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Qunzhou Zhang
University of Pennsylvania

Yidi Wu


David K. Ann

Diana V. Messadi

Tai-Lan Tuan

See next page for additional authors

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At the time of publication, author Qunzhou Zhang was affiliated with the Charles R. Drew Univ. Med. and Sci.. Currently, (s)he is a faculty member at the School of Medical Dentistry at the University of Pennsylvania.

At the time of publication, author Anh D. Le was affiliated with the Charles R. Drew Univ. Med. and Sci., and the University of Southern California, School of Dentistry. Currently, (s)he is a faculty member at the School of Medical Dentistry at the University of Pennsylvania.

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Mechanisms of Hypoxic Regulation of Plasminogen Activator Inhibitor-1 Gene Expression in Keloid Fibroblasts

Abstract

Keloids are an excessive accumulation of extracellular matrix. Although numerous studies have shown elevated plasminogen activator inhibitor-1 (PAI-1) levels in keloid fibroblasts compared with those of normal skin. Their specific mechanisms involved in the differential expression of PAI-1 in these cell types. In this study, the upregulation of PAI-1 expression is demonstrated in keloid tissues and their derived dermal fibroblasts, attesting to the persistence, if any, of fundamental differences between in vivo and in vitro paradigms. We further examined the mechanisms involved in hypoxia-induced regulation of PAI-1 gene in dermal fibroblast derived from keloid lesions and associated clinically normal peripheral skins from the same patient. Primary cultures were exposed to an environmental hypoxia or desferroxamine. We found that the hypoxia-induced elevation of PAI-1 gene appears to be regulated at both transcriptional and post-transcriptional levels in keloid fibroblasts. Furthermore, our results showed a consistent elevation of HIF-1 α protein level in keloid tissues compared with their normal peripheral skin controls, implying a potential role as a biomarker for local skin hypoxia. Treatment with antisense oligonucleotides against hypoxia-inducible factor 1 α (HIF-1 α) led to the downregulation of steady-state levels of PAI-1 mRNA under both normoxic and hypoxic conditions. Conceivably, our results suggest that HIF-1 α may be a novel therapeutic target to modulate the scar fibrosis process.

Keywords

Antisense, Desferroxamine, Half-life, HIF-1 α , Hypoxia, deferoxamine, iron derivative, plasminogen activator inhibitor 1, adult, article, cell culture, clinical article, controlled study, environmental exposure, female, fibroblast, gene expression, human, human cell, human tissue, hypoxia, in vitro study, in vivo study, keloid, male, priority journal, regulatory mechanism, skin culture, transcription initiation, upregulation

Disciplines

Dentistry | Oral and Maxillofacial Surgery | Oral Biology and Oral Pathology | Periodontics and Periodontology

Comments

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Author(s)

Qunzhou Zhang, Yidi Wu, David K. Ann, Diana V. Messadi, Tai-Lan Tuan, A. Paul Kelly, Charles N. Bertolami, and Anh D. Le

Increased Plasminogen Activator Inhibitor-1 in Keloid Fibroblasts May Account for their Elevated Collagen Accumulation in Fibrin Gel Cultures

Tai-Lan Tuan,* Huayang Wu,* Eunice Y. Huang,*
Sheree S. N. Chong,* Walter Laug,[†]
Diana Messadi,[‡] Paul Kelly,[§] and Anh Le^{‡§}

From the Departments of Surgery* and Pediatrics,[†] Childrens Hospital Los Angeles, Keck School of Medicine, University of Southern California, Los Angeles; the School of Dentistry,[‡] University of California Los Angeles, Los Angeles; and the Charles R. Drew University of Medicine and Science,[§] Los Angeles, California

Proteolytic degradation of the provisional fibrin matrix and subsequent substitution by fibroblast-produced collagen are essential features of injury repair. Immunohistochemical studies revealed that although dermal fibroblasts of normal scars and keloids expressed both urokinase type plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1), keloid fibroblasts had a much higher PAI-1 expression. In long-term three-dimensional fibrin gel cultures (the *in vitro* fibroplasia model), normal fibroblasts expressed moderate and modulated activity levels of uPA and PAI-1. In contrast, keloid fibroblasts expressed a persistently high level of PAI-1 and a low level of uPA. The high PAI-1 activity of keloid fibroblasts correlated with their elevated collagen accumulation in fibrin gel cultures. Substituting collagen for fibrin in the gel matrix resulted in increased uPA activity and reduced collagen accumulation of keloid fibroblasts. Furthermore, decreasing PAI-1 activity of keloid fibroblasts in fibrin gel cultures with anti-PAI-1-neutralizing antibodies also resulted in a reduction in collagen accumulation by keloid fibroblasts. Cumulatively, these results suggest that PAI-1 overexpression is a consistent feature of keloid fibroblasts both *in vitro* and *in vivo*, and PAI-1 may play a causative role in elevated collagen accumulation of keloid fibroblasts. (Am J Pathol 2003, 162:1579–1589)

Keloids are scars that show exuberant growth beyond the margins of the original wound that rarely regress throughout time. It is estimated that ~15 to 20% of African-Americans, Hispanics, and Asians develop keloids with a suggested genetic predisposition to keloid formation.^{1,2} Before keloids form, the affected skin shows excessive and prolonged fibroplasia, and keloids differ from other

scars by having multiple collagen nodules in the deep dermis. *In vitro*, keloid fibroblasts exhibit an altered phenotype of either intrinsic or growth factor-stimulated collagen, fibronectin, elastin, and proteoglycan accumulation.^{3–10} These alterations may occur at the level of growth factor production, growth factor receptor expression, or postreceptor signal transduction.^{11–13} Aberrations in cell growth have also been implicated in keloid scar formation and expansion. Accordingly, higher ratios of proliferating and apoptotic cell populations were found in keloid lesions when compared with normal skin cell populations.^{14–16} Using an *in vitro* three-dimensional fibrin matrix gel culture system to simulate fibroplasia of wound repair,^{17,18} we found that keloid fibroblasts exhibit not only an increase in collagen accumulation,¹⁹ but also an altered pattern in fibrin degradation.²⁰ Their defect in fibrin degradation is possibly because of plasminogen activator inhibitor-1 (PAI-1) overproduction.²⁰

Proteolytic degradation of the provisional fibrin matrix and subsequent substitution by fibroblast-produced collagen are essential features of injury repair.²¹ Organ fibrosis is typified by excessive collagen accumulation at the site of injury that involves excessive collagen synthesis or delayed collagen degradation. Therefore, molecules that promote collagen synthesis or inhibit its degradation must be involved. PAI-1 belongs to the serpin family and is the major inhibitor to urokinase-type and tissue-type plasminogen activators (uPA and tPA, respectively).²² Both uPA and tPA convert plasminogen into plasmin. Being the primary protease in fibrinolysis, plasmin also participates in the breakdown of other glycoproteins in the extracellular matrix (ECM), the activation of matrix metalloproteinases (MMPs) from their proenzyme form,²³ and the release of transforming growth factor (TGF)- β from its latency-associated protein.²⁴ In fact, targeted gene manipulations of MMPs display abnormalities in injury repair overlapping with that of the members of the plasminogen activator system.²⁵

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Address reprint requests to Tai-Lan Tuan, Ph.D., Department of Surgery, Childrens Hospital Los Angeles, Keck School of Medicine, University of Southern California, 4650 Sunset Blvd., Mail Stop #35, Los Angeles, CA 90027. E-mail: ttuan@chla.usc.edu.

