JOURNAL OF PATHOLOGY, VOL. **183:** 169–176 (1997)

HUMAN AGEING IMPAIRS INJURY-INDUCED *IN VIVO* EXPRESSION OF TISSUE INHIBITOR OF MATRIX METALLOPROTEINASES (TIMP)-1 AND -2 PROTEINS AND mRNA

GILLIAN S. ASHCROFT[1,2], SARAH E. HERRICK[1], ROY. W. TARNUZZER[3], MICHAEL A. HORAN[2], GREGORY S. SCHULTZ[3] AND MARK W. J. FERGUSON[1]*

[1]*Cells, Immunology and Development Division, School of Biological Sciences, 3.239 Stopford Building, University of Manchester, Oxford Road, Manchester M13 9PT, U.K.*
[2]*Department of Geriatric Medicine, University Hospital of South Manchester, Withington, Manchester M20 2LR, U.K.*
[3]*Department of Obstetrics and Gynecology, University of Florida, Gainesville, FL 32610, U.S.A.*

SUMMARY

Proteolysis is an essential component of wound healing but, if uncontrolled, it may lead to degradation of the neo-matrix and a delay in wound repair. Despite numerous reports of impaired wound healing associated with increasing age, the control of proteolysis is completely unknown. Tissue inhibitor of matrix metalloproteinases (TIMP)-1 and -2 inhibit the activity of matrix metalloproteinases and the pattern of regulation of these molecules determines in part the spatial and temporal regulation of proteolytic activity. This study reports on TIMP-1 and -2 protein localization using immunocytochemistry in healing wounds of healthy subjects of different ages from day 1 to 6 months post-wounding, and has quantified the mRNA levels for both inhibitors using reverse transcriptase-polymerase chain reaction (RT-PCR). TIMP-1 and TIMP-2 proteins are up-regulated from 24 h post-wounding, with a decrease in staining intensity by day 7 for TIMP-2 and by day 14 for TIMP-1. Steady-state mRNA levels for both TIMPs were significantly greater in normal young skin than in aged skin. In the young, there was a significant increase in mRNA expression for TIMP-1 and -2 by day 3 post-wounding, which decreased by day 14 and had returned to basal levels at day 21. In the wounds of the aged subjects, basal levels were observed for TIMP-1 and -2 at all time-points. These results suggest that intrinsic cutaneous ageing is associated with reduced levels of TIMP mRNA both in normal skin and during acute wound repair. These levels may be instrumental in dermal tissue breakdown in normal skin, retarded wound healing, and the predisposition of the elderly to chronic wound healing states. © **1997 John Wiley & Sons, Ltd.**

J. Pathol. 183: 169–176, 1997.
No. of Figures 9. No. of Tables 1. No. of References 29.

KEY WORDS—**wound repair; ageing; proteinase; skin; human**

INTRODUCTION

Wound healing is a highly complex, orchestrated process requiring the action of matrix metalloproteinases (MMPs) for the degradation of the extracellular matrix in order to aid cell migration, remove necrotic debris, and assist the remodelling of the neo-matrix. The accelerated breakdown of connective tissue may, however, contribute to a number of pathological conditions such as tumour invasion, the arthritides, and age-related chronic wound healing states.[1,2] The extent of tissue degradation depends not only on the tissue levels of proteinases, but also on the levels of proteinase inhibitors, one of the major groups described to date being named as the tissue inhibitors of metalloproteinases (TIMPs).

In vitro studies have suggested that the intrinsic ageing process is associated with an imbalance in the MMP/TIMP ratio.[3,4] Despite the importance of therapeutic intervention with respect to both preventing and treating delayed wound healing in the aged, the molecular mechanisms underlying age effects in normal skin and during repair are unknown, and the influence of intrinsic ageing on the *in vivo* levels of TIMP-1 and -2 has been ignored.

