Fibrin-Induced Skin Fibrosis in Mice Deficient in Tissue Plasminogen Activator

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The deposition of fibrin is an integral part of the tissue repair process, but its persistence is also associated with a number of fibrotic conditions. This study addressed the hypothesis that reduced fibrinolysis and fibrin persistence are associated with an enhanced accumulation of collagen and the development of skin fibrosis. Decreased fibrinolysis was confirmed in fibrin gel cultures that contained human dermal fibroblasts plus the specific plasmin inhibitor α_2 -antiplasmin or dermal fibroblasts isolated from plasminogen activator (PA)-deficient mice. Collagen accumulation was significantly increased in the presence of inhibitor and in tPA-deficient, but not uPA-deficient, fibroblasts compared with controls. These findings were also confirmed using a skin fibrosis model in which multiple injections of fibrin were given subcutaneously to PA-deficient mice. Injection sites from tPA-deficient mice displayed significantly increased collagen levels compared with uPA-deficient mice and wild-type controls. Up-regulation of fibroblast procollagen gene expression and reduced activation of pro-MMP-1 appeared to mediate the increase in collagen by human dermal fibroblasts in the presence of α_2 -antiplasmin. These findings suggest that persistent fibrin is associated with enhanced collagen accumulation that may result in the development of fibrotic skin disorders in which reduced fibrinolysis is a feature. (*Am J Pathol* 2005, 167:721–732)

After injury, the deposition of a fibrin-rich matrix in the interstitium is an integral part of the tissue repair process. This insoluble lattice prevents excess blood loss and serves as a provisional matrix for the inward migration of

inflammatory cells, fibroblasts, and endothelial cells that initiates subsequent granulation tissue formation. The process of fibrin matrix formation involves the conversion of plasma-derived fibrinogen into fibrin monomers through the action of α -thrombin. Factor XIIIa subsequently stabilizes the polymerized fibrin monomers by forming cross-links between fibrin and additional plasma proteins, such as fibronectin and vitronectin. As well as providing essential hemostasis and support for cell migration, the fibrin matrix is thought to play a much broader physiological role during the repair process.^{1,2} For instance, fibrin(ogen) is centrally involved in platelet aggregation and activation, its cleavage fragments have been identified as key molecular effectors of the fibroproliferative response and it acts as a storage reservoir for a number of important growth factors, proteases, and their inhibitors. During the repair process, the fibrin-rich matrix is degraded and replaced by a more permanent fibrous matrix predominately composed of collagen. With further matrix remodeling and a concomitant decrease in cellularity and vascularization, scar tissue forms at the injury site.

The fibrin matrix is principally degraded by the serine protease, plasmin, which is generated through proteolytic cleavage of the proenzyme, plasminogen, by the action of plasminogen activators (PAs). Substantial evidence suggests that, as well as being the main fibrinolytic protease, plasmin plays an important role in the activation of latent growth factors such as transforming growth factor- β (TGF)- β^3 and matrix metalloproteinases (MMPs), in particular the major interstitial collagenase, MMP-1.⁴ Two types of PAs, tissue-type (tPA) and urokinase-type plasminogen activator (uPA), are involved in plasminogen activation but, they originate from different genes and have distinct structures with different tissue-specific expression and biological activities.⁵ It is generally agreed that tPA is involved in the clearance of systemic fibrin as it binds to fibrin with high affinity and forms

Supported by Johnson and Johnson Medical Limited and the Medical Research Council, UK.

Accepted for publication June 2, 2005.

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a complex with plasminogen. In contrast, uPA binds to a specific cell surface receptor, uPAR, and has traditionally been associated with cell migration through fibrin-rich substrates. Many of the functions of the two complementary PAs have been elucidated through the use of mice deficient in these fibrinolytic proteases.⁶ Mice lacking tPA show a significantly reduced thrombolytic potential and increased incidence of endotoxin-induced thrombosis, whereas inactivation of the uPA gene produces mice with a normal thrombolytic potential but with a more severe phenotype, with fibrin present in multiple organs and a defective plasmin-mediated macrophage function. Mice with a combined tPA and uPA deficiency have a pronounced phenotype, and suffer from extensive fibrin deposition, delayed skin wound healing, and develop occasional spontaneous peritoneal adhesions. Several studies have shown that fibroblasts produce both tPA and uPA,^{7,8} and so are able to degrade insoluble fibrin matrix into soluble fibrin degradation products, many of which also have biological activity. Due to the diverse functions of these proteases and their degradation products, fibrinolytic activity is tightly regulated by a number of protease inhibitors, in particular PAs by plasminogen activator inhibitors (PAIs) and plasmin by the serpin inhibitors, α_2 -antiplasmin, α_1 -antitrypsin, and α_2 -macroglobulin.⁵

Although deposition of a fibrin matrix is fundamental to normal repair, it is also a common consequence of a number of pathological disorders such as atherosclerosis,⁹ restenosis,¹⁰ postoperative adhesions,¹¹ pulmonary fibrosis,¹² and tumorigenesis.¹³ In such conditions, the presence of fibrin is often integrally associated with excess collagen accumulation and fibrosis. Indeed, Dvorak¹⁴ suggested that the amount of collagenous stroma formed in tumors was directly related to the amount of fibrin initially deposited as a provisional matrix. However, the role of fibrin in regulating collagen production is not clear. *In vitro* studies have demonstrated that the rate of collagen synthesis increases when fibroblasts are grown within a three-dimensional fibrin matrix as opposed to a collagen matrix.^{15,16} Tuan and colleagues^{17,18} found that keloid fibroblasts deposited an increased amount of collagen when grown in fibrin gel culture compared with control fibroblasts and that they also displayed a decreased fibrinolytic activity. The group suggested that reduced fibrinolysis may in part mediate the fibrotic phenotype displayed by these overactive scars. This may also be true of a number of other fibrotic skin disorders, such as scleroderma¹⁹ and lipodermatosclerosis,^{20,21} conditions that are additionally characterized by extensive fibrin deposition and associated with impaired fibrinolysis.

