Fibronectin Is Overproduced by Keloid Fibroblasts during Abnormal Wound Healing

MARY BABU,¹ ROBERT DIEGELMANN,² AND NOELYNN OLIVER^{1*}

Department of Anatomy and Cell Biology, Tufts University Health Sciences Campus, 136 Harrison Avenue, Boston, Massachusetts 02111,¹ and Division of Plastic Surgery, Department of Surgery, Virginia Commonwealth University, Medical College of Virginia, Richmond, Virginia 23298²

Received 4 November 1988/Accepted 9 January 1989

Wound healing in certain individuals leads to the development of keloid tumors which exhibit abnormal collagen metabolism and an increased abundance of extracellular matrix components. Comparison of fibronectin levels in fibroblasts derived from keloids and normal dermis revealed a relative increase in intracellular and extracellular fibronectin in the keloid-derived cells. While fibronectin was similarly processed, compartmentalized, and degraded by both cell types, fibronectin biosynthesis was found to be accelerated as much as fourfold in keloid fibroblasts due to a corresponding increase in the amount of accumulated fibronectin mRNA. These changes account for the elevated steady-state level of the molecule in keloid fibroblasts and suggest that increased fibronectin in keloid lesions is due to overproduction by the wound-healing fibroblasts. Glucocorticoid treatment stimulated fibronectin biosynthesis in both normal and keloid fibroblasts. However, the amount of stimulation was less for the keloid-derived cells, indicating a limitation on maximal rates of fibronectin biosynthesis. These observations suggest that separate mechanisms act to control basal and maximal rates of fibronectin production. Biosynthesis of the 140-kilodalton fibronectin receptor was also found to be increased in keloid fibroblasts, suggesting some level of coordinate regulation for fibronectin and fibronectin receptor expression.

Keloids are dermal fibrotic lesions which can arise spontaneously or during healing of a deep skin wound (14, 15, 18, 27). The tumors are often nodular in shape and extend beyond the boundaries of the original wound. These lesions occur most commonly in individuals of darker pigmentation (27), with blacks and Orientals being the most frequently affected (30, 31, 33). Family studies suggest a genetic predisposition to keloid formation, the basis of which may be complex, since there is evidence for both autosomal dominant (4) and recessive (31) patterns of inheritance. The prevalence of keloids is significant, affecting 1.5% of the American population (41) and as many as 6% of the individuals in some African populations (30, 31). These tumors are benign and do not metastasize; however, therapy is not straightforward, since the lesions generally reappear and are more severe following the trauma of surgical removal (14, 15, 18, 27).

Keloids differ from normal dermis and mature scar tissue in both biochemical and cellular composition. The lesions are characterized by lack of elastic fibers (18), abnormal collagen metabolism (2, 7, 41), and elevated content of some extracellular matrix components, in particular chondroitin sulfate proteoglycan (3, 14, 18) and fibronectin (16). Increased levels of fibronectin could be due to overproduction of the molecule by the fibroblasts recruited for healing or increased local deposition from the circulation. Fibroblasts, also found in increased abundance in keloid tissue (37), are unusual because they proliferate abnormally yet do not metastasize or cause malignant disease. While previous studies suggested that the basic growth characteristics of these cells are not significantly different from those of normal cells (8, 35), more recent work has revealed that subtle differences in growth parameters do exist (36). When

keloid-derived fibroblasts are explanted and cultivated in vitro, they have been observed to overproduce collagen (1, 8, 42). The mechanism of this altered regulation has not been determined. It is also unknown whether these cells produce increased amounts of fibronectin, possibly accounting for the elevated abundance of fibronectin in keloid tissue and contributing to abnormal wound healing.

Fibronectin appears early in wound healing and contributes to the formation of granulation tissue (8-10, 17, 20, 26), a structure with many similarities to embryonic skin (23). Early in healing, fibroblasts migrate into the wound area and rapidly produce a transient matrix of fibronectin (9, 20, 26), which is assembled into a well-ordered, disulfide cross-linked fibrous structure. By means of multiple discrete binding domains (11, 13, 26, 43), fibronectin is able to specifically interact with other matrix proteins and macromolecules. This property is important in the subsequent assembly of other granulation tissue components, in particular collagen type I, heparin sulfate proteoglycan, and chondroitin sulfate. As healing progresses, collagen content increases, the collagen is organized into bundles, and eventually the fibronectin matrix diminishes (20). Granulation tissue is ultimately replaced by neodermis, which has a relatively high content of collagen type I and low content of fibronectin (9, 20). We have suggested that the biochemical stimulus causing increased fibronectin deposition in early granulation tissue is the stress-related transient increase in the level of circulating glucocorticoids (29).

It is likely that fibronectin plays a significant role in wound healing, since the molecule is abundant during the initial repair phase. Keloid fibroblast cultures have provided a system to investigate the role of fibronectin during normal and abnormal healing and to establish a correlation between fibronectin overproduction and the resulting abnormal physiology.

* Corresponding author.

MATERIALS AND METHODS

Tissue sources. The fibroblast cultures used for these studies were obtained from the Wound Healing Center at the Medical College of Virginia (Richmond, Va.). Keloid tissue specimens were obtained by surgical removal under local anesthesia after informed consent was obtained. Of the six patients (two females, four males) providing tissue for this study, four were black, one was white, and one was Asian. Patient ages ranged from 23 to 42 years. The lesions were diagnosed as keloids on the basis of clinical appearance, persistence for several years, extension beyond the original wound margins, and histopathology. Four of the keloids were from the upper thorax or face, one was from the upper back, and one was from the ear lobe. Normal tissue provided by two white patients (31-year-old male and 44-year-old female) was from the abdominal region, obtained during elective surgery.