TIMP-1 is a 29 kD *N*-glycosylated protein synthesized by macrophages and mesenchymal and endothelial cells and forms high-affinity, non-covalent complexes with pro-MMP-9 and active MMP-1, -3 and -9.[2,5] TIMP-2 is a 22 kD protein which possesses 40 per cent sequence identity to TIMP-1 and forms complexes with pro- and active MMP-2.[2,6] Differential regulation of TIMP-1 and -2 has been observed,[7] thus potentially influencing the differential activity of specific proteinases. In addition to the well-described direct inhibitory effects on MMPs, TIMP-1 influences endothelial cell proliferation and stimulates MMP-1 secretion *in vitro* from human dermal fibroblasts, while TIMP-2 inhibits neovascularization.[8–10] Moreover, both TIMP-1 and TIMP-2 possess cell growth-promoting activity, which is cell-specific.[11–13] Thus, the levels of these inhibitors may have profound effects on wound healing, in terms of both cell proliferation and matrix turnover.

*Correspondence to: Professor Mark W. J. Ferguson, School of Biological Sciences, 3.239 Stopford Building, University of Manchester, Oxford Road, Manchester M13 9PT, U.K.

CCC 0022–3417/97/100169–08 $17.50

Received 19 November 1996
Accepted 9 May 1997

The spatial and temporal distribution of TIMPs during wound repair is critical for controlled proteolysis. Cell-specific patterns are emerging for the proteinase inhibitors which may have wide-ranging effects on specific cell-type proliferation, migration, and local tissue remodelling.[14,15] Temporally, TIMP-1 protein has been reported to be up-regulated in acute wound fluid 2 days post-mastectomy[16] and mRNA in porcine burn wounds at day 6,[17] but the time course of TIMP-2 appearance is unknown. Moreover, the relationship between protein and message levels of TIMP-1 and -2 has not been investigated previously. Potential for the differential regulation of TIMP-1 and -2 at the transcriptional level was suggested by the report that TIMP-1 expression, in contrast to TIMP-2, is regulated by the *ras* oncogene.[15] We have therefore determined the age-related changes *in vivo* of TIMP-1 and -2 protein and mRNA expression in acute healing wounds of healthy subjects, using immunocytochemical techniques and quantitative RT-PCR.

MATERIALS AND METHODS

Health-status-defined panel subjects

This research project was approved by the South Manchester Health Authority Ethics Committee. Informed consent was obtained from all subjects. One hundred and thirty-two Caucasian subjects of both sexes with defined health status (screened using the SENIEUR protocol of Ligthart *et al.*[18]), aged between 19 years and 96 years of age, were divided into three age groups (19–39, 40–59, and 60+ years). Subjects were non-smokers, on no medication, with no past medical history of note. Medical examination in all cases was normal and all subjects aged over 50 years had normal CXR, ECG, haematological and biochemical profiles. Isopropyl alcohol was used to clean the skin of the left upper inner arm (a non-sun-exposed site) and 1 per cent lignocaine was infiltrated into the skin. Two 4 mm punch biopsies (Steifel Labs, Bucks., U.K.) were taken from the anaesthetized area and haemostasis was obtained by pressure for 10 min. Each biopsy of normal skin was bisected and one-half embedded in Optimal Cutting Temperature compound (Miles Inc., Elkhart, IN, U.S.A.), frozen over liquid nitrogen, and stored at -70°C; the other half was snap-frozen in liquid nitrogen and stored at -70°C. The wounds were covered with a Multisorb dry gauze dressing (Smith & Nephew, York, U.K.) for 24 h and then left uncovered until re-biopsy. At fixed time-points from day 1 to 6 months post-wounding, the left upper inner arm was cleaned with isopropyl alcohol, elliptical excisions of the wounds were made following 1 per cent lignocaine infiltration, and two sutures were used to close the wound. All biopsies were always taken 1 min following anaesthetic injection and were immediately snap-frozen. Each excised wound was bisected and processed as described above. For normal tissue and wounds required for molecular analysis, the minimal subcutaneous fat present was removed by micro-dissection and the wounds were excised carefully to avoid the wound margin, in order to prevent any potential contamination with normal skin.