A number of gene-targeting studies using experimental animal model systems have also highlighted the importance of a regulated fibrinolytic system in the development of tissue fibrosis. After bleomycin-induced lung injury, mice deficient in plasminogen, uPA, or tPA, or those that overexpress PAI-1, show extensive fibrin deposition, collagen accumulation, and accelerated pulmonary fibrosis when compared with control mice.^{22,23} These studies concluded that reduced fibrinolysis results in fibrin accumulation at the site of injury and subsequent

fibrosis ensues. However, despite evidence in other systems, an association between the persistence of fibrin and increased collagen accumulation in the skin has not been shown.

The aim of the current study was to address the hypothesis that reduced fibrinolysis and a persistence of fibrin are associated with an enhanced accumulation of collagen resulting in skin fibrosis. The effect of reduced fibrinolysis and fibrin persistence on collagen levels was assessed in dermal fibroblast-populated fibrin gels using two alternative methods. The first involved inhibition of plasmin activity using a specific plasmin inhibitor, α_2 -antiplasmin in human fibroblast cultures, and the other involved using fibroblasts derived from PA-deficient mice. *In vitro* findings were confirmed *in vivo* using a skin fibrosis model in which fibrinogen and thrombin were simultaneously injected on multiple occasions into the dorsal skin of fibrinolytic-deficient mice. To begin to understand the mechanism by which reduced fibrinolysis and fibrin persistence may lead to an increase in collagen accumulation, procollagen gene expression and pro-MMP-1 activation, indicating a change in the synthesis and/or degradation of collagen respectively, were also assessed in human fibroblast cultures.

Materials and Methods

Animals and Cell Strains

Breeding pairs of mice deficient in tPA and uPA and wild-type littermates on a mixed genetic background (25% B6:129/75% C57BL/6J) were obtained from The Centre for Transgene Technology and Gene Therapy, Flanders Interuniversity Institute for Biotechnology, Katholieke Universiteit Leuven, Leuven, Belgium. Adult female and male mice, 8 to 12 weeks old, were used in the studies after their genotype was confirmed by polymerase chain reaction using set primers.⁶ All experiments were performed according to Home Office regulations and approval. Murine (uPA^{-/-}, tPA^{-/-}, and WT) dermal fibroblasts were isolated from the skin of adult mice by enzymatic digestion. Briefly, washed skin explants were transferred to standard Dulbecco's modified Eagle's medium (DMEM; Gibco, Paisley, UK) containing 2 mmol/L L-glutamine, and antibiotics (1000 U/ml penicillin and 50 μ g/ml streptomycin) with added 0.25% trypsin (Gibco) and incubated for 3 hours at 37°C before removing the epidermis. Dermis was minced and transferred into a solution of 1 mg/ml bacterial collagenase type II (Worthington Biochemical Corp., Lakewood, NJ) in standard DMEM media and incubated with shaking for 2 hours at 37°C. The resulting cell suspension was filtered through a 100- μ m pore mesh and diluted 1:1 with heat-inactivated fetal calf serum (CSL UK Ltd., Andover, UK), before centrifugation and resuspension in standard DMEM with 20% fetal calf serum and 2.5 μ g/ml fungizone (Gibco). Normal human dermal fibroblasts were derived from skin biopsies (2 mm²) harvested from the distal leg of an adult male patient undergoing coronary artery bypass surgery after ethical approval and informed con-

sent. Human fibroblasts were isolated and cultured as for murine fibroblasts. Cells were maintained after the first passage in standard DMEM with 10% fetal calf serum in a humidified atmosphere of 10% CO₂ in air at 37°C and were used in studies between passages 6 to 9.

Fibrin Gel Preparation

Human plasma fibrinogen (9 μ mol/L final, essentially plasminogen-free; Sigma, Poole, UK) was prepared in standard serum-free DMEM containing 50 μ g/ml ascorbate (Sigma) and 0.2 mmol/L proline (Sigma). Human or mouse dermal fibroblasts (5×10^5 cells/ml) were suspended in the fibrinogen solution and human thrombin was added (2 nmol/L final, Sigma). After mixing, 1 ml of fibrin-cell suspension was aliquoted into each well of a 24-well plate and gels set for 30 minutes before adding 1 ml of incubation media consisting of serum-free supplemented DMEM, 50 μ g/ml ascorbate, and 0.2 mmol/L proline. Another set of cultures consisted of human or mouse dermal fibroblasts (5×10^5 cells/well) plated as monolayers on untreated wells containing 2 ml of incubation media. Cultures were incubated for 24, 48, and 72 hours before being processed for collagen analysis ($n = 6$), fibrin gel degradation analysis ($n = 6$), or digested with trypsin/ethylenediamine tetraacetic acid and assessing the number of viable cells ($n = 4$). After each 24-hour incubation period, 10 μ l of stock ascorbate (Sigma) solution was added to a final concentration of 50 μ g/ml to restore reduced ascorbate levels. To assess the role of fibrin persistence on collagen accumulation two methods were used. One involved the addition of an inhibitor of plasmin, α_2 -antiplasmin (1 μ mol/L final, Sigma) to human fibroblast cultures. Studies have shown that α_2 -antiplasmin becomes reversibly cross-linked to fibrin(ogen) resulting in the half-life of plasmin activity in a fibrin matrix of 0.1 second.²⁴ Alternatively, fibrin matrix gels were prepared containing either murine uPA^{-/-} or tPA^{-/-} or WT peritoneal fibroblasts. Bovine TGF- β 1 (R&D Systems, Abingdon, UK) was activated with 4 mmol/L HCl and 1 ng/ml (final concentration) was added to cell cultures at the start of the incubation period.