Fibroblast isolation and cell culture. Epidermis and subdermal fat were removed from sterile biopsies of normal skin and keloids. The specimens were minced into pieces of 1 to 2 mm³ in sterile tissue culture dishes and gently overlaid with Dulbecco modified Eagle medium (4.5 g of glucose per liter) (DMEM) supplemented with 10% fetal calf serum (Gemini Bioproducts, Calabasas, CA), 500 U of penicillin per ml, and 100 µg of streptomycin per ml (DMES). Explants were incubated at 37°C in a humidified CO₂ incubator for 10 to 14 days and fed every third day. After 12 to 14 days, fibroblasts were harvested from the primary cultures by trypsin treatment and replated. Visual examination and electron microscopy confirmed that cultures contained fibroblasts. Cultures were screened for mycoplasma contamination by Hoechst 33258 staining (5). Both normal and keloid cultures were used at passages 2 to 6 for the studies described. For collagen biosynthesis assays, fibroblasts were cultured in DMES supplemented with ascorbic acid (50 µg/ml). For steroid regulation assays, the synthetic corticosteroid triamcinolone acetate (TA) was added 36 to 48 h prior to assay at a final concentration of 50 nM. TA was solubilized at 10 mM in 100% ethanol and diluted for use as a stock solution of 10 µM in phosphate-buffered saline (PBS).

Fibronectin immunofluorescence. Fibroblast cultures were grown in 35-mm-diameter tissue culture plates to approximately 50% confluence. The medium was removed, the plates were rinsed with warm DMEM, and cells were fixed in 4% formaldehyde for 5 min at 37°C. Fixed cells were washed with PBS and permeabilized by treatment with 0.1% Triton X-100 in a buffer containing 40 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.0), 50 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid), pH 6.9], 75 mM KCl, 0.1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid], and 1 mM MgCl₂ for 90 s at room temperature. Intact cells and permeabilized cells were washed with PBS, and fibronectin was localized by treatment with a 1:250 dilution of affinity-purified rabbit anti-human fibronectin antibody (kindly provided by J. McCarthy and L. Furcht, University of Minnesota) at room temperature for 60 min. In order to visualize areas of fibronectin immunoreactivity, samples were then treated for 60 min at room temperature with a 1:50 dilution of rhodamine-conjugated second antibody, goat anti-rabbit immunoglobulin G (IgG) (Cappel Laboratories, Cochranville, Pa.). Negative controls in which rabbit gamma globulin (RAGG; Antibodies Inc., Davis, Calif.) was used in place of the primary antibody revealed no background immunofluorescence. Fibronectin immunofluorescence was recorded on

Kodak Tri-X 100 film with a Zeiss IM-35 inverted fluorescence light microscope equipped with a 40× Planapochromat water immersion objective lens. Film speed was rated at 1000 ASA, and negatives were developed in Acufine film developer for 4.5 min.

Metabolic labeling of newly synthesized protein. Fibroblast cultures grown to near confluence in 60-mm-diameter tissue culture dishes were rinsed with PBS and labeled for 1 h with 0.5 ml of methionine-free DMEM supplemented with L-glutamine and 200 µCi of L-[³⁵S]methionine (New England Nuclear; 1,100 Ci/mmol) per ml. For fibronectin receptor biosynthesis assays, cultures were labeled with methionine- and cysteine-free medium with 200 µCi of L-[³⁵S]methionine plus L-[³⁵S]cysteine (Amersham; 1,000 Ci/mmol) per ml. After labeling, cells were lysed and proteins were solubilized by the addition of 0.5 ml of cold cell lysis buffer (40 mM Tris hydrochloride [pH 8.8], 4% deoxycholate, 4 mM phenylmethylsulfonyl fluoride [PMSF], 4 mM EDTA, 2.0% Nonidet P-40) to the culture. Lysed cells were then scraped from the dish, forced repeatedly through a 23-gauge needle to shear DNA, and centrifuged at 10,000 × *g* at 4°C for 15 min to remove insoluble material. Incorporation of radioactivity into newly synthesized protein was determined by trichloroacetic acid (TCA) precipitation, and each sample was adjusted to contain the same number of TCA-insoluble counts before immunoprecipitation. Samples were cleared before immunoprecipitation with RAGG complexed with affinity-purified goat anti-RAGG (GARGG; Antibodies Inc., Davis, Calif.).

Immunoprecipitation. Fibronectin in the cell lysates was allowed to complex with excess affinity-purified rabbit anti-human fibronectin antibody overnight at 4°C. The immune complexes were precipitated at room temperature for 2 h with carrier RAGG and affinity-purified GARGG as the second antibody. Precipitates were pelleted at 4°C in an Eppendorf microfuge and washed three times with 20 mM Tris hydrochloride (pH 8.8)–0.5% deoxycholate–0.5% Nonidet P-40–50 mM NaCl–2 mM PMSF–2 mM EDTA. Washed pellets were suspended in Laemmli sample buffer (21) containing 5% β-mercaptoethanol and heated at 100°C for 2 min.