Furthermore, inhibition of both the plasminogen activator/plasmin and MMP systems leads to complete arrest of wound healing and wound closure.²⁶ On the other hand, increased PAI-1 has been a hallmark of organ fibrosis in which it has been associated with thrombotic diseases and metabolic disorders that are linked with the development of arteriosclerosis and organ fibrosis in liver, lung, kidney, blood vessels, and skin.²⁷⁻³⁰

Definitive proof of the plasminogen activator system involvement in ECM metabolism during tissue injury repair comes from targeted gene interruption or overexpression in mice. In this regard, it has been shown that bleomycin-induced pulmonary fibrosis is more severe in transgenic mice overexpressing PAI-1 or in mice deficient of plasminogen, uPA, or tPA.^{28,31} Administration of uPA into the lungs of wild-type or PAI-1 transgenic mice after bleomycin injury decreases lung fibrosis.³² Accordingly, PAI-1 knockout mice are protected against bleomycin-induced pulmonary fibrosis.³³ Furthermore, plasminogen knockout mice exhibit delayed skin wound repair,³⁴ and PAI-1-deficient mice show accelerated wound closure.³⁵ These observations suggest that members of the plasminogen activator system play an essential role in ECM metabolism during wound repair. Increased PAI-1, which inhibits PA activity and the extent of plasminogen activation, may result in fibrin accumulation at the site of tissue injury and in fibrosis. In contrast to acute wounds, chronic wounds contain very high levels of active uPA and multiple species of gelatin- and casein-degrading proteases.³⁶ Furthermore, the activity of these proteases seems to regress as the chronic wound heals.³⁶ Therefore, abnormal healing may result from changes in either the activity of proteases, their inhibitors, or in the regulatory machinery that controls the expression of these molecules.

The complex nature of the repair process and a lack of proper model systems have made it difficult to investigate the underlying mechanism(s) of fibrosis after injury repair in humans. We have developed an *in vitro* three-dimensional fibrin matrix gel culture system for this purpose.¹⁸ This system models the key features of fibroplasia, ie, cell proliferation, fibrin reorganization and degradation, and collagen synthesis and deposition. It is suitable for the cellular and molecular studies of the intricate interactions between the cell and its surrounding ECM in wound repair. In continued experiments we demonstrated that PAI-1 overexpression is a consistent feature of keloid fibroblasts both *in vitro* and *in vivo*. We also studied collagen production and uPA/PAI-1 expression of normal and keloid fibroblasts in long-term fibrin matrix cultures, and investigated the effect of PAI-1 overproduction on collagen accumulation of keloid fibroblasts.

Materials and Methods

Cell Isolation

Fibroblasts were established from donors of human normal skin, scar, and keloid using the explant method. The protocol for skin and scar collections was approved by

the institutional review boards of both the Childrens Hospital Los Angeles and the Charles R. Drew University of Medicine and Science. The raised core region of keloid scars was used for fibroblast isolation. Fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Inc., Grand Island, NY) containing 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% fetal calf serum (Life Technologies, Inc.). Cultures were incubated in a humidified incubator in an atmosphere of 5% CO₂ and 95% air. Fibroblasts were harvested from cultures using 0.25% trypsin containing 0.05% ethylenediaminetetraacetic acid in Hanks' solution (Life Technologies, Inc.) and passaged once a week. Early passages (2 to 10) of fibroblasts were used in the experiments. Cell passage is defined as weekly expansion of cells from primary cultures. The source of each strain of fibroblasts used in the study is listed in Table 1.

Preparation of Fibrin Gels

Human fibrinogen (Calbiochem, San Diego, CA) was used for the preparation of fibrin gels. Fibrinogen was reconstituted in distilled H₂O, adjusted to 10 mg/ml, and stored at -20°C. The clottability of fibrinogen was determined by mixing 1 to 5 mg/ml of fibrinogen with 1 to 2 U/ml of human thrombin and incubating for 30 minutes at 37°C. The clots that formed were detached from test tube walls. Tubes were centrifuged at 12,000 \times *g* for 15 minutes to pellet the clot and to collect soluble fibrinogen. The soluble nonclottable fibrinogen remaining in the supernatant was determined by protein concentration at OD₂₈₀. All fibrinogen used was 95 to 98% clottable.

The method for fibrin gel preparation has been described in a previous publication.¹⁷ Briefly, human skin fibroblasts in DMEM were added to a fibrinogen solution at 24°C. Final concentrations of fibrinogen and fibroblasts were 2.5 mg/ml and 0.5 \times 10⁶ cells/ml, respectively. Aliquots (180 μ l) of the fibroblast/fibrinogen mixtures were placed in wells of 24-well tissue culture plates (Costar, Cambridge, MA) with 1 U of thrombin per sample. Each aliquot occupied an area outlined by a 16-mm-diameter circular score within the well. The preparations were incubated at 37°C for 1 hour in a humidified incubator containing 5% CO₂ to ensure polymerization of fibrin. At the end of the incubation period, 1.0 ml of DMEM containing 10% fetal calf serum was added to each well to cover the gel.

Samples selected for uPA and PAI studies were first thoroughly rinsed (five times) with DMEM and incubated in DMEM for an additional 24 hours. Conditioned culture media were collected and subjected to fibrin overlay and reverse fibrin overlay assays.

Preparation of Collagen Gels

Collagen gels were prepared according to the method previously described by Tuan and colleagues³⁷ using Vitrogen (Cohesion Technologies, Inc., Palo Alto, CA), a preparation of predominantly type I collagen. Briefly, the collagen was adjusted to physiological ionic strength and