Fibrin Gel Degradation Assay

Human fibrinogen was conjugated to fluorescein isothiocyanate (FITC) according to the manufacturer's instructions (Fluoro Tag kit, Sigma). Fibrin gel degradation was assessed by measuring the level of fluorescence released from the gels. Initially, a standard curve of fibrin-FITC degradation was established by adding increasing concentrations of plasmin (25 nmol/L to 500 nmol/L) to cell-free fibrin-FITC gels and incubating for 1 hour at 37°C. There was a linear distribution at concentrations up to 200 nmol/L plasmin at which point total fibrin gel degradation was observed. Human plasma fibrinogen used in these studies was essentially plasminogen-free but contained ~ 0.03 U of plasminogen/mg fibrinogen (according to the manufacturer; Sigma), therefore if all contaminating plasminogen was activated by incorporated

cells, sufficient amounts were present to allow complete fibrin gel degradation. Addition of α_2 -antiplasmin (250 nmol/L to 2 μ mol/L) showed that 1 μ mol/L α_2 -antiplasmin was sufficient to completely inhibit fibrinolysis by added plasmin at concentrations up to 500 nmol/L and therefore, in all inhibitor experiments 1 μ mol/L α_2 -antiplasmin was routinely used. Slow release of fluorescence occurred throughout time from gels that did not contain cells due to the presence of nonclottable fibrinogen in our initial preparation. This value was therefore subtracted from the values obtained from gels that contained cells at each time point.

Skin Fibrosis Model

Mice (uPA^{-/-}, tPA^{-/-}, or WT) were anesthetized with 4% halothane in air and after shaving, injection sites (3 cm from the base of the skull and 1 cm either side of the spine) were tattooed with permanent ink. Care was taken to avoid subcutaneous fat pads. On the right side, 25 μ l of murine fibrinogen (9 μ mol/L, Sigma) and 25 μ l of murine thrombin (140 nmol/L, Sigma) were injected subcutaneously through two adjacent syringes. All solutions were made in sterile phosphate-buffered saline (PBS) with 2.4 mmol/L CaCl₂, pH 7.3. On the left side, 50 μ l of sterile PBS/CaCl₂ were injected into a corresponding site in a similar manner (25 μ l from each syringe). In another set of animals, 25 μ l of murine thrombin (140 nmol/L) and 25 μ l of PBS/CaCl₂ were injected using the same procedure. Injections were repeated at the same sites twice a week for 3 weeks. One week after the final set of injections, animals were killed and a full-thickness 8-mm punch biopsy was taken at each injection site. Samples from each treatment were fixed for wax histology ($n = 3$) or snap-frozen in liquid nitrogen before collagen analysis ($n = 7$).

Histological Assessment

Samples were fixed in 4% paraformaldehyde in PBS, pH 7.3, processed to paraffin wax, sectioned (7 μ m), and stained using standard histological procedures. Measurements of skin (from the surface of the epidermis to abdominal muscle) and fascia depth (base of the panniculus carnosus layer to abdominal muscle), were performed by image analysis using the KS300 imaging system (Carl Zeiss Ltd., Welwyn Garden City, UK) on a precalibrated screen. Collagen and fibrin densities in the fascial layer of Martius Scarlet Blue-stained slides were measured by full-color thresholding analysis, in which the density of collagen was identified by blue staining and fibrin by the density of red staining. Blue/red thresholding was performed as a function of the imaging system software package, gated, and maintained at the same values throughout the study. Thresholding for quantitated image processing was defined to exclude the muscle layer and nuclear staining, the latter being a darker blue/black color compared to the blue of the collagen. Slides were batch stained to ensure uniformity of color saturation and reference slides were included in each run and thresh-

olded to the same values to confirm that there was no variation between batches. Five measurements were taken per section on five sections, each 200- μ m apart, from each skin sample by two independent investigators.

Determination of Fibroblast Collagen Accumulation in Vitro and Total Skin Collagen in Vivo

Collagen accumulation in fibrin gels and total skin collagen in biopsies were assessed by quantitation of hydroxyproline levels in intact collagen using reverse-phase high-performance liquid chromatography (HPLC) as described previously.^{25,26} Briefly, fibrin gels and media were collected, proteins precipitated with ethanol, and samples filtered (0.45- μ m pore size) to separate proteins from free amino acids and small peptides. Frozen skin biopsies were minced to a powder and together with the filters hydrolyzed in 6 mol/L HCl overnight and then prepared for HPLC analysis (Beckman Systems, High Wycombe, UK) after derivatization with 7-chloro-4-nitrobenzofuran (Sigma). Collagen accumulation was expressed as the amount of hydroxyproline produced per 10^5 cells for *in vitro* studies or the amount of hydroxyproline per 8-mm² full-thickness skin biopsy for animal studies.

Northern Analysis of $\alpha 1$ (I) Procollagen Gene Expression

Procollagen gene expression by dermal fibroblasts in fibrin gels was assessed by Northern blot analysis. In brief, the media was discarded from each fibrin gel and 1 ml of TRIzol reagent was added according to the manufacturer's instructions (Gibco). Isolated total RNA (5 μ g) was mixed with RNA loading buffer (Sigma) and electrophoresed on a 1% (w/v) formaldehyde/agarose gel in 1 $\times$ 3-(*N*-morpholinopropanosulfonic acid) (MOPS) running buffer. The integrity of the RNA and the uniformity of the loading were visualized and quantified by fluorescent scanning of the ethidium bromide-stained bands of 18S and 28S ribosomal RNA (FLA 3000; Fuji, Japan). RNA was transferred to a nylon membrane (Hybond N; Amersham Int., High Wycombe, UK) and then fixed by UV cross-linking (Stratalinker; Stratagene, Cambridge, UK). After transfer, the membrane was hybridized overnight in standard Denhardt's solution at 65°C in the presence of cDNA probe for procollagen $\alpha 1$ (I), preradiolabeled with [³²P] dCTP using a random-priming DNA labeling kit as per the manufacturer's instructions (Megaprime DNA labeling kit, Amersham Int.). After hybridization, the membrane was rinsed at low stringency (2 $\times$ standard saline citrate, 0.1% sodium dodecyl sulfate) for 10 minutes at room temperature followed by 20 minutes at 65°C. The blot was subsequently washed at high stringency (0.1 $\times$ standard saline citrate/0.1% sodium dodecyl sulfate) for 20 minutes at 65°C with constant agitation. The membrane was wrapped in cling-film and exposed to phosphorimager storage screens (Fujifilm, Japan) for 2 to 4

hours and mRNA levels quantitated by phosphorimager analysis using Advanced Image Data Analysis software (Raytek Ltd., Milton Keynes, UK). Absorbance values of the signal representing the bands for $\alpha 1$ (I) procollagen mRNA were normalized relative to loading of total RNA in the same sample and procollagen gene expression was expressed as the ratio obtained from procollagen/18S RNA readings (in arbitrary units).