Immunocytochemistry

Stepped sections were cut at 7 μm on a Leitz cryostat at -20°C, placed on poly-L-lysine-coated slides (Sigma, Poole, U.K.), air-dried, and fixed in acetone for 10 min. Sections were then washed in 4-chloronaphthol (2·8 mM in methanol/PBS with 0·01 per cent H_2O_2, 10 min) to block non-specific binding of fluorescein to eosinophils[19] and then washed in PBS (3×5 min). Antibodies to TIMP-1 and TIMP-2 were obtained from Oncogene Science[20,21] and used at 2 μg/ml in 10 mM PBS supplemented with 1 per cent BSA. Primary antibody incubation was for 30 min at room temperature, followed by three washes with PBS. Goat anti-mouse IgG fluorescein-conjugated antibody was used at a dilution of 1:40 for 20 min, followed by three PBS washes. For dual immunostaining, specimens were incubated with rabbit anti-von Willebrand factor (Dako, Bucks., U.K.), 1:40, for 1 h, followed by three PBS washes and anti-rabbit-TRITC antibody (Dako, Bucks., U.K.), 1:40, for 20 min. Staining then proceeded as described for TIMPs. Sections were mounted in a non-fading medium, containing DABCO [1,4-diazobicyclo-(2,2,2)-octane], and photographed using a Leitz Aristoplan microscope under standard conditions. For each primary antibody, control sections were stained using the above protocol but substituting normal sheep serum (1:1000) for the primary antibody in the case of anti-TIMP-1 and -2, and normal rabbit serum at 1:40 for the anti-von Willebrand factor; in each case, this produced minimal levels of background staining. The intensity of staining, relative to normal, in each case was scored on the following scale by two independent observers blinded to the identity of each specimen: -5 = no staining; -4 = 0–25 per cent; -3 = 26–50 per cent; -2 = 51–75 per cent; -1 = 76–99 per cent; 0 = normal; 1 = 101–125 per cent; 2 = 126–150 per cent; 3 = 151–175 per cent; 4 = 176–200 per cent; 5 = 200 per cent+. In all cases where regions of negative immunoreactivity were found within wounds, adjacent areas of normal epidermis and/or dermis revealed positive immunoreactivity (dependent on the primary antibody used) and thus served as internal semi-quantitative controls.

Quantitative RT-PCR

Cellular RNA was isolated from tissue specimens using the method of Chomczynski and Sacchi.[22] Wound tissue (two samples from each age, sex and time-point—days 1, 3, 7, 14, and 21, and 6 months post-wounding) was homogenized in a Dounce homogenizer with a solution containing 4 M guanidinium isothiocyanate, 25 mM sodium citrate (pH 7), 0·5 per cent sarcosyl, and 0·1 M 2-mercaptoethanol. Solubilized tissue was acidified using 2 M sodium acetate, pH 4, extracted with equal volumes of water-saturated phenol–chloroform–isoamyl alcohol (1:0·2:0·05) and centrifuged at 10 000 rpm for 10 min. RNA in the

aqueous phase was precipitated twice with an equal volume of isopropanol. The purity of extraction was assessed using the $A_{260/280}$ ratio spectrophotometrically, which in all cases was above 1·75. Day 1 pooled-wounds yielded inadequate quantities of RNA for further analysis.

Quantitative RT-PCR was performed as described by Tarnuzzer *et al.*[23] Briefly, the reverse transcriptase reaction was carried out using eight decreasing dilutions of template with 1 μg of authentic cellular RNA. β-Actin was used as a positive control. Polymerase chain reactions were carried out on the reverse transcription reaction. Electrophoresis was then carried out on a 2 per cent agarose gel containing 25 ng/ml ethidium bromide at 100 V for 1 h using an Electro 4 tank (Hybaid, Teddington, U.K.) and photographed using a dual intensity transilluminator (Genetic Research instrumentation, Dunmow, U.K.) with a Polaroid MP 4+ camera and 665 Polaroid black and white film. Photographic images were captured using a PC Image Software system (Foster Finley, Newcastle, U.K.) on a 486 DX2 Dan computer (Dan, U.K.) and a CCD camera (Swift, U.K.). The band intensities were determined by image analysis using a Macintosh computer and NIH software program. Band intensity values were normalized based on the molecular weights of the products. The log of the ratio of band intensities within each lane was plotted against the log of the copy number of template added per reaction. Quantities of target messages were determined where the ratio of template and target band intensities were equal to 1. Copy numbers per cell were calculated by assuming 26 pg RNA/cell.[23] The cellularity of the tissue did not differ significantly between different groups (data not shown).