Table 1. Normal Skin, Scar, and Keloid Fibroblasts Used in the Study

Type	Cell strain	Ethnic background	Gender	Age	Anatomical site
Normal skin	N65	Hispanic	M	8	Ear
Normal skin	N77	African-American	M	4	Ear
Normal skin	N86	African-American	M	28	Ear
Normal skin	N123	Caucasian	F	23	NK*
Normal skin	N127	Iranian	M	33	Post ear
Normal skin	N141	Caucasian	M	Newborn	Foreskin
Normal skin	N143	Caucasian	M	Newborn	Foreskin
Normal skin	N144	Caucasian	M	Newborn	Foreskin
Normal skin	K7N	African-American			
Normal scar	NSC14	Hispanic	F	25	Elbow
Normal scar	NS70	Caucasian	M	8	Foot
Normal scar	NS75	African-American	M	4	Cheek
Keloid	K9	African-American	F	NK*	Ear lobe
Keloid	K10	African-American	F	NK*	Flank
Keloid	K74	African-American	M	28	Ear lobe
Keloid	K76	African-American	M	4	Ear
Keloid	K80	Hispanic	M	5	Supra-pubic
Keloid	K86	African-American	M	28	Ear
Keloid	K109	African-American	M	14	Ear
Keloid	K134	Asian	F	6	Chest
Keloid	K135	Hispanic	F	6	Hand
Keloid	K139	African-American	F	47	Ear
Keloid	K142	African-American	F	7	Sternal area
Keloid	K147	African-American	M	18	Ear
Keloid	K148	African-American	M	14	Ear
Keloid	K150c	African-American	F	23	Chest
Keloid	K165	Hispanic	M	5	Ear

*NK, not known.

pH with 10× minimum essential medium (Sigma Chemical Co., St. Louis, MO) and 0.1 N of NaOH at 4°C. The final collagen concentration was 1.5 mg/ml. Fibroblasts were incorporated into the reconstituted collagen at a final concentration of 0.5×10^6 cells/ml. Samples of the collagen/fibroblast suspension were dispensed into 24-well culture plates. Each 180- μ l aliquot was contained within a circle of 16-mm diameter scored onto the base of the well. The culture plates were then placed in an incubator at 37°C with 5% CO₂ for 45 minutes to allow collagen to polymerize.

Fibrin and Collagen Mixture Gels

Gels were prepared by mixing fibrinogen and collagen in different ratios (fibrin:collagen, 100%:0%; 50%:50%; 0%:100%). Fibroblasts were incorporated into the matrix at a final density of 0.5×10^6 cells/ml. Aliquots (180 μ l) of gel-fibroblast mixtures were placed in wells of 24-well tissue-culture plates with 1 U of thrombin per sample following a similar format described above.

Fibrin Overlay and Reverse Overlay

Briefly, aliquots (25 μ l) of serum-free conditioned culture media were subjected to electrophoresis using a 10% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS, Sigma). The gel was washed for 1 hour at room temperature in 2.5% Triton X-100 to remove SDS. After a brief rinse in distilled water, the gel was placed on an indicator gel layer (fibrin overlay assay for PA detection) that contained 1% low-temperature gelling agarose, hu-

man plasminogen (9 μ g/ml, Sigma), thrombin (0.7 U/ml, Sigma), and fibrinogen (2 mg/ml). To detect PAI, SDS-polyacrylamide gels were washed in 2.5% Triton X-100 for 1 hour at room temperature and placed on top of a substrate gel similar to the indicator gel (above) with the addition of uPA (0.2 U/ml, Sigma) (reverse fibrin overlay assay). Both preparations were placed in a humidified chamber at 37°C. Activity of PA appeared as clear zones in the opaque fibrin indicator layer indicating fibrinolysis. Activity of PAI appeared as opaque zones in a cleared reverse overlay substrate layer indicating inhibition of fibrinolysis. The results were photographed.

Chromogenic Substrate Assay

A two-stage, indirect enzymatic assay, Spectrolyse (pL PAI (no. 101201; American Diagnostica, Greenwich, CT), was used for the quantitative determination of PAI-1 activity in plasma. In stage one, a fixed amount of tissue plasminogen activator (tPA) was added to the sample and allowed to react with PAI-1 present. The sample was then acidified to destroy α -2-anti-plasmin and other potential plasmin inhibitors that would otherwise interfere with the tPA assay. In stage two, the residual tPA activity was measured by adding the sample to a mixture of Glu-plasminogen, poly-D-lysine and chromogenic substrate at neutral pH. The residual tPA activity in the sample catalyzes the conversion of plasminogen to plasmin, which in turn hydrolyzes the chromogenic substrate. The amount of color developed is proportional to the amount of tPA activity in the sample. Poly-D-lysine is present as a stimulator of the tPA-catalyzed conversion of plasmino-

gen to plasmin. The PAI content of the sample is then identified as the difference between the amount of tPA added and the amount of tPA recovered. One U of PAI activity (U) is defined as the amount of PAI that inhibits 1 IU of a human single chain tPA as calibrated against the International Standard for tPA lot 86/670 distributed by NIBSAC, Holly Hill, London, UK.

Collagen Synthesis, Purification, and Phenotype Analysis

[³H]Proline was used to label newly synthesized collagen by fibroblasts. Samples in triplicates were labeled for 48 hours with L-(5-[³H]proline) (50 μ Ci/ml; Amersham, Arlington Heights, IL) in DMEM-10% fetal calf serum supplemented with β -aminopropionitrile (62.5 μ g/ml). At the end of labeling, all samples were adjusted to 0.5 mol/L acetic acid and treated with 1 mg/ml of pepsin (PM grade; Worthington, Freehold, NJ) for 24 hours at 4°C to digest proteins other than intact collagen. Pepsin was inactivated by adding Tris to 50 mmol/L and titration to pH 7.4. Collagen was purified by sequential neutral salt (4 mol/L NaCl) and acid salt (2 mol/L NaCl) precipitation as described previously.¹⁸ The final collagen pellet was rinsed in 50 mmol/L of Tris and 40% ethanol and dissolved in 0.5 mol/L of acetic acid. Samples were subjected to SDS-polyacrylamide gel electrophoresis and followed by fluorography. Samples designated for cell count were treated with trypsin and collagenase, and viable cell numbers were estimated using a hemocytometer in the presence of Trypan Blue. Purified collagen was expressed as cpm/cell. Data presented is an average of three replicate samples. Statistical differences between and within groups were assessed using one-way analysis of variance.

Northern Blots

Standard Northern blot analysis was used to study RNA expression.³⁸ Briefly, RNA samples were extracted using guanidinium thiocyanate and separated by centrifugation through cesium chloride. Total RNA (20 μ g/lane) was separated by electrophoresis, transferred to nylon filters, and baked at 80°C under vacuum for 2 hours. After prehybridization, the radioactive-labeled DNA probes were hybridized to filters for 20 hours at 40°C, washed, and visualized by exposure to X-ray film at -70°C. The cDNA probes were labeled according to the method of Feinberg and Vogelstein.³⁹ All samples were standardized to the level of expression of β -actin in each cell strain. Specific human cDNA probes for uPA nucleotides 623 to 1039 and PAI-1 cDNA (full length) were used as hybridization probes.⁴⁰

Immunohistochemistry

Freshly collected skin and scar samples were rinsed in ice-cold phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde (pH 7.5, Sigma) at 4°C for 24 hours.