Fibronectin receptor was immunoprecipitated by using rabbit anti-mouse fibronectin receptor antiserum (kindly provided by V. Patel, Northwestern University) as described above. In competition experiments, 10 µg of human plasma fibronectin was added to the lysates before the antibody was added, to compete with the labeled fibronectin for receptor binding.

Type I collagen was immunoprecipitated by using sheep anti-human type I collagen antibody (kindly provided by H. Kleinman, National Institutes of Health) and rabbit anti-sheep IgG (Cappel Laboratories, Cochranville, Pa.) as described above.

PAGE. Polyacrylamide gel electrophoresis (PAGE) with the Hoefer Mighty Small Electrophoresis System with 5% resolving gels and 3.5% stacking gels was done as described previously (28). Each gel was run at 20 mA constant current, and molecular weights were determined by using ¹⁴C-labeled markers (New England Nuclear). Gels were fixed in 40% methanol–7% acetic acid for 30 min, treated with Autofluor (National Diagnostics, Manville, N.J.), washed, dried, and exposed to Kodak X-Omat R film at –80°C. Gels were generally exposed for 15 to 30 min for fibronectin and overnight for collagen and fibronectin receptor. Fibronectin, collagen, or fibronectin receptor was located on gels by using the developed fluorographs; the bands were excised and

radioactivity was determined by scintillation spectrophotometry.

Northern analysis. Total RNA was isolated from the keloid and normal fibroblasts according to the method of Chirgwin et al. (6) and quantitated spectrophotometrically. Samples containing 10 μ g of total RNA in 10 mM NaH_2PO_4 (pH 6.5)–0.5 mM EDTA–2.2 M formaldehyde–50% formamide were denatured at 65°C for 15 min and resolved on a 1% agarose gel containing 2.2 M formaldehyde. Electrophoresis was at 100 V constant voltage for 6 h. The section of the gel to be transferred to nitrocellulose was cut out carefully, washed with 20 $\times$ SSPE, and soaked for 1 h in 50 mM NaOH–10 mM NaCl at room temperature. The gel was neutralized by soaking for 1 h in 0.1 M Tris (pH 7.5) and 1 h in 20 $\times$ SSPE and then transferred to nitrocellulose overnight. The positions of 18S and 28S rRNAs were determined by staining duplicate samples with acridine orange (33 μ g/ml) dissolved in 10 mM Na_2PO_4 (pH 6.5). The blot was baked for 1 h at 80°C in a vacuum oven.

Probe preparation and hybridization conditions. A 100-ng amount of CsCl-purified plasmid cDNA clones encoding human fibronectin, pFH154 (19), and human 18S rRNA, pB (kindly provided by J. Sylvester, University of Pennsylvania), were used for nick translation (25) with [32 P]dCTP (New England Nuclear). Unincorporated nucleotides were removed by spin column chromatography (24). The specific activity of each probe was 10⁹ cpm/ μ g, and both were greater than 85% TCA precipitable. Nitrocellulose blots were prehybridized for 2 h at 42°C in 200 μ g of denatured salmon sperm DNA per ml in 50% formamide–5 $\times$ SSPE–5 $\times$ Denhardt solution (24)–0.1% sodium dodecyl sulfate (SDS). Hybridization was done overnight at 42°C with 10⁶ cpm of the fibronectin probe per ml. Blots were washed at 65°C in 0.1 $\times$ SSPE–0.1% SDS, dried, and exposed to Kodak X-Omat film at –80°C. To assay the same samples for 18S rRNA, blots were washed three times with boiling water containing 0.1% SDS and then rehybridized with 5 $\times$ 10⁵ cpm of the human 18S rRNA probe per ml.

Quantitation of fibronectin mRNA levels. A microcomputer-based image acquisition system was used to quantify the fibronectin mRNA levels in normal and keloid cells. Developed fluorographs of Northern blots were placed on a light box in the view of a DAGE-MTI-65 Newvicon video camera. Bands were digitized automatically with an IBM PC-XT as the system controller. The PC is equipped with Tecmar's Graphics Master and Video VanGogh A/D cards, allowing near real-time imaging and analog-to-digital conversion with a 250 $\times$ 240 line resolution and 0 to 256 grey level sensitivity. The library of subroutines needed for frame grabbing, image averaging, and background subtraction have been modified from Tecmar's Video VanGogh software support (2a). Background was determined by using a region of X-ray film that lacked RNA. Each band of interest was digitized 16 times; the results were averaged and the background was subtracted.

Samples of increasing amounts of total RNA (containing increasing amounts of fibronectin mRNA and 18S rRNA) were used as standards and to confirm a linear correspondence between signal intensity and RNA level. Pixel values for the integrated areas found under the digitized peaks for the keloid and normal samples were compared with values for the standard samples. Only values within the linear range for signal intensity and amount of RNA were used. Ratios of values for fibronectin and 18S rRNA were used to compare fibronectin mRNA levels in keloid and normal fibroblasts.

FIG. 1. Type I collagen biosynthesis in normal and keloid fibroblasts. Newly synthesized type I collagen was detected and resolved by metabolic pulse labeling, specific immunoprecipitation, and SDS-PAGE as described in the text. (A) Resolution by SDS-PAGE of type I collagen immunoprecipitates for representative keloid and normal samples. Because the cells were labeled for 1 h, the newly synthesized type I collagen was mostly contained in a single band representing $\alpha 1$ and $\alpha 2$ procollagens. (B) Collagen biosynthesis. Relative rates of type I collagen production are a function of the counts per minute in the collagen-containing portions of the gel, since immunoprecipitation reactions all contained the same number of TCA-precipitable counts. For comparison, a value of 1.0 was assigned to the normal sample with the lowest number of type I collagen counts. Each fibroblast culture was tested in at least two separate experiments, and the results of a single representative determination are presented.