Statistical analysis

All data are presented as mean ± SD. Differences between means were evaluated by three-factor ANOVA. Regression analysis was used to analyse the log ratio of band intensities to log of the copy number of template. In all circumstances, $P<0·05$ was considered to be significant.

RESULTS

Age-related decrease in TIMP wound immunostaining, with differential spatial regulation of TIMP-1 and -2

In normal skin, faint TIMP-1 staining was present around the endothelium with no staining in aged subjects. TIMP-2 was absent from the matrix and was localized faintly to papillary fibroblasts in all age groups (Fig. 1).

At days 1 and 3 post-wounding, the clot stained brightly for TIMP-1 in all age groups, with scattered inflammatory cell staining. At day 7, the migrating neo-epidermis stained brightly in the young and middle-aged groups only (Fig. 2), with diffuse staining in a band under the leading edge of the epidermis in all age groups. At days 14 and 21 post-wounding, faint basal neo-epidermal staining was apparent in the wounds of the young, with faint matrix staining in the upper wound (less intense in the aged) and bright pericellular staining of all blood vessels within the wound space (Fig. 3). At day 84 and 6 months post-wounding, there was persistent faint staining of blood vessels. The temporal changes in TIMP-1 staining were highly significant ($P<0·0001$), as was the decreased staining associated with ageing ($P<0·01$).

At days 1 and 3 post-wounding, TIMP-2 was present faintly in the clot in all age groups. At days 3 and 7, a band of keratinocytes at the leading edge of the neo-epidermis stained brightly, whereas the underlying granulation tissue stained for TIMP-2 in all age groups (Fig. 4). At days 14 and 21, when re-epithelialization was complete in all age groups, no epidermal staining was apparent. In the young, faint fibroblast staining was apparent, with faint matrix staining throughout the wound granulation tissue. By contrast, in the aged, neither matrix nor fibroblasts stained within the wounds. At day 84 post-wounding, TIMP-2 staining was absent from the wounds. The temporal changes in TIMP-2 staining were highly significant ($P<0·0001$), as was the decreased staining associated with ageing ($P=0·023$).

TIMP-1 steady-state mRNA levels are increased to a greater extent than TIMP-2 in the acute wounds of young subjects, with an age-related decrease in TIMP-1 and -2 mRNA levels

Normal young skin expressed greater TIMP-1 mRNA than aged skin when expressed as copy number/cell (mean of 25 copies/cell in the young subjects and 1 copy/cell in the aged) ($P=0·002$), and greater TIMP-2 levels (mean of 13 copies/cell in the young and 1 copy/cell in the aged) ($P=0·03$) (Figs 5 and 6).

At days 3 and 7 post-wounding, TIMP-1 message peaked in the young (57 copies/cell at day 3 and 163 copies/cell at day 7) (Fig. 7), remaining approximately two times greater than normal skin until 3 months post-wounding, when the copies/cell had declined to 3. In the wounds of the aged, only a non-significant increase in message occurred at day 14 post-wounding (mean of 8 copies/cell). In general, increasing age was associated with a significant decrease in TIMP-1 levels from day 3 to day 21 post-wounding ($P<0·0001$).

TIMP-2 levels were greater from day 3 to day 14 in the wounds of the young subjects compared with the aged ($P<0·0001$). In the young subjects, TIMP-2 mRNA levels were greater than normal skin at days 3 (25 copies/cell), 7 (21 copies/cell), and 14 (19 copies/cell), and by day 21 they had decreased to less than the expression in normal young skin (2 copies/cell) (Fig. 8). In the wounds of the aged, mRNA levels increased at day 7 (7 copies/cell) and had declined by day 14 (1 copy/cell). At 6 months post-wounding, mRNA levels for both TIMP-1 and TIMP-2 in the young were significantly lower than in normal skin. The up-regulation of TIMP-1 mRNA in the wounds of young subjects was approximately two times greater than TIMP-2 expression at all time-points, except at day 7 where the increase was 10-fold. There were no significant sex differences observed at any time-point for TIMP-1 and -2 (data not