Samples were treated with 70% ethanol for 24 hours before dehydration. After dehydration, samples were embedded in paraffin (60°C), and 5- μ m-thick sections were prepared using a microtome. Sections were rehydrated and treated with H₂O₂. To minimize nonspecific binding, sections were first treated with 1.5% bovine serum albumin/PBS for 30 minutes at room temperature. Mouse monoclonal antibody against human uPA at 1:50 dilution (no. 3698 and no. 394, American Diagnostica Inc.) and murine monoclonal antibody against human PAI-1 at 1:25 dilution (no. 3785, American Diagnostica Inc.) were used to detect uPA and PAI-1, respectively. After primary antibody treatment, sections were washed three times with PBS and incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech Limited, Buckinghamshire, UK) for 50 minutes. After thorough rinsing with PBS, sections were treated with 3,3'-diaminobenzidine (Sigma) to reveal antibody-antigen reaction. Sections were also stained lightly with hematoxylin for nuclear staining.

Results

PAI-1 Expression Is Increased in Fibroblasts of Keloid Lesions

Keloid fibroblasts exhibit elevated PAI-1 expression in culture.^{20,29} To examine if PAI-1 overexpression also occurs *in vivo*, protein expressions of both PAI-1 and uPA were studied in keloid lesions ($n = 5$) using antibodies against PAI-1 or uPA in immunohistochemistry. The results were compared with normal skin ($n = 3$) and normal scar ($n = 3$) samples. Keloids are characterized by their overabundance of collagen deposition in the dermis, thus our results also include the deep dermal region of keloid lesions (Figure 1, j, k, and l). Besides collagen, fibroblasts and blood vessels of various sizes were the major visible structural components in the dermis (Figure 1). In normal skin, staining of PAI-1 and uPA was localized to the blood vessels (Figure 1, b and c). In normal scars and keloids, although both blood vessels and fibroblasts stained positive for uPA and PAI-1, the intensity of their staining was quite different. PAI-1 staining appeared much stronger in keloid fibroblasts than in normal scar fibroblasts (Figure 1, h and k *versus* e); and uPA staining was stronger in normal scar fibroblasts than in keloid fibroblasts (Figure 1, f *versus* i and l). The high level of PAI-1 staining was observed in four of five (80%) keloid specimens. The epidermis was also positive for uPA and PAI-1 (Figure 1, asterisks) and again the epidermis of keloids showed a stronger PAI-1 staining than that of normal skin or normal scar. Keloid and normal skin samples were collected from African-American patients where melanocytes in the basal layer of epidermis appeared dark brown in immunohistochemistry. Staining was negative in all control groups (Figure 1, a, d, g, and j).

To compare if PAI-1 overexpression also occurred at the mRNA level, skin fibroblasts were isolated from normal skin, normal scar, and keloid samples and analyzed using Northern blot technique. Results showed that PAI-1

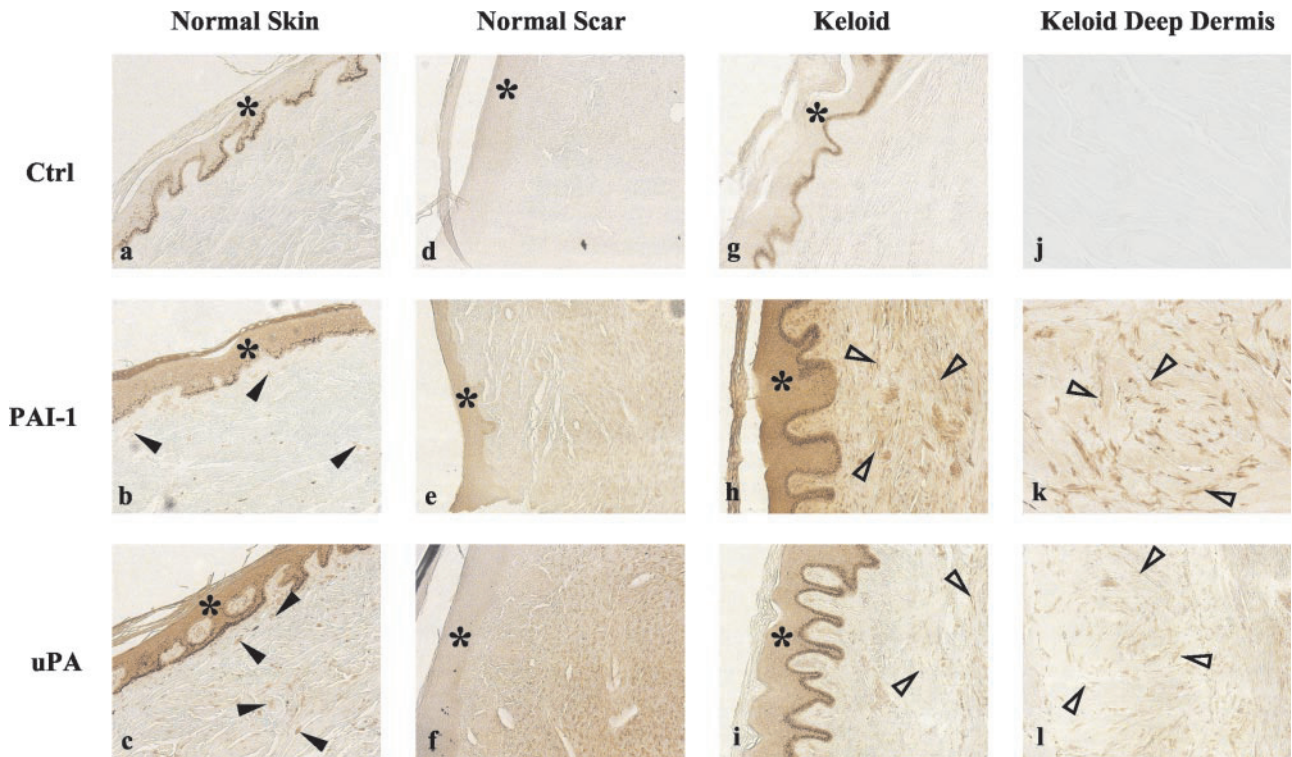


Figure 1. Immunohistochemistry study of uPA and PAI-1 expressions in normal skin, normal scar, and keloid. Keloid and normal skin samples were from African-American patients where melanocytes appeared dark brown in immunohistochemistry. Ctrl, control without primary antibodies. *, Epidermis. **j, k, and l** are from deep dermal region of keloid scar. **Solid arrowheads** in **b** and **c** indicate blood vessels. **Open arrowheads** in **h, k, i, and l** indicate fibroblasts. Photo images were taken at $\times 100$ magnification.

had two RNA messages, 3.0 kb and 2.2 kb, respectively (Figure 2). The 2.2-kb PAI-1 mRNA in keloid fibroblasts was consistently higher than both normal skin and normal scar fibroblasts. Therefore, PAI-1 overexpression is a consistent feature of keloid fibroblasts both *in vitro* and *in vivo*.