Total and Active MMP-1 Analysis

MMP-1 activity in the media of human fibroblast-populated fibrin gels was assessed using a specific MMP-1 substrate cleavage assay according to the manufacturer's instructions (Calbiochem, Nottingham, UK). The assay system was initially standardized using serial dilutions of active MMP-1 standard (data not shown). Aliquots of media were left untreated or were pretreated with aminophenylmercuric acid (final concentration of 2 mmol/L, Calbiochem) for 1 minute at room temperature to assess total MMP-1 levels. In brief, treated and untreated conditioned media (50 μ l) was mixed with 50 μ l of MMP-1 substrate solution (10 mg/ml) in each Optiplate 96-well plate (Packard Bioscience, Pangbourne, UK). The increase in fluorescence at 398 nm was measured against time using a Cytofluor multiwell plate reader series 4000 (Perceptive Biosystems, MA).

Statistical Analysis

All data are represented as mean $\pm$ SEM. Statistical analysis was performed using an unpaired Student's *t*-test for single group comparisons and analysis of variance for multiple-group comparisons. Differences were considered statistically significant at *P* < 0.05 or below.

Results

To investigate the effect of reduced fibrinolysis and fibrin persistence on collagen accumulation *in vitro*, two methods were used. The first involved the addition of a specific plasmin inhibitor, α_2 -antiplasmin to human dermal fibroblast-populated fibrin gels, and the other, incorporation of dermal fibroblasts isolated from PA-deficient mice into fibrin gels. Reduced fibrinolysis in both systems was initially confirmed using gels containing FITC-conjugated fibrinogen and measuring the level of fluorescence released into the supernatant. Cell number and collagen accumulation were then assessed at 24, 48, and 72 hours in both culture systems.

Collagen Accumulation by Human Dermal Fibroblasts Grown in Fibrin Gels in the Presence of a Plasmin Inhibitor

After 72 hours, human dermal fibroblasts had lysed ~52% of the original fibrin-FITC gel whereas addition of α_2 -antiplasmin significantly reduced fibrinolysis to 14% and was significantly reduced at all time points com-

Figure 1. Collagen accumulation by human dermal fibroblasts grown in fibrin gels in the presence of a plasmin inhibitor. **A:** Degradation of FITC-labeled fibrin gels by human dermal fibroblasts with and without 1 μ mol/L α_2 -antiplasmin (α_2 -AP). Gels containing plasmin (200 nmol/L) without cells acted as a positive control (mean $\pm$ SEM, $n = 6$, $*P < 0.01$). **B:** Collagen accumulation by human dermal fibroblasts grown in fibrin gels with and without 1 μ mol/L α_2 -AP for 72 hours, measured by HPLC analysis of hydroxyproline (mean $\pm$ SEM, $n = 6$, $*P < 0.01$).

pared with cultures without inhibitor (Figure 1A). The same cells (5×10^5 cells/well) have been found using enzyme-linked immunosorbent assays to produce both uPA (2.28 ± 0.22 ng/ml) and tPA (0.83 ± 0.06 ng/ml) when grown on tissue culture plastic, but similar and lower amounts of both uPA ($<0.16 \pm 0.01$ ng/ml) and tPA (0.20 ± 0.01 ng/ml) when grown in fibrin gels, all in the presence of TGF- β 1. Therefore, α_2 -antiplasmin is likely to inhibit plasmin produced by both uPA and tPA from the contaminating plasminogen in the fibrinogen solution. The fibrinolysis that was still observed in the inhibitor-containing cultures may be due to plasmin-independent fibrin-degrading proteases also produced by the fibroblasts. Fibroblast-populated fibrin gels under reduced fibrinolytic conditions produced significantly increased levels of collagen compared with those without inhibitor after 48 hours and 72 hours (Figure 1B). Inhibitor alone showed no effect on collagen accumulation when fibroblasts were grown on tissue culture plastic (data not

Figure 2. Collagen accumulation by PA-deficient murine dermal fibroblasts grown in fibrin gels. **A:** Degradation of FITC-labeled fibrin gels by PA-deficient dermal fibroblasts (tPA^{-/-} or uPA^{-/-}) or wild-type dermal fibroblasts (WT). Plasmin (200 nmol/L) without cells represented a positive control (mean $\pm$ SEM, $n = 6$, $*P < 0.01$ compared with wild type). **B:** Collagen accumulation by PA-deficient fibroblasts (tPA^{-/-} or uPA^{-/-}) or wild-type fibroblasts (WT) grown in fibrin gels for 72 hours, measured by HPLC analysis of hydroxyproline (mean $\pm$ SEM, $n = 6$; $*P < 0.05$ and $**P < 0.01$ compared with wild type).

shown), suggesting that increased collagen was due to the culture system rather than the inhibitor itself. Moreover, there was no difference in fibroblast number in the presence of α_2 -antiplasmin in either fibrin gels or on tissue culture plastic throughout time. At all time points, fibroblasts grown in fibrin gels produced significantly more collagen with and without the inhibitor than an equivalent number of fibroblasts cultured on tissue culture plastic (data not shown).