Fig.1

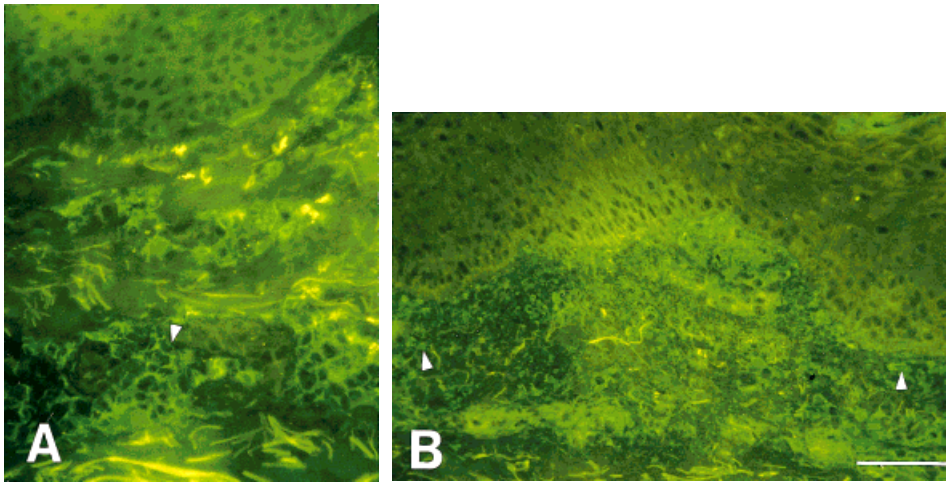

Fig.2

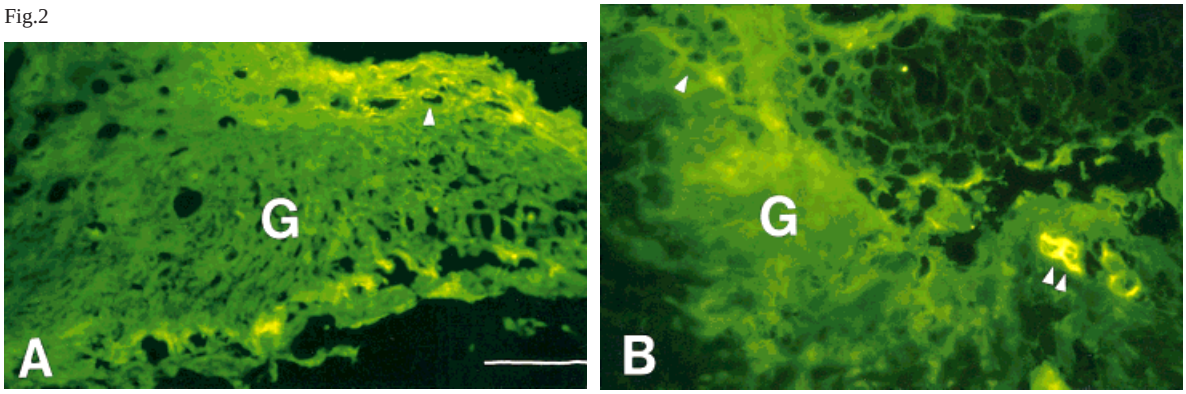

Fig.3

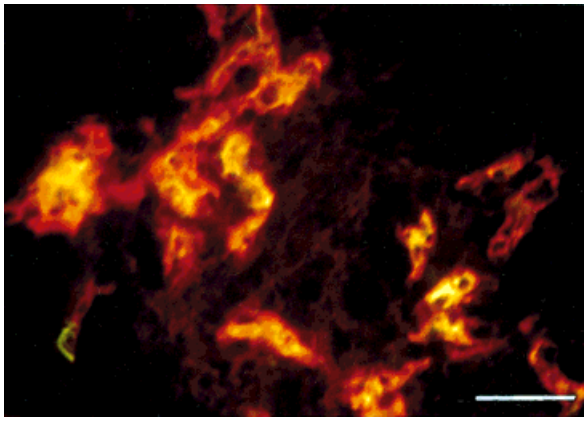

Fig.4

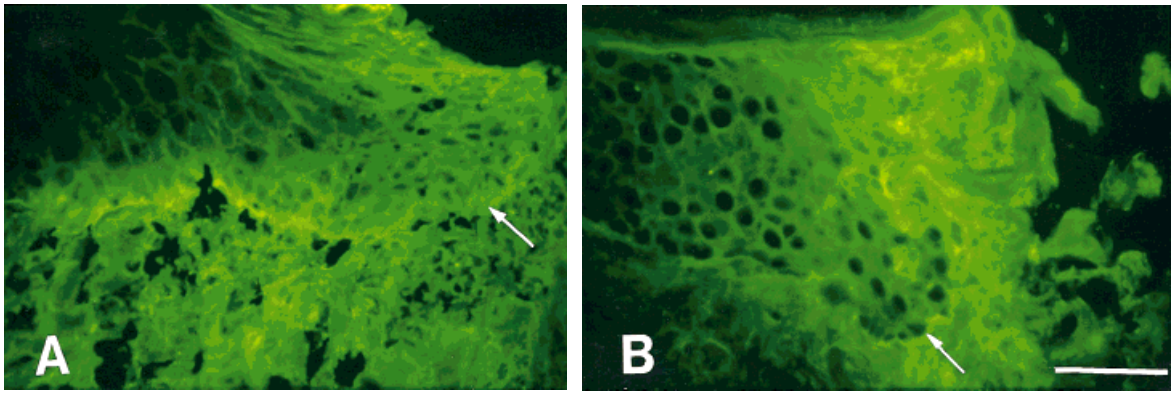

Table I—Semi-quantitative analysis of TIMP-1 and -2 immunostaining

	Y		M		O	
TIMP-1						
Day 1	5	(0)	5	(0·3)	5	(0·3)
Day 3	4·8	(0·3)	4·7	(0·6)	4·8	(0·3)
Day 7	4·8	(0·3)	4·5	(0·6)	2·35*	(0·3)
Day 14	1·5	(0·3)	1·5	(0·3)	1·45*	(0·8)
Day 21	1·7	(0·5)	1·3	(0·8)	0·7*	(0·5)
Day 84	0·85	(0·8)	1	(0·3)	0·85	(0·3)
6 months	0·85	(0·8)	0·85	(0·8)	0·85	(0·8)
TIMP-2						
Day 1	3	(0)	2·85	(0·3)	2·7	(0·6)
Day 3	2·7	(0·6)	2·7	(0·6)	2·7	(0·6)
Day 7	1·4	(0·3)	1·5	(0·6)	1·4	(0·3)
Day 14	1·85	(0·3)	0·85	(0·6)	0·3*	(0·6)
Day 21	0·7	(0·6)	0·3	(1)	0*	(0·6)
Day 84	0·15	(0·3)	0	(0·3)	0	(0·6)
6 months	0	(0·8)	0	(0·6)	0	(0·8)

Figures represent mean values with $n = 6$ (combined male and female data); figures in parentheses represent the SD of the mean. The scores for TIMP-1 at days 1–7 represent clot and epidermal staining, and at days 14 and 21 they represent matrix staining. The scores for TIMP-2 represent clot and matrix staining.

Y = young (20–39 years); MA = middle-aged (40–59 years); O = old (60+ years).

*Significant decrease in scoring ($P < 0·05$).

shown), nor between young and middle-aged groups. Figure 9 shows the log ratio of band intensity versus log copy number for a representative sample.

