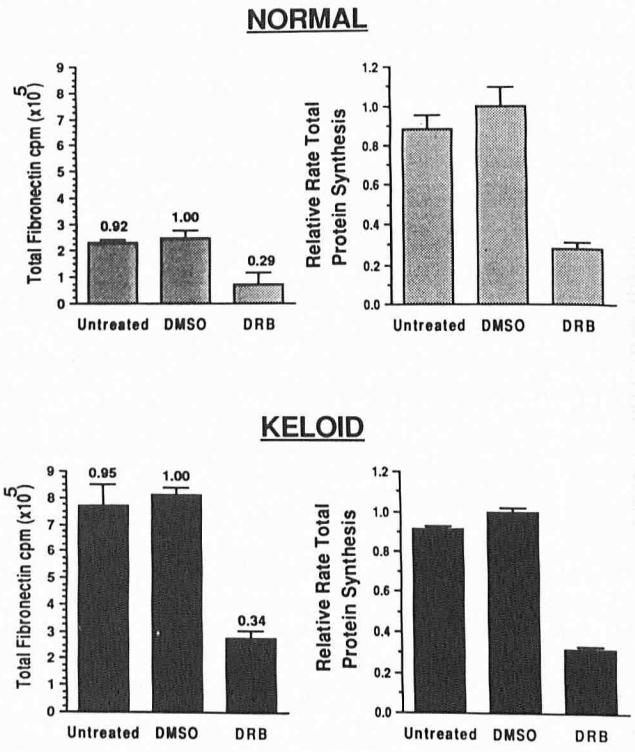

Figure 5. Translational competence of "aged" fibronectin mRNA. Cells were treated with DRB or DMSO for 12 h (approximately one fibronectin mRNA half-life) prior to metabolic labeling. Cultures were pulse-labeled for 1 h using ^{35}S-methionine and then cells were lysed. Newly synthesized fibronectin was specifically immunoprecipitated and quantitated by scintillation counting to give a relative measure of the rate of fibronectin biosynthesis from mRNA that has already undergone one half-life. Rates of total protein synthesis were determined by precipitation with trichloroacetic acid.

rected by a novel regulatory pathway or, more likely, by a normal regulatory pathway that is inappropriately engaged in abnormal wound healing. Due to an inherent abnormality of keloid fibroblasts, this could involve altered regulation or constitutive expression of the pathway. Thus, in keloid fibroblasts the normal controlling factors might no longer affect utilization or operation of the pathway. Glucocorticoid hormones [25,26,43,44] and γ-interferon [45] affect production of extracellular matrix, simultaneously stimulating fibronectin production and repressing collagen production. One or both of these regulatory pathways may promote the early development of granulation tissue [46], which is associated with a transient increase in the content of fibronectin and a transient decrease in the content of type I collagen [20]. Inappropriate utilization of these pathways is unlikely to explain the keloid phenotype because keloid fibroblasts overproduce both fibronectin [7] and type I collagen [4–6]. Coordinate overproduction of collagen and fibronectin further suggests that abnormal wound healing is not the result of keloid fibroblasts being arrested in an early stage of wound healing.

Other potentially relevant transcriptional regulatory pathways for fibronectin and collagen involve neoplastic transformation, the agents that promote this state, and growth factors. In neoplastic transformation of fibroblasts, decreased expression of fibronectin and collagen seems to be the rule [47]. This is exactly opposite from the extracellular matrix overproduction of keloids, emphasizing that there are fundamental differences between keloids and malignantly transformed cells. Many different growth factors, including PDGF, bFGF, EGF, IGF, TGF-α, and TGF-β, can improve impaired healing in vivo [48]. TGF-β is known to have profound effects on the extracellular matrix [49,50] and is the only one of these factors that can clearly regulate production of both fibronectin and collagen via a transcriptional mechanism [25,51,52]. In addition, TGF-β mRNA has been observed in some dermal fibroblasts of affected areas in patients with generalized morphea or Shulman's syndrome, other cutaneous disorders also characterized by increased extracellular matrix content [53]. Finally, this pathway is a good candidate for mediating overproduction of extracellular matrix components in abnormal wound healing because it is known that keloid fibroblasts respond differently to TGF-β treatment than normal fibroblasts [54]. In keloid fibroblasts TGF-β treatment enhances DNA synthesis in the presence of EGF, whereas in normal fibroblasts treatment with TGF-β plus EGF results in diminished DNA synthesis [54].

TGF-β is known to increase production of fibronectin, collagen [55], and the core proteins of chondroitin and dermatan sulfate proteoglycans [56]. For type I collagen and fibronectin, enhanced production results, at least in part, from transcriptional stimulation of the respective genes [52,57]. Transcriptional stimulation of collagen α_2I depends on the presence of an NF-1 consensus sequence in the promoter [57]; however, the nature of the connection of NF-1 with TGF-β is unclear. TGF-β regulation of collagen α_2I expression is likely to be complex because the increase in mRNA can be inhibited by TNF-α at the level of transcription, whereas γ-interferon also inhibits the increase in mRNA, but by a post-transcriptional mechanism [58]. The fibronectin promoter can also mediate transcriptional regulation by TGF-β [25]; however, the identities of the DNA elements and protein factors are not known. Depending on the cell type, TGF-β enhanced production of fibronectin can involve post-transcriptional control [25,51,59], suggesting that this regulatory pathway is also likely to be complex. Although inappropriate utilization of the TGF-β regulatory pathway is the most likely explanation for extracellular matrix overproduction in keloids, TGF-β itself might not activate this regulatory pathway in keloids, because transcriptional stimulation could be indirect, involving other intermediates or regulatory factors.

Overproduction of fibronectin may be particularly detrimental to successful healing due to the different structural and regulatory functions of the molecule. Extracellular matrix assembly and communication of regulatory signals may rely on properly controlled gene expression. Examination of dermal lesions associated with other fibrotic conditions such as scleroderma has also revealed the presence of excess extracellular matrix material [36,37], further supporting the idea that overproduction of extracellular matrix is not beneficial and that expression of genes encoding components of the extracellular matrix must be properly regulated for normal healing to occur. Keloid fibroblast cultures have allowed us to establish a correlation between enhanced fibronectin gene transcription and abnormal wound healing. Now this system provides a means of identifying factors that contribute to keloid formation by focusing on fibronectin gene expression. Study of the regulatory pathways and molecules involved in keloid formation will contribute to a better understanding of normal wound healing and facilitate development of new strategies for the management or cure of keloids and other fibrotic conditions.

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