Collagen Accumulation by PA-Deficient Murine Dermal Fibroblasts Grown in Fibrin Gels

Fibroblasts isolated from tPA-deficient mice showed significantly reduced fibrin-FITC gel degradation compared with wild-type and uPA-deficient fibroblasts at all time points (Figure 2A). At 72 hours, fibroblasts deficient in uPA also showed a significant decrease in fibrin gel degradation compared with wild-type fibroblasts. Collagen accumulation by tPA-deficient fibroblasts in fibrin gels was significantly greater than that produced by both uPA-deficient and wild-type dermal fibroblasts at 48 and 72 hours (Figure 2B). However, there was no correspond-

Figure 3. The effect of subcutaneous injections of fibrin on collagen deposition in PA-deficient mice. **A:** The effect of repeated subcutaneous injections of fibrinogen (9 μmol/L) and thrombin (140 nmol/L) or PBS alone on total skin depth in PA-deficient mice (mean ± SE, $n = 3$, $*P < 0.05$). **B:** Histological appearance of fibrin injection sites compared with PBS injection sites in PA-deficient and wild-type mice. Fibrin (*) was observed in the fascia layer at the injection site (arrow) of tPA-deficient mice (E, epidermis; D, dermis; Sc, subcutaneous layer; Pc, panniculus carnosus; F, fascia). Arrow indicates fascia thickness. **C:** The effect of multiple subcutaneous injections on fibrin and collagen density in the fascial layer (mean ± SE, $n = 3$, $*P < 0.05$). **D:** The effect of multiple subcutaneous injections of fibrinogen on collagen deposition in the skin of PA-deficient and wild-type mice as assessed by HPLC analysis of hydroxyproline (mean ± SE, $n = 7$, $*P < 0.05$). Scale bar, 100 μm.

ing significant increase in collagen accumulation by uPA-deficient fibroblasts at any time point compared with wild-type fibroblasts. Collagen accumulation by all three cell strains was significantly increased when grown in fibrin gels compared with equivalent cells grown on tissue culture plastic and there was no significant difference in basal levels of collagen accumulation between the three cell strains grown on plastic (data not shown). Furthermore, there was no difference in fibroblast number between the three genotypes throughout time in either fibrin gels or on tissue culture plastic.

The Effect of Subcutaneous Injections of Fibrin on Collagen Deposition in PA-Deficient Mice

Findings from the *in vitro* studies suggested reduced fibrinolysis and fibrin persistence associated with tPA-deficiency led to increased collagen accumulation by

dermal fibroblasts grown in a fibrin matrix. To confirm this finding *in vivo*, a skin fibrosis model was developed whereby physiological concentrations of fibrinogen and thrombin were simultaneously injected subcutaneously into the dorsal skin of groups of PA-deficient and wild-type mice, twice a week, throughout a 3-week period. The same volume of sterile PBS with or without thrombin was injected into the opposite flank of each animal to act as a control. One week after the final injection, the two injection sites and an area of untreated skin were harvested and processed for histological analysis or total skin collagen content by HPLC analysis of hydroxyproline.

Histological analysis of skin depth in untreated control mice of all three strains was similar. However, after simultaneous fibrinogen and thrombin injections, skin depth was dramatically increased in all three groups, with skin of both PA-deficient animals being significantly thicker than that of wild-type mice (Figure 3, A and B). There was

no significant difference in total skin thickness at the fibrin injection site between tPA-deficient and uPA-deficient animals. Both strains displayed a dramatic increase in the fascia layer below the panniculus (Figure 3B) suggesting that fibrin was also injected into this layer. Equivalent injections of PBS or thrombin (2 nmol/L; data not shown) showed little effect on total skin thickness and appeared similar to untreated control skin in the three groups. Fibrin injection sites in all animals appeared to be associated with a mild inflammatory cell response and the level of vascularity and cellularity found in the fascia layer at the fibrin injection site appeared similar when hematoxylin and eosin-stained sections were assessed. Semiquantitative analysis of Martius Scarlet Blue-stained sections demonstrated that there was a significantly greater density of residual fibrin in the fascial layer of tPA-deficient animals, compared with uPA-deficient and wild-type mice 1 week after the final injection (Figure 3C). Furthermore, the density of collagen in this layer was significantly enhanced in the fibrin-injected skin of tPA-deficient animals but not uPA-deficient or wild-type mice (Figure 3C).

Total skin collagen in biopsies from PBS-injected and normal untreated skin was found to be similar in all of the three strains of mice as measured by HPLC analysis of hydroxyproline. Furthermore in all mice, total collagen levels in fibrin-injected sites was significantly higher compared with the respective PBS-injected control sites (Figure 3D). In agreement with histological findings, the amount of collagen in the skin of tPA-deficient mice was significantly increased compared with that of wild-type mice in response to repeated subcutaneous injections of fibrin (Figure 3D). Indeed, tPA-deficient mice demonstrated almost a threefold increase in the amount of collagen after fibrin injection compared with PBS injection. However, there was no significant difference in the amount of collagen in fibrin-injected skin between uPA-deficient and wild-type mice.

Effect of Reduced Fibrinolysis and Fibrin Persistence on Procollagen Gene Expression and Pro-MMP-1 Activation in Human Fibroblast Cultures

The mechanism by which reduced fibrinolysis and fibrin persistence may lead to an increase in collagen deposition was investigated further *in vitro*. Human dermal fibroblasts were grown in fibrin gels in the presence or absence of the plasmin inhibitor, α_2 -antiplasmin as previously described. Procollagen gene expression was assessed by Northern blot analysis to determine whether there was an up-regulation in collagen synthesis. In addition, the level and activity of MMP-1 were determined to show whether there was a possible reduction in collagen degradation.