DISCUSSION

We have shown using immunolocalization that TIMP-1 and TIMP-2 proteins are up-regulated markedly from 24 h post-wounding, with a decrease in staining intensity by day 14. At 6 months post-wounding, TIMP-2 staining intensity was absent from the wounds, whereas TIMP-1 continued to stain more intensely than normal skin. TIMP-1 was present in the clot, in the neo-epidermis and, from day 14 to 6 months, predominantly around blood vessels. Our results are consistent with previous reports which showed that TIMP-1 mRNA was detected at the epithelial migrating edge, in fibroblasts and macrophages, at day 6 post-wounding in a porcine burn model.[17] We report that discrete areas of migrating keratinocytes at the wound edge stained intensely for TIMP-1 in the young, compared with the fainter staining of adjacent keratinocytes in normal skin resting upon an intact basement membrane. This is consistent with previous *in vitro* studies showing that TIMP expression by keratinocytes is inhibited by basement membrane laminin but is stimulated by collagen I.[24] The reduction in TIMP-1 staining in the migrating epidermal tip in the aged subjects may be important in the age-related delay in re-epithelialization observed: it is possible that an imbalance in proteolytic activity in the wounds of the aged may degrade newly-synthesized basement membrane components. The presence of TIMP-1 in endothelial cells has been demonstrated previously.[17] *In vitro* studies have shown that endothelial cells are capable of producing large quantities of MMPs required for the process of angiogenesis and matrix breakdown, in addition to TIMP-1, which down-regulates this response.[9] Thus, the prolonged expression of TIMP-1 in the wound endothelium of all age-groups may be necessary to down-regulate the angiogenic response *in vivo*. There was a significant age-related decrease in TIMP-2 protein staining from day 14 to 21 post-wounding. The consequences of this reduction in proteinase inhibitor may be a decrease in cell proliferation,[7] in addition to a possible increase in proteolytic activity.

We report differential spatial localization of TIMP-1 and -2, with TIMP-2 present in the clot, fibroblasts, coating the matrix, and at the migrating epidermal edge, whereas TIMP-1 is located at the migrating epidermal edge, in the matrix, and around endothelium. In normal skin, TIMP-1 was localized to the endothelium, whereas TIMP-2 was restricted to papillary fibroblasts. These results suggest that the proteinase inhibitor involved in tissue neovascularization and in normal endothelial biology is TIMP-1, whereas TIMP-2 is the predominant form secreted by wound fibroblasts. The differential expression of TIMP-1 and -2 in specific cell types provides further evidence that the TIMPs possess different physiological roles.

Levels of mRNA for both TIMPs were significantly greater in normal young skin than in aged skin. This

Fig. 1—Differential localization of TIMP-1 and TIMP-2 in normal skin. (A) Normal skin from a 25-year-old female illustrating endothelial staining for TIMP-1 (arrow). (B) Normal skin staining for TIMP-2 from a 25-year-old female illustrating the faint cellular staining, which did not co-localize with either granulocyte or macrophage markers (arrows). Scale bar = 100 μm

Fig. 2—TIMP-1 localizes to the migrating edge of the epidermis at day 7 post-wounding, with an age-related reduction in staining. (A) Section from a 24-year-old female. (B) Section from a 75-year-old female. Bright staining of the two-cell-deep migrating epidermal lip is shown in A, with faint epidermal staining in B. The arrow represents the migrating keratinocytes. G = granulation tissue. The double arrows in B represent inflammatory cell staining. Scale bar = 100 μm

Fig. 3—TIMP-1 localizes to wound endothelium at day 21 post-wounding in all age groups. The section represents the mid-wound region from an 83-year-old male. Blood vessel staining appears red with TIMP staining green; where the two co-localize, an orange colour is observed. Scale bar = 100 μm

Fig. 4—TIMP-2 localizes to the migrating epithelial tip at day 7 post-wounding in all age groups. (A) Section taken from a 24-year-old male. (B) Section from a 63-year-old male. The arrow indicates the keratinocytes at the edge of the migrating epidermis staining for TIMP-2, below which is a band of diffuse staining in the granulation tissue. Scale bar = 100 μm