RESULTS

Confirmation of keloid phenotype. Although cell cultures were derived from explants of keloid lesions, it was essential to establish that these cells demonstrated the biochemical and metabolic properties typical of keloid fibroblasts. Since it has been well documented that keloid fibroblasts exhibit significantly elevated rates of collagen biosynthesis compared with normal skin fibroblasts (8, 42), overproduction of collagen was used as a distinguishing feature of the keloid phenotype. We measured the relative rates of collagen biosynthesis in six cultures derived from keloid tissue and two cultures from normal dermis by metabolic pulse labeling and immunoprecipitation with anti-type I collagen antibody. Immunoprecipitates were resolved by SDS-PAGE (Fig. 1A), and the labeled, newly synthesized type I collagen was located by fluorography and quantitated by scintillation spectrophotometry (Fig. 1B). All keloid cultures showed a two- to threefold increase in rates of collagen biosynthesis compared with normal fibroblasts, confirming the keloid phenotype. For these and all subsequent experiments, both normal and keloid cultures were used before the sixth passage.

Fibronectin accumulation. Keloids and keloid-derived fibroblasts have been observed to contain higher levels of fibronectin than normal dermis or normal fibroblasts (16).

FIG. 2. Fibronectin accumulation in normal and keloid fibroblasts. Intracellular and extracellular fibronectin in intact and permeabilized normal and keloid fibroblasts was detected by immunofluorescence as described in the text. Representative cells were first located by phase-contrast microscopy, photographed, and then observed for fibronectin distribution by fluorescence microscopy. Magnification and enlargement are the same for all samples. (A) Intact cells stained for extracellular fibronectin. (B) Permeabilized cells stained for intracellular and extracellular fibronectin.

However, it is unclear whether excess fibronectin results from overproduction by the resident fibroblasts or increased local deposition of plasma fibronectin (either from the circulation or the serum in the medium). Fibronectin accumulation was examined in normal and keloid fibroblasts by immunofluorescence with affinity-purified anti-human fibronectin antibody. Relative to the normal cells, levels of

fibronectin in the extracellular matrix were found to be increased for the keloid cells (Fig. 2A). Since extracellular fibronectin could be either produced by the cells or deposited from the serum contained in the medium, we also examined intracellular levels of fibronectin in permeabilized cells. These experiments also revealed increased amounts of intracellular fibronectin in the keloid cells (Fig. 2B). Thus, as

FIG. 3. Fibronectin biosynthesis in normal and keloid fibroblasts. Newly synthesized fibronectin (FN) was detected and resolved by metabolic labeling, specific immunoprecipitation, and SDS-PAGE as described in the text. (A) Fluorographs of representative keloid (K) and normal (N) samples. (B) Fibronectin biosynthesis. Relative rates of fibronectin production were determined as described for type I collagen in the legend to Fig. 1B. Each fibroblast culture was tested in at least two separate experiments, and the results of a single representative determination are presented.

in keloid tissue, elevated amounts of fibronectin are present in the fibroblasts derived from these lesions.

Fibronectin biosynthesis. Increased accumulation of fibronectin in the cell layer can result either from changes in rates of biosynthesis and degradation or from altered cellular compartmentalization. To distinguish between these possibilities, relative rates of fibronectin production were determined in cultures of normal and keloid fibroblasts by metabolic labeling and immunoprecipitation with affinity-purified anti fibronectin antibody. Since cells were pulse labeled, the amount of [35 S]methionine incorporated into newly synthesized fibronectin is a function of biosynthetic rates, and because equivalent samples were used for immunoprecipitation, the amount of [35 S]methionine incorporated into fibronectin indicates the relative rate of synthesis. As shown in Fig. 3A, the amount of radioactivity contained in the fibronectin band was considerably higher for the representative keloid cells, indicating significantly elevated rates of fibronectin biosynthesis relative to normal cells. Relative rates of fibronectin production in six keloid and two normal fibroblast cultures were determined and compared as described above (Fig. 3B). Fibronectin biosynthesis rates were consistently observed to be accelerated in keloid cultures, ranging from 1.4 to 4.4 times higher than normal rates (Table 1). Thus, like collagen, production of fibronectin is clearly increased in fibroblasts associated with abnormal wound healing.

TABLE 1. Fibronectin biosynthesis^a

Fibroblasts	Specimen no.	Relative rates of fibronectin production		TA-induced stimulation
		Untreated	TA treated	
Normal	1	1.0	3.9	3.9
	3	1.1	4.7	4.3
Keloid	1	3.3	4.9	1.5
	2	2.0	4.1	2.0
	3	3.0	6.9	2.3
	4	2.2	5.1	2.3
	5	1.4	5.1	3.7
	6	4.4	5.0	1.1
HT1080C		0.05	0.42	8.4

^a Relative rates of fibronectin production were determined as described for type I collagen in the legend to Fig. 1B. TA-induced stimulation of fibronectin production is the ratio of the rate of fibronectin production for the treated and untreated samples. Each fibroblast culture was tested in at least two separate experiments, and the results of a single representative determination are presented. For comparison with malignant transformation, the relative rate of fibronectin production in human fibrosarcoma cells (HT1080C) (34) was also measured.