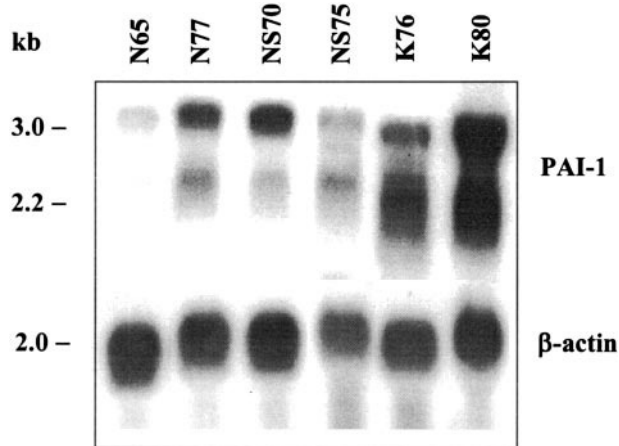


Figure 2. Northern blot analysis of mRNA of PAI-1 from fibroblasts of normal skin, normal scar, or keloid origins. Normal skin (N65 and N77), normal scar (NS70 and NS75), and keloid (K76 and K80) fibroblasts were cultured at a density of 8×10^3 cells/cm² and extracted for Northern blot analysis (see Materials and Methods). Twenty μ g of total RNA was loaded on each lane. Samples were standardized to the level of β -actin.

Keloid Fibroblasts Exhibit Elevated Collagen Accumulation and Persistently High PAI-1 Activity in Long-Term Fibrin Gel Cultures

PAI-1 has been implicated in tissue fibrosis. To examine if PAI-1 overexpression in keloid fibroblasts correlated with their collagen overproduction, PA/PAI and collagen production were studied throughout a 2-week period using the *in vitro* fibroplasia model.¹⁸ Experiments were conducted each time using one keloid and one normal strain of fibroblasts, and a minimum of six keloid and six normal strains of fibroblasts were examined.

Total collagen produced by fibroblasts was purified as described in Materials and Methods. In fibrin gels, keloid fibroblasts grew at a similar rate as normal fibroblasts (data not shown). Although a small amount of type III (γ) and type V (ν) collagen was detected, type I collagen (α_1 and α_2) was the predominant collagen made by keloid and normal fibroblasts (data not shown). Results of a typical experiment are shown in Figure 3. The quantity of total collagen was normalized to the cell number and expressed as cpm/cell. In the 2-week study, general patterns of collagen accumulation were highly reproducible among different strains of each cell type. For normal fibroblasts, collagen accumulation increased gradually in the first 10 days. It peaked at ~ 13 to 15 days, and decreased at the end of the culture period (16th day) (Figure 3, normal). Keloid fibroblasts showed a similar

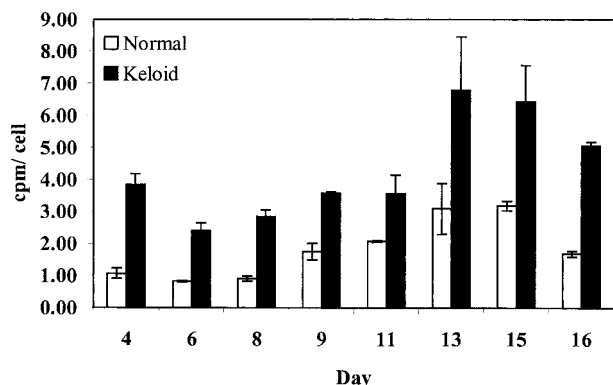


Figure 3. A time course study of collagen accumulation comparing normal and keloid fibroblasts cultured in fibrin gels throughout a 16-day period. Collagen synthesized by either normal or keloid fibroblasts was purified according to the procedure described in "Material and Methods." The amount of purified collagen at each time point is expressed in cpm/cell.

increase in collagen accumulation, however, the level was persistently twofold to threefold higher than that of the normal fibroblasts (Figure 3, keloid).

For the detection of uPA and PAI, conditioned media were collected from cultures at designated time points and subjected to fibrin overlay and reverse overlay assays. A minimum of four strains each of normal and keloid fibroblasts were examined. The result of a typical experiment is shown in Figure 4. Fibrin overlay assay revealed that normal fibroblasts expressed both the two-chain form (50 kd) and the catalytic fragment single chain form (30 kd) of uPA (Figure 4, normal, top). The 50-kd uPA was expressed in early culture periods (3 to 5 days) and reappeared in late culture periods (after 12 days). The 30-kd uPA was expressed in a low level throughout most of the culture period and increased to a high level in the later culture period (Figure 4, normal, top). In contrast, keloid fibroblasts exhibited a moderate level of 30-kd uPA, which only appeared in the late culture periods (Figure 4; keloid, top). Reverse fibrin overlay assay revealed that normal fibroblasts expressed PAI-1 in a variable activity level (Figure 4; normal, bottom). In drastic contrast, keloid fibroblasts expressed a persistently high level of PAI-1 throughout the entire culture period (Figure 4, keloid, bottom). The PAI-1 activity was also measured

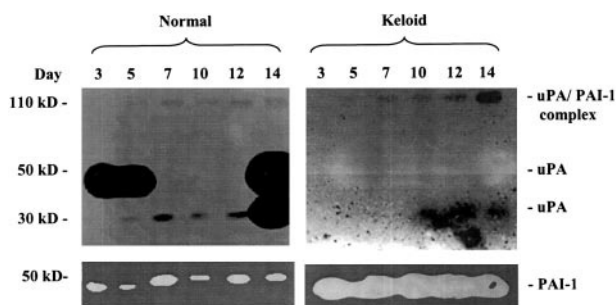


Figure 4. Expressions of uPA and PAI-1 throughout a 14-day period comparing normal and keloid fibroblasts. **Top:** Fibrin overlay assay demonstrating uPA activities. **Bottom:** Reverse fibrin overlay assay demonstrating PAI-1 activities. The two chain uPA is present with a molecular weight of ~50 kd. The single chain uPA is present with a molecular weight of 30 kd. The high molecular weight proteins (~110 kd) are uPA/PAI-1 complex. Human PAI-1 shows a molecular weight at ~50 kd.

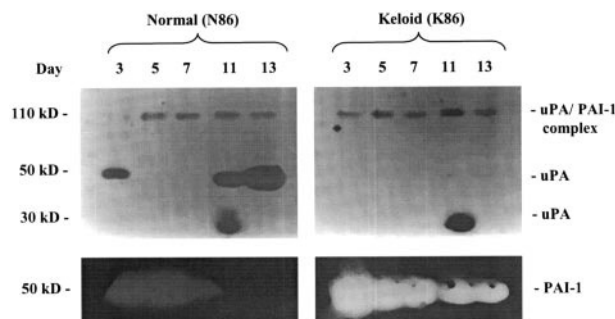


Figure 5. Expressions of uPA and PAI-1 from donor- and anatomical site-matched normal (N86) and keloid (K86) fibroblasts throughout a 13-day culture period. **Top:** Fibrin overlay assay demonstrating uPA activities. **Bottom:** Reverse fibrin overlay assay demonstrating PAI-1 activities. The two chain uPA is present with a molecular weight ~50 kd. The single chain uPA is present at approximately the molecular weight of 30 kd. The high molecular weight proteins (~110 kd) are uPA/PAI-1 complex. Human PAI-1 shows a molecular weight at ~50 kd.