Procollagen type I gene expression was maximal at 24 hours, with no difference in expression in the presence or absence of the inhibitor (Figure 4, A and B). At 48 and 72 hours, there was an up-regulation of procollagen type I

Figure 4. The effect of reduced fibrinolysis and persistent fibrin matrix on procollagen α_1 (I) mRNA expression by dermal fibroblasts grown in three-dimensional fibrin gels. **A:** Representative Northern blot of procollagen α_1 (I) mRNA in the presence or absence of α_2 -antiplasmin (α_2 -AP) at 24, 48, and 72 hours (representative lane shown for each condition on tissue culture plastic at 48 hours). **B:** Densitometric analysis of mRNA transcripts from **A**. **C:** Repeat Northern blot of procollagen α_1 (I) mRNA in the presence or absence of α_2 -AP at 48 hours. **D:** Densitometric analysis of mRNA transcripts from **C** (mean $\pm$ SE, $n = 3$, $*P < 0.05$). Values for procollagen α_1 (I) mRNA are normalized for loading with respect to 28S rRNA band and expressed in arbitrary units.

gene expression in the presence of inhibitor (reduced fibrinolysis), with the greatest difference at 48 hours. However, there was no increase in procollagen mRNA levels in presence of inhibitor in plastic monolayer cultures, suggesting that the increase in procollagen gene expression by fibroblasts in fibrin gels was not due to α_2 -antiplasmin stimulation. The increase in procollagen gene expression at 48 hours was confirmed by densitometric analysis of Northern blots using mRNA isolated from three separate cultures representing each condition. Data confirmed that there was a significant increase in procollagen type I gene expression by dermal fibroblasts in fibrin gels supplemented with inhibitor compared with cells in fibrin gels without inhibitor (Figure 4, C and D). Furthermore, procollagen gene expression was significantly higher when fibroblasts were grown in fibrin gels compared with equivalent cells grown on tissue culture plastic, with or without inhibitor.

Media from human fibroblast-populated fibrin gels with and without α_2 -antiplasmin, was assessed for MMP-1 activity. A significant decrease in the level of active MMP-1 was found in the presence of the plasmin inhibitor at all time points compared with cultures without inhibitor (Figure 5A). In addition, active MMP-1 levels were significantly greater at 48 and 72 hours compared with at 24 hours. However, analysis of total MMP-1 levels after the addition of aminophenylmercuric acid (pro-MMP activator) showed that the total amount of MMP-1 (pro- and active) was similar in media from cultures with or without α_2 -antiplasmin at all time points (Figure 5B). In summary, these findings suggest that both an up-regulation of procollagen gene expression and a reduction in pro-MMP-1 activation are involved in the increased amount of colla-

Figure 5. Effect of reduced fibrinolysis and fibrin persistence on pro-MMP-1 activation. **A:** Active MMP-1 levels in conditioned media from human dermal fibroblasts in fibrin gels with and without α_2 -antiplasmin (α_2 -AP) (mean $\pm$ SE, $n = 12$, $*P < 0.01$). **B:** Total MMP-1 levels in conditioned media from human dermal fibroblasts in fibrin gels with and without α_2 -AP. Conditioned media was treated with aminophenylmercuric acid for total MMP activation (mean $\pm$ SE, $n = 12$).

gen found in fibroblast-populated fibrin gels under conditions of reduced fibrinolysis at later time points.

Discussion

Fibrin is the most abundant extracellular matrix protein during the initial stages of tissue repair and provides a provisional matrix promoting inward migration of tissue repair cells and prevents excessive blood loss. In addition, fibrin and its cleavage products are likely to play a much greater role during tissue repair by regulating such processes as angiogenesis,²⁷ re-epithelialization,² fibroblast migration, proliferation,^{28,29} and wound contraction.³⁰ Despite this, a role of fibrin in regulating collagen production has not been clearly demonstrated.

In the present study, dermal fibroblasts grown in fibrin gels under certain conditions of reduced fibrinolysis (the presence of a plasmin inhibitor with human cells or by using fibroblasts derived from mice deficient in tPA) were found to produce significantly greater amounts of collagen compared with similar cultures exhibiting normal fibrinolysis. These findings were confirmed *in vivo* using a skin fibrosis model developed in PA-deficient mice. Total skin collagen was found to be significantly increased in the tPA-deficient animals compared with either uPA-deficient or wild-type mice, 1 week after a final injection of subcutaneous fibrin. The increase in hydroxyproline levels in the fibrin-injected tPA-deficient skin was also reflected by a significant increase in skin depth as well as fibrin and collagen density. These results demonstrate that reduced fibrinolysis and fibrin persistence mediated by a lack of tPA is associated with an enhanced accumulation of collagen and the development of skin fibrosis.

The finding that fibrin injection increased collagen levels in the skin of wild-type animals compared to PBS-treated skin confirms previous *in vivo* studies in which an injection of fibrin into the cornea of rabbits,³¹ or subcutaneous implantation of fibrin-filled Plexiglas chambers subcutaneously in rats,³² results in neovascularization and fibroblast proliferation as well as collagen deposition. Yamamoto and colleagues demonstrated that multiple

subcutaneous injections of bleomycin into mice caused skin thickening and increased collagen deposition at the injection site.³³ Fibrin deposition is a common consequence of bleomycin-induced pathologies, therefore these fibrotic effects may be the result of vascular damage and leakage of fibrinogen in bleomycin-treated skin. An association between reduced fibrinolysis and the development of fibrosis has been highlighted in other pathological experimental model systems in studies similar to ours using mice with overexpression or underexpression of various components of the fibrinolytic system.^{22,23,34,35}

Eitzman and colleagues²² found that overexpression of PAI-1, resulted in enhanced deposition of collagen in the lungs of bleomycin-treated mice, whereas PAI-1-deficient mice produced levels of collagen comparable to wild-type controls. Of particular interest, a study by Swaisgood and colleagues²³ also using PA-deficient mice, showed that bleomycin-instillation resulted in approximately a twofold increase in the amount of collagen in the lungs of tPA-deficient mice compared with uPA-deficient and wild-type mice directly correlating with findings in the present study. Tuan and colleagues^{17,18} also demonstrated the importance of a regulated fibrinolytic system in the control of fibrosis using human dermal fibroblasts derived from keloid scars. When grown in fibrin gels, these cells express a persistently high PAI-1 to uPA ratio compared with normal dermal fibroblasts. The same cells show an elevated synthesis of collagen compared with control cells suggesting that reduced fibrinolysis, due to raised PAI-1 levels, may play a causative role in the development of fibrosis in these overactive scars.