These cells were also studied for fibronectin production following treatment with TA, a synthetic glucocorticoid. TA treatment resulted in stimulation of fibronectin production in both normal and keloid cells (Fig. 3A and B). Untreated keloid cells clearly showed elevated fibronectin production relative to normal cells; however, the relative rates of TA-stimulated fibronectin biosynthesis were very nearly the same in both cell types. This was especially evident when fibronectin biosynthesis in normal cells was compared with that in the keloid sample showing the greatest fibronectin overproduction (Fig. 3B). Glucocorticoid stimulation in these keloid cells only minimally enhanced the rate of fibronectin biosynthesis. In contrast, glucocorticoid treatment of normal cells enhanced fibronectin biosynthesis to a much greater extent. Hence, the amount of stimulation resulting from TA treatment (Table 1) was greater in normal cells (>4-fold) than in keloid cells (1.5- to 3.7-fold). For comparative purposes, fibronectin production by a human fibrosarcoma cell line, HT1080C, was also measured (Table 1). Basal fibronectin production was low but detectable in untreated HT1080C cells relative to normal and keloid fibroblasts, and following TA treatment, the rate of fibronectin biosynthesis was greatly elevated, showing at least an eightfold stimulation over untreated cells (Table 1).

Degradation of newly synthesized fibronectin. We next investigated fibronectin turnover in normal and keloid cells to determine whether increased rates of biosynthesis are accompanied by changes in rates of fibronectin degradation. Four cultures containing the same number of cells of one keloid and one normal specimen were incubated at 37°C for 1 h with medium containing [35 S]methionine. The radioactive medium was discarded, and the cells were washed with PBS. Cells of one sample in each group was solubilized to determine the amount of labeled, newly synthesized fibronectin initially present in the cell layer. The rest of the samples were incubated at 37°C for an additional 2, 4, or 8 h in medium containing excess cold methionine (1 mM). Cell layers as well as supernatants were then solubilized, fibronectin was immunoprecipitated and resolved by SDS-PAGE, and the labeled fibronectin was quantitated. For both normal and keloid cells, the amount of labeled fibronectin

FIG. 4. Kinetics of fibronectin degradation in normal (□) and keloid (●) fibroblasts. Newly synthesized fibronectin was detected by metabolic labeling, specific immunoprecipitation, and SDS-PAGE, quantitated, and expressed as a proportion of the amount of fibronectin present prior to chasing.

decreased steadily with time (Fig. 4). The percentage of the initially labeled fibronectin remaining at increasing times was similar for both normal and keloid cells. The time required for loss of 50% of the newly synthesized fibronectin was approximately 4 h in both cases. Thus, there was no significant difference in relative rates of fibronectin degradation.

Fate of newly synthesized fibronectin. To understand whether fibronectin is used and processed similarly by normal and keloid fibroblasts, we studied the fate of newly synthesized fibronectin in these cells. Fibronectin was labeled by exposure of the cultures to [35 S]methionine for 1 h, and the labeled fibronectin was chased for 1 and 2 h with excess cold methionine. The proportion of labeled fibronectin present in the cell layer or secreted into the medium was determined by separate immunoprecipitation of each of these cellular compartments. S1 represents the proportion of labeled, newly synthesized fibronectin secreted into the medium after a 1-h chase, and S2 is fibronectin secreted after an additional 1-h chase (Fig. 5). CL is the proportion of labeled fibronectin present in the cell layer following a 2-h chase (Fig. 5). For the normal cells, quantitation of the labeled fibronectin revealed that 23% of newly synthesized fibronectin was secreted into the medium in 1 h and an additional 33% was secreted during the second 1-h chase (Fig. 5). Similarly, for the keloid cells, 18% of the labeled,

FIG. 5. Fate of the newly synthesized fibronectin. Newly synthesized fibronectin was detected by metabolic labeling, specific immunoprecipitation, and SDS-PAGE, quantitated, and expressed as a proportion of the amount of fibronectin present prior to chasing. S1, Amount secreted into medium after 1-h chase; S2, amount secreted into medium after an additional 1-h chase; CL, amount present in cell layer after 2-h chase.

FIG. 6. Accumulation of fibronectin (FN) RNA in normal and keloid fibroblasts. Total RNA from a representative set of normal and keloid cells was isolated, and fibronectin RNA was detected by Northern analysis as described in the text. A 32 P-labeled human fibronectin cDNA probe was used to detect the 7.9-kilobase (Kb) RNA species encoding fibronectin.

newly synthesized fibronectin was secreted into the medium in 1 h and an additional 40 to 42% was secreted during the second 1-h chase (Fig. 5). In both normal and keloid cultures, approximately 45% of the labeled, newly synthesized fibronectin was associated with the cell layer after a 2-h chase (Fig. 5). Hence, there was no significant difference in the compartmentalization of newly synthesized fibronectin by normal and keloid fibroblasts.

Fibronectin mRNA accumulation. Increased rates of fibronectin biosynthesis in keloid cells can result either from elevated levels of fibronectin mRNA or from enhanced translation of fibronectin mRNA. To distinguish between these possibilities, we studied the steady-state levels of fibronectin mRNA by Northern analysis. Samples containing 10 μ g of total RNA isolated from normal and keloid cells were resolved by agarose gel electrophoresis, transferred to nitrocellulose, and probed for fibronectin mRNA. Image analysis of Fig. 6 revealed two- to threefold more fibronectin mRNA in the keloid cells than in the normal cells. The same blot was stripped of the fibronectin probe and reprobed for 18S rRNA to be certain that the same amount of total RNA was used for each sample. The amount of 18S rRNA was found to be nearly identical in all of the samples (data not shown).