using the Chromogenic Substrate Assay (American Diagnostica). In the assay, keloid fibroblasts typically showed twofold to threefold higher levels in PAI-1 activity than normal fibroblasts (K:N, 45:10; 80:45; 40:16 IU/ml in three separate measurements). A small amount of uPA/PAI-1 complex was detected in cultures of both normal and keloid fibroblasts (Figure 4, top, uPA/PAI-1 complex). The complex was catalytically inactive *in situ*, and its fibrinolytic activity, which appeared in the fibrin overlay, was because of an artifact of SDS treatment during the SDS-polyacrylamide gel electrophoresis procedure.⁴¹

Activities of uPA and PAI-1 were also examined in a pair of donor- and anatomical site-matched samples, N86 and K86. The result is shown in Figure 5. With a slight difference in the time and level of expression, N86 exhibited a very similar pattern of uPA expression (Figure 5; N86, top) when compared to other normal fibroblasts (Figure 4; normal, top). N86 is different, however, in its PAI-1 expression, which appeared very high in the early half of the culture period and disappeared in the later half (Figure 5; N86, bottom). The expression patterns of uPA and PAI-1 by K86 were very similar to other keloid fibroblasts in which uPA of the 30-kd form appeared in a moderate amount at day 11, and PAI-1 was overexpressed throughout the entire culture period (Figure 5, K86). The presence of the uPA/PAI-1 complex (~110 kd) in keloid samples (Figures 4 and 5) indicated that the uPA secreted by keloid fibroblasts was primarily bound by PAI-1. Therefore, in long-term fibrin gel cultures, although normal fibroblasts exhibit regulated expressions of uPA and PAI-1, keloid fibroblasts exhibit a low level of uPA and a persistently high level of PAI-1. Furthermore, PAI-1 overexpression by keloid fibroblasts correlates with the elevated collagen accumulation.

The High PAI-1 Activity Is Causal in Elevated Collagen Accumulation of Keloid Fibroblasts

Fibroblasts in fibrin matrix actively reorganize the matrix and produce collagen to replace fibrin.¹⁸ To determine whether the expression pattern of uPA or PAI-1 by fibroblasts in fibrin gels was influenced by the changing ECM

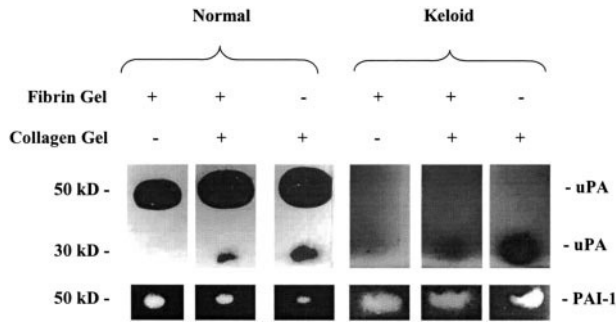


Figure 6. Expressions of uPA and PAI-1 of normal or keloid fibroblasts cultured in fibrin, fibrin-collagen, or collagen gels. **Top:** Fibrin overlay assay demonstrating uPA activities. **Bottom:** Reverse fibrin overlay assay demonstrating PAI-1 activities. The two chain uPA is present with a molecular weight of ~50 kd. The single chain uPA is present at approximately the molecular weight of 30 kd. Human PAI-1 shows a molecular weight at ~50 kd.

environment (ie, from fibrin to collagen), fibrin, fibrin-collagen, or collagen gels were used in cell cultures to mimic the matrix phenotype of early, mid, or late stage during *in vitro* fibroplasia. Results showed that in normal fibroblasts, uPA expression transitioned from the 50-kd two-chain form to the 50-kd and 30-kd forms in the presence of collagen (fibrin-collagen and collagen gels) (Figure 6, normal, top). Interestingly, the level of PAI-1 expression decreased as the concentration of collagen in the gel matrix increased from 50 to 100% (Figure 6, normal, bottom). Keloid fibroblasts responded to the presence of collagen in the matrix by expressing 30-kd uPA; however, there was no significant change in their PAI-1 level (Figure 6, keloid). Therefore, although expressions of both uPA and PAI-1 of normal fibroblasts were modulated by ECM, only uPA expression of keloid fibroblasts was subjected to ECM modulation. There was no difference in cell growth among cultures of fibrin, collagen, or fibrin-collagen hybrid gels (data not shown). Therefore, PAI-1 overexpression is an intrinsic characteristic of keloid fibroblasts.

Collagen accumulation of normal or keloid fibroblasts was also studied and compared between cultures of fibrin and collagen gels. Results showed that the level of collagen accumulation by normal fibroblasts was similar between cultures of fibrin and collagen gels (Figure 7, normal). In contrast, when keloid fibroblasts were cultured in collagen gels, their usually high level of collagen accumulation observed in fibrin gels was reduced to a level comparable to normal fibroblasts (Figure 7, keloid). A similar reduction in collagen accumulation was found in keloid fibroblasts when they were cultured in fibrin-collagen gels (data not shown). Similar data were obtained in two additional strains of keloid fibroblasts. Therefore, the results so far indicate that the high PAI-1 activity is necessary in sustaining the elevated collagen accumulation by keloid fibroblasts, because an increase in uPA activity by culturing keloid cells in collagen or fibrin-collagen hybrid gels reduced collagen accumulation of keloid fibroblasts.

To further test if the high PAI-1 activity led to increased collagen accumulation, collagen accumulation of keloid fibroblasts in cultures of fibrin gels was studied in the

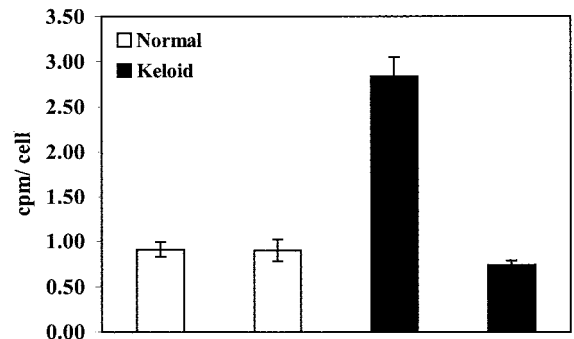


Figure 7. Collagen accumulation of normal or keloid fibroblasts cultured in fibrin or collagen gels. Collagen synthesized by fibroblasts was purified as described in "Material and Methods" and expressed as cpm/cell.