The mechanisms by which a fibrin matrix modulates an accumulation of collagen *in vivo* are likely to be multiple. Firstly, fibrin serves as a provisional scaffold for the inward migration of repair cells,^{36–38} and fibrin(ogen) cleavage products are known to possess chemotactic and mitogenic properties.^{39–41} Therefore, fibrin deposition and its persistence at the injection site may result in an increased fibroblast proliferation and migration leading to enhanced collagen accumulation compared with PBS-injected sites. Furthermore, fibrin acts as a reservoir for growth factors including thrombin,⁴² basic fibroblast growth factor,⁴³ platelet-derived growth factor, and TGF- β ³ suggesting there may be a continual supply of potent mediators stimulating a repair response at the fibrin injection site. In this regard, Coustry and colleagues¹⁵ found that when fibroblasts were grown in collagen gels, they produced significantly less collagen in response to TGF- β 1 than when grown in fibrin gels with equivalent amounts of TGF- β 1, implying that fibrin provides a more suitable matrix for the availability of growth factors. With a reduction of fibrinolysis due to tPA deficiency and a persistence of fibrin, the above effects may be exacerbated leading to an enhanced collagen accumulation over and above that with just fibrin.

Another reason for a further increase in collagen accumulation with reduced fibrinolysis and fibrin persistence mediated by a lack of tPA may be due to a direct up-regulation of procollagen type 1 gene expression induced by fibroblast-fibrin matrix interactions. Indeed, findings from the current fibrin gel studies showed that

after 48 hours, human dermal fibroblasts grown in fibrin gels with addition of a plasmin inhibitor, showed an increase in procollagen type 1 gene expression compared with cultures lacking inhibitor. Fibroblasts are known to be able to directly bind to fibrin through cell surface integrins such as $\alpha_v\beta_3$, and $\alpha_5\beta_1$, and blocking studies have shown that these cell-matrix interactions regulate the migration of fibroblasts through a fibrin matrix.^{44–46} It is possible that fibrin-mediated integrin ligation on the surface of fibroblasts causes a downstream intracellular signaling response via MAPK/ERK pathways that could potentially lead to an increase in collagen synthesis.^{46,47} A similar process has been proposed to regulate the down-regulation of collagen synthesis by fibroblasts grown in collagen gels.⁴⁸ A persistence of fibrin due to reduced fibrinolysis may result in prolonged or enhanced cell-matrix interactions resulting in a further stimulation of procollagen gene expression.

Fibrin matrices used in the present study were produced using physiological concentrations of fibrinogen that was 98% pure. Therefore, it is also possible that other contaminating matrix proteins, such as fibronectin and vitronectin, may be stimulating fibroblast procollagen gene expression.⁴⁹ The fibrin matrix also acts as a storage reservoir for a number of important growth factors, several of which are known to play a role in regulating fibroblast procollagen gene expression. For instance, thrombin has been shown to be a potent inducer of procollagen type I gene up-regulation via proteolytic activation of protease activated receptor-1 (PAR-1),⁵⁰ but at concentrations 10-fold higher than were used in this study. Pardes and colleagues⁵¹ analyzed procollagen type I gene expression in response to fibrinogen cleavage products, fibrinopeptides A and B, and demonstrated a significant increase in procollagen mRNA in response to fibrinopeptide A. However, we found no effect on the amount of collagen in human dermal fibroblast cultures on plastic in response to either fibrinopeptides A or B at physiological concentration (10^{-6} mol/L) and this is likely to be the same as generated in the current study (10^{-5} to 10^{-7} mol/L). However, these findings do not discount a possible role for these factors in synergy with other factors or in the presence of a fibrin matrix that persists.

A further mechanism by which fibrin deposition and fibrinolysis may modulate the accumulation of collagen is through a reduction in its degradation. Although the principal role of plasmin is thought to be the degradation of fibrin into soluble degradation products,⁵² plasmin is also a potent activator of MMPs, in particular, the interstitial collagenase MMP-1.^{4,53} Indeed, addition of the plasmin inhibitor, α_2 -antiplasmin, to human fibroblast-populated fibrin gels resulted in a significantly lower level of active MMP-1 being produced compared with cultures without the inhibitor. Moreover, as the total levels of MMP-1 (including latent and active) produced by human fibroblasts in fibrin gels were similar, irrespective of the presence of the inhibitor, it is likely that plasmin directly affects pro-MMP-1 activation rather than its synthesis. A reduction in pro-MMP-1 activation is likely to result in reduced collagen degradation as shown by an overall increase in

collagen levels in these cultures at later time points. At 24 hours however, there was a decrease in MMP-1 activation in inhibitor-treated cultures compared with untreated cultures but both systems had a similar level of collagen accumulation. The reasons for this finding are not clear but there are several possible explanations. The increase in active MMP-1 at 24 hours in the untreated cultures may not be sufficiently elevated to result in a major decrease in collagen accumulation. Alternatively, as procollagen gene expression was elevated at 24 hours in both sets of cultures, collagen accumulation may outweigh collagen degradation and the effect of a relatively small increase in active MMP-1 in untreated cultures at this time point may be masked.

Although a reduction in pro-MMP-1 activation was apparent in human fibroblast populated fibrin cultures under reduced fibrinolytic conditions, it is not known whether a similar reduction in pro-MMP activation occurs with tPA-deficient cells in fibrin gels or in tPA-deficient mice. Furthermore, because mice have a different MMP system compared with humans, there are problems extrapolating the present MMP-1 studies performed with human cells to that which occurs with murine cells or in experimental murine models. However, there is evidence to suggest that fibrotic scenarios develop in mice if MMP activity is reduced. Mice with a targeted mutation in the collagenase cleavage site in the collagen type I molecule^{54,55} show a number of alterations in tissue remodeling, reflecting the inability to degrade type I collagen, including a thickening of the dermis and fascia similar to fibrin-injected skin in the present study. Haraguchi and colleagues⁵⁶ showed that when rats with experimental glomerulonephritis were treated with recombinant tPA, a significant increase in plasmin was observed that resulted not only in reduced fibrin deposition but also a significant increase in MMP activation and a concomitant reduction of collagen accumulation in the glomeruli. These results suggest that the development of tissue fibrosis mediated through an imbalance in the fibrinolytic system is also likely to involve an alteration in the MMP system.