Biosynthesis of the 140-kDa fibronectin receptor. Since fibronectin biosynthesis is elevated in the keloid cells, it was of interest to compare production of the 140-kilodalton (kDa) fibronectin receptor (12, 22, 32) in normal and keloid cells. Following metabolic labeling and immunoprecipitation of the fibronectin receptor, samples were resolved by SDS-PAGE and the fibronectin receptor was identified by molecular mass (Fig. 7A). Since these gels were run under reducing conditions, the α and β subunits of the fibronectin receptor were not separated. Fibronectin was observed to bind to the receptor and coprecipitate (Fig. 7A), since competition by excess cold fibronectin added to the cell lysates prior to immunoprecipitation reduced the amount of labeled fibronectin bound to the receptor (data not shown). Rates of synthesis of the fibronectin receptor were determined by excision of the 140-kDa band and quantitation of radioactivity. Keloid fibroblasts exhibited a twofold increase in the rate of fibronectin receptor synthesis relative to normal cells, showing some correlation with the increase in fibronectin production (Fig. 7B). Unlike fibronectin, fibronectin receptor synthesis was not regulated by glucocorticoids, since no increase in relative rates was observed for TA-treated cultures (Fig. 7A and B).

FIG. 7. Biosynthesis of the 140-kDa fibronectin receptor in normal and keloid fibroblasts. (A) Fibronectin receptor (FN-R) immunoprecipitates resolved by SDS-PAGE for representative keloid and normal fibroblast samples. Fibronectin receptor was identified as a 140-kDa protein. Fibronectin (FN) coprecipitated with fibronectin receptor. (B) Biosynthesis of fibronectin receptor. The fibronectin receptor bands were excised, and the radioactive content was determined. Relative rates of fibronectin receptor production were determined as described for type I collagen in the legend to Fig. 1B. Each fibroblast culture was tested in at least three separate experiments, and the results of a single representative determination are presented. Solid bars, No TA; shaded bars, with TA.

DISCUSSION

Keloid fibroblasts accumulate more cytoplasmic and cell surface-associated fibronectin than normal fibroblasts as a result of alterations in fibronectin metabolism. Specifically, we observed a 1.4- to 4.4-fold increase in the rate of fibronectin biosynthesis relative to that in normal cells, which can be accounted for by an increase in the level of fibronectin mRNA. Increased amounts of fibronectin mRNA could result either from an increase in the rate of fibronectin gene transcription or from an increase in the stability of fibronectin mRNA. Although fibronectin biosynthesis is clearly stimulated, keloid cells continue to process, compartmentalize, and degrade fibronectin normally. Increased accumulation of fibronectin in these fibroblasts is probably the cause of elevated fibronectin levels in keloid lesions.

While fibronectin production in keloid fibroblasts can be stimulated more than fourfold over normal, a range of values for fibronectin overproduction was observed. Although a number of factors can account for this variation, we favor the hypothesis that fibroblast cultures derived from keloids are heterogeneous. Only keloid tissue is used to isolate fibroblasts; however, since this may contain normal as well as abnormal cells, keloid cultures probably also contain a mixture of both. Additionally, there may be individual variation either in fibronectin production or in the physiology of the disease. Possibly, the site of the lesion or patient age also contribute to the observed variation.

Fibronectin biosynthesis is accelerated in both normal and keloid fibroblasts as a result of glucocorticoid treatment. Although the keloid cells overproduce fibronectin relative to

normal cells, glucocorticoid-enhanced rates of fibronectin biosynthesis were approximately equivalent in both cell types. Thus, the stimulation of fibronectin production resulting from glucocorticoid treatment was lower for keloid cells than for normal cells. This finding suggests a limitation on the maximal rate of fibronectin production in fibroblasts and may reflect a physiological limit to the amount of fibronectin that these cells require or can produce.

Increased fibronectin production in keloid cells could result from the inappropriate activation of a physiologically normal regulatory process. Since fibronectin biosynthesis is not stimulated to the same extent by glucocorticoids in keloid cells as in normal cells, it is possible that the mechanism by which glucocorticoids control fibronectin expression is already partially activated in these abnormal cells. Because glucocorticoids are known to downregulate production of type I collagen (38, 39), this hypothesis predicts that collagen production in keloid cells would be decreased relative to that in normal cells. However, keloid fibroblasts exhibit increased production of type I collagen, instead suggesting that some single, alternative process which elevates both collagen and fibronectin has been activated in keloid cells. This idea supports our previous suggestion that multiple mechanisms act to regulate fibronectin expression (28).

Production of fibronectin and the 140-kDa fibronectin receptor appears to be under some degree of coordinate control. Since fibronectin and the fibronectin receptor interact with each other, it is not unreasonable to expect that either of these molecules may be involved in regulating production of the other or that their expression involves common regulatory elements. However, it is likely that control of fibronectin receptor production is even more intricate because it belongs to a complex family of related molecules, the integrins (12, 22, 32). Production of fibronectin receptor was observed to be increased in the keloid cells relative to the normal cells. The elevated levels of both fibronectin and the fibronectin receptor in keloid fibroblasts suggest coordinate regulation in abnormal wound healing. However, it is unlikely that increased receptor biosynthesis is due only to elevated fibronectin levels, since glucocorticoid treatment and the resultant increase in fibronectin did not stimulate production of the fibronectin receptor.