Figure 7. Collagen accumulation of normal or keloid fibroblasts cultured in fibrin or collagen gels. Collagen synthesized by fibroblasts was purified as described in "Material and Methods" and expressed as cpm/cell.

presence of PAI-1-neutralizing antibodies. According to the manufacturer, the antibodies (rabbit anti-PAI-1 antibody, no. 395R; American Diagnostica) react with all forms of human PAI-1. At the 50% inhibition point, 1 mg of this antibody can inhibit ~1000 IU of PAI-1. Results showed that anti-PAI-1 antibodies, but not nonimmune IgG, decreased PAI-1 activity (Figure 8, inset) and reduced collagen accumulation of keloid fibroblasts (Figure 8, keloid in fibrin gel + anti-PAI-1). Two additional strains of keloid fibroblasts were also tested for the effect of anti-PAI-1-neutralizing antibodies on collagen accumulation. Studies of collagen accumulation in fibrin gel cultures of normal fibroblasts or collagen gel cultures of keloid fibroblasts were also conducted at the same time for comparison (Figure 8, normal in fibrin gel and keloid in collagen gel).

Discussion

Our study demonstrates that PAI-1 overexpression is a consistent feature of keloid fibroblast both *in vitro* and *in vivo*. In long-term fibrin gel cultures, although normal fibroblasts exhibit regulated levels of uPA and PAI-1 as well as collagen accumulation, keloid fibroblasts exhibit persistently high levels of PAI-1 and collagen accumula-

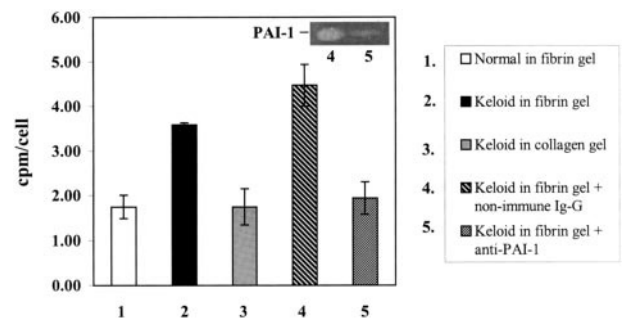


Figure 8. The effect of anti-PAI-1-neutralizing antibodies on collagen accumulation of keloid fibroblasts cultured in fibrin gels. Collagen synthesized by fibroblasts was purified according to the procedure described in "Material and Methods" and expressed as cpm/cell. **Inset:** Reverse fibrin overlay demonstrating PAI-1 activity.

tion. Conditions that would reduce PAI-1 activity abolish the elevated collagen accumulation of keloid fibroblasts. These conditions include increasing uPA by culturing fibroblasts in collagen or fibrin-collagen gels, or decreasing PAI-1 activity by adding PAI-1-neutralizing antibodies to fibroblasts in cultures of fibrin gels. Therefore, the increased PAI-1 activity of keloid fibroblasts may account for their elevated collagen accumulation in fibrin gel cultures.

In contrast to hypertrophic scars, which are caused by trauma to the deep dermis, keloids can occur from minor skin injuries, such as scratches, acne, and ear piercing of predisposed individuals.² Excision of keloid lesions is often considered to be the last resort of treatment because new keloid formation can occur as a result of the procedure itself and it is unethical to elicit or cause unnecessary skin injuries in these patients. Therefore, one limitation to our data is that the ideal multiple comparisons between lesional and nonlesional, donor- and site-matched specimens were not possible. The specimens used in the study (Table 1) represent a research effort in sample procurement throughout an 8-year period. Each experiment presented in the study was conducted and compared between multiple strains of normal and keloid fibroblasts in pairs matching donor age and anatomical site whenever it was possible.

Fibroplasia is a dynamic process that incorporates constant interactions and feedbacks between participating cell, ECM, and soluble mediators.²¹ It was previously shown that normal skin fibroblasts can actively reorganize the fibrin matrix and remodel it into a collagen-containing scar-like tissue.¹⁸ From this study, it is evident that as normal fibroblasts synthesized and deposited collagen into the fibrin matrix, the activity levels of uPA and PAI-1 were also regulated (Figures 4 and 5). This ECM-mediated change in uPA and PAI-1 expressions was proven in subsequent experiments using fibrin, fibrin-collagen mixture, or collagen gels (Figure 6). Integrins are the likely candidates in mediating such dynamic reciprocity between fibroblasts and the ECM, because integrin engagement or disengagement from ECM may mediate an integrin species-specific change in the phenotype of cells.⁴²⁻⁴⁶ The evidence can be further drawn from studies of cells in collagen gels. In collagen gels, the binding of $\alpha_2\beta_1$ integrin to collagen increases cell survival and ECM production; in contrast, the disruption of $\alpha_2\beta_1$ binding to collagen induces MMP2 production/activation, therefore, matrix degradation.⁴⁷ It has been shown that fibroblasts are able to bind to fibrin using integrins containing the α_v subunit.^{17,48,49} It is, however, not excluded in the current study that fibronectin may be involved in the binding of fibroblasts to the fibrin gel matrix through $\alpha_5\beta_1$ integrin,²¹ because fibrinogen used in the study contains a trace amount of fibronectin (< 0.1 $\mu\text{g}/\text{mg}$ of fibrinogen), and 10% fetal calf serum (which contains fibronectin) was used in the collagen synthesis assay. Therefore, the difference in uPA and PAI-1 expression between fibrin and collagen gels may be mediated by a difference in α_v -containing integrin or $\alpha_5\beta_1$ binding to fibrin/fibronectin and/or $\alpha_2\beta_1$ binding to collagen.

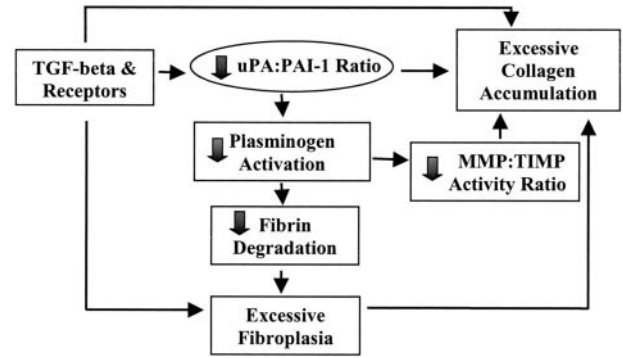


Figure 9. A schematic diagram summarizing the major findings of keloid fibrosis and connecting them to key events/components of tissue injury repair. The plasminogen activator/plasmin and PAI-1 system is central to matrix remodeling. It regulates fibrin degradation, influences TGF- β and MMP activities, and modulates cell adhesion/migration to ECM.^{22,23,57,65} Keloid fibroblasts not only exhibited an elevated level of collagen accumulation (Younai et al¹⁹ and this study), which could be further increased on exposure to TGF- β ,¹⁹ but also a defect in fibrin degradation that attributed to their increased PAI-1 and decreased uPA activities (Tuan et al²⁰ and this study). The connection between increased PAI-1 activity and excessive collagen accumulation of keloid fibroblasts was established when PAI-1-neutralizing antibodies were seen to reduce collagen accumulation of keloid fibroblasts to a level comparable to the normal fibroblasts or when the uPA activity was increased by culturing keloid fibroblasts in collagen-containing matrix gels (this study). **Filled block arrows** indicate activity levels.