Although enhanced collagen accumulation was associated with reduced fibrinolysis and persistent fibrin mediated by a lack of tPA both *in vitro* and *in vivo*, it was obvious from the present study that uPA deficiency exerted different effects. Surprisingly, collagen accumulation was significantly increased only in the tPA-deficient fibroblast cultures compared with wild-type controls, even though fibroblasts derived from both PA-deficient mice showed significantly reduced fibrinolysis at 72 hours. The reason for this difference is not known but several explanations are possible. It is thought that the major physiological role of tPA is in the removal of fibrin from the systemic circulation due to its specificity for fibrin, and clot-restricted plasminogen activation. In contrast, uPA binds to a specific cell surface receptor (uPAR) and is generally thought to be involved in cell-mediated proteolysis, and removal of fibrin at extravascular sites.⁵⁷ There is now considerable evidence that uPA-mediated plasmin generation and MMP activation occurs at the cell surface.⁴ Therefore, absence of this interaction with uPA-

deficient fibroblasts within a fibrin matrix may prevent pericellular collagenolysis and thus deposited collagen accumulates around each cell. Fibroblasts grown in collagen gels show down-regulation of collagen gene expression and decreased collagen accumulation⁴⁸ and this may be a reason for a lack of enhanced collagen accumulation by uPA-deficient fibroblasts. Alternatively, collagen gene expression may be down-regulated by these cells compared with tPA-deficient cells due to a difference in cell-matrix interactions. It is known that uPA, uPAR, PAI-1, and certain integrins interact on the cell surface and that this interaction may initiate various downstream intracellular signaling events. It is therefore possible that lack of uPA binding to uPAR causes a change in the intracellular signaling response associated with fibrin matrix-induced up-regulation of procollagen gene expression. A further explanation may be related to the fact that uPA-deficient cells showed decreased fibrinolysis at 72 hours compared with wild-type cells but not at 24 and 48 hours in contrast to tPA-deficient cells. Collagen accumulation may therefore not be significantly increased until a later time point due to a delayed effect of reduced fibrinolysis in uPA-deficient fibroblasts cultures.

Urokinase-deficient mice showed an increase in skin thickness but a similar amount of fibrin and collagen after multiple fibrin injections to wild-type mice, in contrast with tPA-deficient mice. As stated previously, Swaisgood and colleagues²³ found similar effects in the lungs of uPA-deficient mice after bleomycin treatment. Histologically, the lungs appeared fibrotic with major structural changes in the alveoli and abundant inflammatory cell infiltrate, but HPLC analysis of the hydroxyproline content in these tissues showed that collagen levels remained unchanged compared with their wild-type counterparts. Furthermore, we previously showed that after peritoneal surgery, tPA-deficient mice are more susceptible to fibrous adhesion formation compared with uPA-deficient or wild-type animals.³⁵ These findings suggest that reduced fibrinolysis and fibrin persistence due to a lack of the tPA but not uPA is associated with collagen accumulation after injury. One explanation for a lack of collagen accumulation in uPA-deficient mice may be the absence of fibrin persistence at the injection site compared with tPA-deficient mice. Mice lacking tPA appeared unable to completely degrade subcutaneously injected fibrin implying that the presence of uPA or another fibrinolytic protease was not able to compensate for the lack of tPA. However, uPA-deficient mice were able to clear injected fibrin suggesting that other proteases probably produced by inflammatory cells, are capable of degrading the fibrin. Indeed, Carmeliet and colleagues⁶ found that tPA but not uPA deficiency was associated with reduced thrombolytic potential. By degrading the fibrin matrix, the stimulation for cells to up-regulate procollagen gene expression may be diminished. Another possibility is that uPA-deficient fibroblasts are unable to migrate into the fibrin injection sites. Indeed, several reports have demonstrated a reduced migration of cells derived from uPA-deficient mice,^{6,58,59} however from histological assessment, there did not appear to be a difference in cellularity at the fibrin-injected

site between the three mouse strains. Furthermore, *in vitro* studies showed a lack of enhanced collagen accumulation even when cells were within a fibrin matrix. As well as the reasons outlined above, other factors such as a difference in the formation of the fibrin matrix, a change in mechanical stress surrounding the fibrin matrix, presence of inflammatory cells, or the availability of other proteolytic systems and growth factors may all contribute to a difference in collagen accumulation in these two mouse strains.

In summary, results of the current study have demonstrated that under certain conditions of reduced fibrinolysis and fibrin matrix persistence, excess collagen accumulation occurs that may in part explain the development of a number of fibrotic conditions in which fibrin deposition is a characteristic feature. These findings do however appear contradictory to the studies showing that fibrinogen-deficient mice are as susceptible to lung fibrosis as wild-type mice after bleomycin administration^{60–62} and are also able to produce granulation tissue and heal full-thickness skin wounds.^{63,64} One explanation for this phenomenon is that fibrin(ogen) itself is not essential for collagen accumulation but that its persistence, or that of another component of the fibrin matrix, promotes and prolongs collagen accumulation. In addition, the type of fibrin matrix deposited at sites of tissue damage are likely to vary considerably in terms of composition, degree of crosslinking, mechanical stress, as well as cellularity, and these properties may influence the behavior of fibroblasts and the resultant amount of collagen formed. Alternatively as in the present study, by affecting just the fibrinolytic system, plasmin-mediated pro-MMP activation and hence collagen degradation is reduced, thus leading to an overall enhanced collagen accumulation irrespective of the presence of fibrin(ogen). Findings from this study suggest that therapeutic interventions designed to enhance fibrinolysis, such as administration of either PA or inhibitors of PAI-1, may prove useful in preventing the development of fibrosis in which fibrin deposition and reduced fibrinolysis are key features.

Acknowledgments

We thank Maria De Mol for technical assistance and Grenham Ireland and Robin McNulty for helpful comments.

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