Inappropriately accelerated cellular proliferation is almost always accompanied by a decrease in fibronectin production (11, 13, 26, 40, 43). This clearly is not the case for fibroblasts derived from keloids, since these cells proliferate abnormally but exhibit increased rather than diminished fibronectin production. One explanation for this difference is that control of proliferation is complex and loss of this control can occur in stages. It is possible that decreased fibronectin production is characteristic only of fully transformed fibroblast cells, and keloid fibroblasts, which do not metastasize, are just partially transformed. While we have found that keloid fibroblasts exhibit decreased population doubling times and require longer passage in culture to become senescent (M. Babu and N. Oliver, unpublished observations), other studies indicate that growth of keloid fibroblasts is not significantly different from that of normal cells. Alternatively, keloid fibroblasts may have an inherent inability to progress beyond the early phases of wound healing, when the immigrant fibroblasts proliferate and produce elevated amounts of fibronectin (9, 20, 26). In this case, keloid fibroblasts could be considered to resemble embryonic cells, which also exhibit accelerated proliferation, rather than transformed cells.

A number of distinct biological processes have been observed to affect fibronectin production in fibroblast cells. These include neoplastic transformation and wound healing, biological processes whose relatedness is unclear. Examination of fibronectin metabolism in fibroblasts derived from normal dermis and keloids has revealed aberrant fibronectin production and suggested multiple mechanisms controlling fibronectin expression. Clarification of the underlying regulatory mechanisms may serve to establish common features in the processes of tissue repair and neoplastic transformation.

ACKNOWLEDGMENTS

We are grateful to Kelman I. Cohen for providing keloid biopsies and William Lindbald for establishing explant cultures (Wound Healing Center, Medical College of Virginia). We thank Steven Adair, Ira Herman, and Jennifer Martin for stimulating discussion and critical assessment of our results.

This work was supported by grant 1552-C from the American Cancer Society, Massachusetts Division, and Public Health Service grants CA43860 from the National Cancer Institute and GM20298 from the National Institute of General Medical Sciences.