Increased PAI-1 activity has been a hallmark of tissue and organ fibrosis. There is evidence that a direct correlation exists between the genetically determined level of PAI-1 expression and the extent of collagen accumulation that follows inflammatory lung injury. The support was drawn from studies of bleomycin-induced pulmonary fibrosis in transgenic mice.²⁸ These studies were based on the rationale that excessive PAI-1 activity leads to fibrin accumulation, which in turn elicits a fibrogenic effect on lung repair. Fibrin is the best-known substrate of plasmin and its breakdown products are chemotactic to inflammatory cells.^{21,22,50} Therefore, accumulation of fibrin at the site of tissue injury is causal for tissue fibrosis. In our studies, the difference in the uPA:PAI-1 ratio between normal and keloid fibroblasts was reflected in the degree of fibrin matrix degradation, whereas, in a short-term assay, normal fibroblasts caused fibrin matrix degradation but keloid fibroblasts did not.²⁰ Furthermore, the treatment of normal fibroblasts with TGF- β 1, a potent inducer of PAI-1,⁵¹ prevented fibrin degradation. Clinical observations by Paul Kelly, M.D., have revealed that before keloids form the affected area is preceded by a prolonged inflammatory reaction. In addition, most keloids have three distinctive areas: an erythematous outer border (area of expansion/growth), an inner nonerythematous raised border (classical keloid), and a central regressing area. As fibrin is involved in inflammation, it is likely that keloid lesions, especially the outer border, may contain greater accumulations of fibrin. It is yet to be elucidated, however, if the overexpression of PAI-1 by keloid fibroblasts is the cause. Nevertheless, the notion of fibrin in causing fibrosis has been challenged recently in lung injury-repair studies. In mutant mice lacking the A α or γ chain of fibrinogen and with no intact fibrinogen in the circulation, the degree of lung fibrosis after bleomycin treatment was comparable to the wild-type mice.^{33,52,53}

These studies indicate that although fibrin may promote fibrosis, it does not appear to be a prerequisite of fibrosis. In the present study the reduction of collagen accumulation of keloid fibroblasts by adding PAI-1-neutralizing antibodies into fibrin cultures or by culturing cells in fibrin-collagen or collagen gels (which induces uPA expression), strongly suggests that the overexpression of PAI-1, instead of fibrin, may be the key to excessive collagen accumulation in keloid fibrosis. The fact that both PAI-1 overexpression (Figure 2)^{20,29} and collagen overproduction^{3,54} have been found in cultures of keloid fibroblasts on plain cell culture surfaces in the absence of fibrin, gives further support of the involvement of PAI-1 in keloid fibrosis.

It is noteworthy that the collagen purification protocol used in this study by pepsin treatment recovers only intact collagen and reflects collagen accumulation (see Materials and Methods).⁵⁵ Because collagen production may be modulated posttranslationally by proteases of the MMP family,⁵⁶ it is possible that the reduction of collagen accumulation by keloid fibroblasts cultured in collagen or fibrin-collagen gels is because of collagen degradation caused by plasmin-mediated MMP activation (pathways summarized in Figure 9). Alternatively, an integrin-mediated mechanism may be involved, because PAI-1, aside from its effect on cell growth and apoptosis, is able to modulate integrin-mediated cell adhesion and migration through its binding to uPA and to vitronectin.⁵⁷ Therefore, the change in uPA:PAI-1 ratio of keloid fibroblasts under conditions mentioned above might affect the binding of keloid fibroblasts to the gel matrix and, subsequently, alter the state of fibroblast differentiation and collagen synthesis.^{43-47,58} These are testable hypotheses for the future, using the *in vitro* fibroplasia model.

When cultured in collagen gels, it was interesting to note that although normal fibroblasts exhibited an increase in uPA and a decrease in PAI-1 levels, there was no change in the levels of collagen accumulation (Figures 6 and 7). We conclude that it might be because of the isometric tension developed in the matrix during fibroblast contraction of gels. It has been previously shown that the isometric tension developed in the ECM matrix as a result of cell-matrix interaction may dictate the metabolic state of the cell.⁵⁹⁻⁶² Accordingly, fibroblasts in collagen gels detached from the tissue culture dish, allowing the fibroblasts to contract the collagen matrix under relatively little tension, produce very little collagen. In contrast, as fibroblasts in attached collagen gels contract the matrix and generate increasing tension, the basal collagen synthesis is maintained.⁶¹⁻⁶⁴ In the current study, both fibrin and collagen gels were attached to culture dishes; therefore, no difference in collagen accumulation was observed.

An array of soluble factors are able to modulate PAI-1 expression. Among these, TGF- β , implicated in tissue and organ fibrosis, exerts the most profound effect on PAI-1 up-regulation.⁶⁵ TGF- β not only augments the production of ECM molecules by cells, but also slows down their removal by up-regulating protease inhibitors (TIMP-1 and PAI-1) and down-regulating protease (MMP-1 and uPA) expressions.⁶⁵ Previous reports have

shown that TGF- β is up-regulated in keloids.^{66,67} However, it is unlikely that the high levels of PAI-1 detected in keloid fibroblasts in this study is caused by TGF- β secreted by the fibroblasts and acting as autocrine or paracrine factors, because the assay was performed under serum-free conditions. Under the conditions, we have shown that the level of TGF- β produced by either normal or keloid fibroblasts was similar.^{19,20} In addition, TGF- β is secreted by cells in its latent form and requires activation before binding to its receptors on the cell surface. As plasmin has been shown *in vitro* to be physiological activator of TGF- β ,²⁴ the persistently high level of PAI-1 expressed by keloid fibroblasts would have inhibited TGF- β activation through inhibition of uPA activity. With our increasing knowledge of TGF- β receptor and postreceptor signaling events, it is possible, however, that the persistently high expression of PAI-1 in keloid fibroblasts may reflect altered response(s) at the level of the TGF- β receptor and/or in the postreceptor signaling system.^{12,13}

The epidermis of keloids also showed a stronger PAI-1 expression than that of normal skin and normal scar (Figure 1). This may also have some clinical implications because human adult keratinocytes do not normally express PAI-1. Its expression accompanies epidermal migration and only occurs during wound repair.⁶⁸ Other serine or MMP protease inhibitors such as α -1 anti-trypsin, α -2 macroglobulin, and tissue α -globulins⁶⁹ were also detected in keloid lesions.⁷⁰ The effect of these proteins on keloid fibroplasia can also be tested in the future using the *in vitro* model system. In conclusion, using the three-dimensional matrix gel systems, we have demonstrated that PAI-1 overexpression correlates with elevated collagen accumulation by keloid fibroblasts. When PAI-1 activity is inhibited or reduced, the abnormal collagen accumulation is abolished, thus proving a causal relationship between the two. A schematic diagram depicting the major findings in keloid fibrosis and connecting them to key events/components of tissue injury repair is presented in Figure 9.

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