LITERATURE CITED

1. Abergel, P., M.-L. Chu, E. A. Bauer, and J. Uitto. 1987. Regulation of collagen gene expression in cutaneous diseases with dermal fibrosis: evidence for pretranslational control. *J. Invest. Dermatol.* **88**:727-731.
2. Abergel, R. P., D. Pizzurro, C. A. Meeker, G. Lask, L. Y. Matsuoka, R. R. Minor, M.-L. Chu, and J. Uitto. 1985. Biochemical composition of the connective tissue in keloids and analysis of collagen metabolism in keloid fibroblast cultures. *J. Invest. Dermatol.* **84**:384-390.
- 2a. Askey, D. B., and I. M. Herman. 1988. Computer-assisted analysis of the vascular endothelial cell migratory response to injury. *Computers Biomed. Res.* **21**:551-561.
3. Bazin, S., C. Nicoletis, and A. Delaunay. 1973. Intercellular matrix of hypertrophic scars and keloids, p. 571-578. *In* E. Kulonen and J. Pikarainen (ed.), *Biology of the fibroblast*. Academic Press, Inc., London.
4. Bloom, D. 1956. Heredity of keloids. Review of the literature and report of a family with multiple keloids in five generations. *N.Y. State J. Med.* **56**:511-519.
5. Chen, T. R. 1977. *In situ* detection of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain. *Exp. Cell Res.* **104**:255-262.
6. Chirgwin, J. J., A. E. Przbyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**:5294-5299.
7. Clore, J. N., I. K. Cohen, and R. F. Diegelmann. 1979. Quantitative assay of types I and III collagen synthesized by keloid biopsies and fibroblasts. *Biochim. Biophys. Acta* **586**:384-390.
8. Diegelmann, R. F., I. K. Cohen, and B. J. McCoy. 1979. Growth kinetics and collagen synthesis of normal skin, normal scar and keloid fibroblasts *in vitro*. *J. Cell. Physiol.* **98**:341-346.
9. Grinnell, F. 1984. Fibronectin and wound healing. *J. Cell. Biochem.* **26**:107-116.
10. Grinnell, F., R. F. Billingham, and L. Burgess. 1981. Distribution of fibronectin during wound healing in vivo. *J. Invest. Dermatol.* **76**:181-189.
11. Hynes, R. O. 1985. Molecular biology of fibronectin. *Annu. Rev. Cell Biol.* **1**:67-90.
12. Hynes, R. O. 1987. Integrins: a family of cell surface receptors. *Cell* **48**:549-554.
13. Hynes, R. O., and K. M. Yamada. 1982. Fibronectins: multifunctional modular glycoproteins. *J. Cell Biol.* **95**:369-377.
14. James, W. D., C. D. Besanceney, and R. B. Odom. 1980. The ultrastructure of a keloid. *J. Am. Acad. Dermatol.* **3**:50-57.
15. Ketcham, L. D., I. K. Cohen, and F. W. Masters. 1974. Hypertrophic scars and keloids: a collective review. *Plast. Reconstr. Surg.* **53**:140-154.
16. Kischer, C. W., and M. J. C. Hendrix. 1983. Fibronectin (FN) in hypertrophic scars and keloids. *Cell Tissue Res.* **231**:29-37.
17. Kischer, C. W., M. J. C. Hendrix, and R. O. Hynes. 1981. Immunofluorescent localization of fibronectin (FN) in normal skin, hypertrophic scars and granulation tissue. *J. Cell Biol.* **91**:144a.
18. Kischer, C. W., M. R. Shetlar, and M. Chvapil. 1982. Hypertrophic scars and keloids: a review and new concept concerning their origin. *Scanning Electron Microsc.* **4**:1699-1713.
19. Kornblihtt, A. R., K. Vibe-Pedersen, and F. E. Baralle. 1984. Human fibronectin: cell-specific alternative mRNA splicing generates polypeptide chains differing in the number of internal repeats. *Nucleic Acids Res.* **12**:5853-5868.
20. Kurkinen, M., A. Vaheri, P. J. Roberts, and S. Stenman. 1980. Sequential appearance of fibronectin and collagen in experimental granulation tissue. *Lab. Invest.* **43**:47-51.
21. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
22. Leptin, M. 1986. The fibronectin receptor family. *Nature (London)* **321**:728-730.
23. Linder, E., S. Stenman, V.-P. Lehto, and A. Vaheri. 1978. Distribution of fibronectin in human tissues and relationship to other connective tissue components. *Ann N.Y. Acad. Sci.* **312**:151-159.
24. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. Meinkoth, J., and G. Wahl. 1984. Hybridization of nucleic acids immobilized on solid supports. *Anal. Biochem.* **138**:267-284.
26. Mosher, D. F. 1984. Physiology of fibronectin. *Annu. Rev. Med.* **35**:561-577.
27. Murray, J. C., S. V. Pollack, and S. R. Pinnell. 1981. Keloids: a review. *J. Am. Acad. Dermatol.* **4**:461-470.
28. Oliver, N., R. F. Newby, L. T. Furcht, and S. Bourgeois. 1983. Regulation of fibronectin biosynthesis by glucocorticoids in human fibrosarcoma cells and normal fibroblasts. *Cell* **33**:287-296.
29. Oliver, N. A. 1987. Altered production of fibronectin and collagen in hypercortisolism may inhibit tissue repair. *Arch. Dermatol.* **213**:570-571.
30. Oluwasanmi, J. O. 1974. Keloids in the African. *Clin. Plast. Surg.* **1**:179-195.
31. Omo-Dare, P. 1975. Genetic studies on keloids. *J. Natl. Med. Assoc.* **67**:428-432.
32. Pytela, R., M. D. Pierschbacher, and E. Ruoslahti. 1985. Identification and isolation of a 140-kilodalton cell surface glycoprotein with properties expected of a fibronectin receptor. *Cell* **40**:191-198.
33. Ramakrishnan, K. M., K. P. Thomas, and C. R. Sundararajan. 1974. Study of 1000 patients with keloids in South India. *Plast. Reconstr. Surg.* **53**:276-280.
34. Rasheed, S., W. A. Nelson-Rees, E. M. Toth, P. Arnstein, and M. B. Gardner. 1974. Characterization of a newly derived human sarcoma cell line (HT-1080). *Cancer* **33**:1027-1033.
35. Russell, J. D., and W. S. Witt. 1976. Cell size and growth characteristics of cultured fibroblasts isolated from normal and keloid tissue. *Plast. Reconstr. Surg.* **57**:207-212.
36. Russell, S. B., K. M. Trupin, S. Rodriguez-Eaton, J. D. Russell, and J. S. Trupin. 1988. Reduced growth factor requirement of keloid-derived fibroblasts may account for tumor growth. *Proc. Natl. Acad. Sci. USA* **85**:587-591.
37. Ryan, G. B., W. J. Cliff, G. Gabbiani, C. Irle, D. Montandon, P. R. Statkov, and G. Majao. 1974. Myofibroblasts in human granulation tissue. *Hum. Pathol.* **5**:55-67.
38. Shull, S., and K. R. Cutroneo. 1983. Glucocorticoids coordinately regulate procollagens type I and type II synthesis. *J. Biol. Chem.* **258**:3364-3369.
39. Sterling, K. M., M. J. Harris, J. J. Mitchell, T. A. DiPetrillo, G. L. Delaney, and K. R. Cutroneo. 1983. Dexamethasone decreases the amounts of type I procollagen mRNAs *in vivo* and in fibroblast cell cultures. *J. Biol. Chem.* **258**:7644-7647.

40. Tyagi, J. S. H., G. T. Merlino, and I. Pastan. 1983. Transcriptional control of the fibronectin gene in chick embryo fibroblasts transformed by Rous sarcoma virus. *J. Biol. Chem.* **258**:5787-5793.
41. Uitto, J. 1986. Regulation of collagen gene expression. *Ann. Intern. Med.* **105**:740-756.
42. Uitto, J., A. J. Perejda, R. P. Abergel, M.-L. Chu, and F. Ramirez. 1985. Altered steady-state ratio of type I/III procollagen mRNAs correlates with selectively increased type I procollagen biosynthesis in cultured keloid fibroblasts. *Proc. Natl. Acad. Sci. USA* **82**:5935-5939.
43. Yamada, K. M., S. K. Akiyama, T. Hasegawa, E. Hasegawa, M. J. Humphries, D. W. Kennedy, K. Nagata, H. Urshiharda, K. Olden, and W.-T. Chen. 1985. Recent advances in research on fibronectin and other cell attachment proteins. *J. Cell. Biochem.* **28**:79-97.