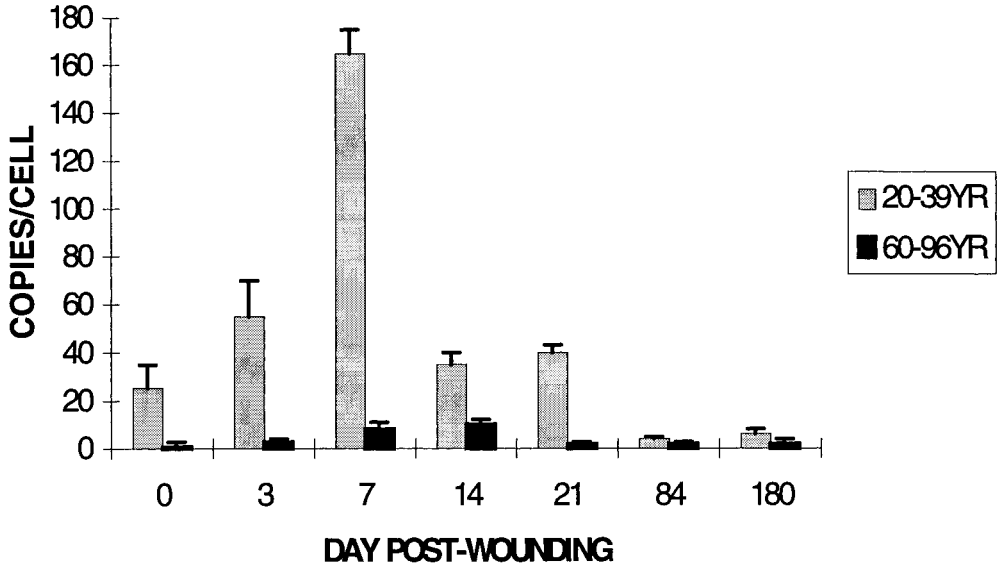

Fig. 5—TIMP-1 mRNA is greater in normal young skin and in the wounds of young subjects than in aged subjects. Quantitative RT-PCR (as described in the Materials and Methods section) was used to determine the amount of mRNA in normal skin and wound tissue. For clarity, only the data for females are shown, as there were no significant sex differences observed. $n=10$ for each age and gender group for normal skin; $n=2$ for wound tissue. Data represent mean ± SD

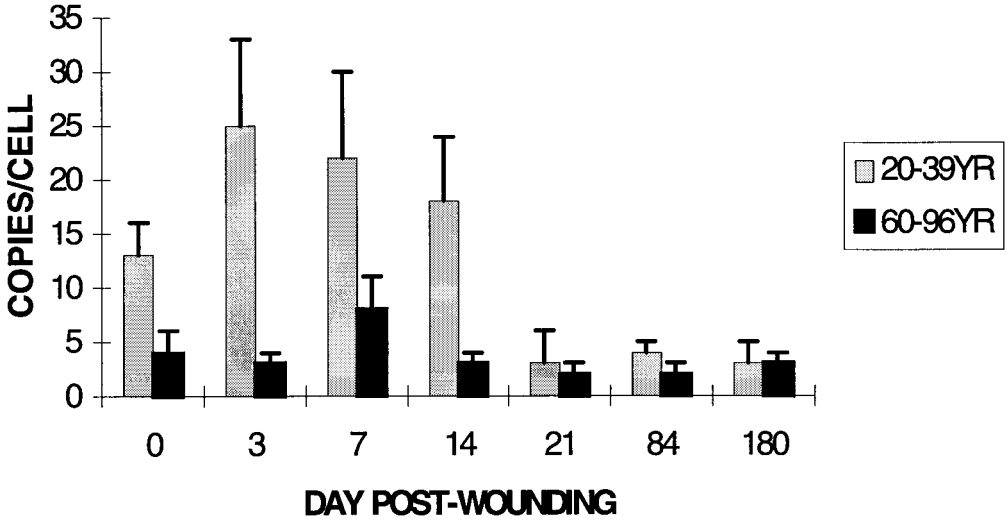

Fig. 6—TIMP-2 mRNA is markedly up-regulated in the wounds of young subjects. There were no sex differences observed for any of the values and therefore for clarity, only the female data are presented. $n=10$ for each age and gender group for normal skin; $n=2$ for wound tissue. Data represent mean ± SD

difference was not observed for TIMP-2 in protein staining, possibly because quantitative RT-PCR is a more sensitive technique than immunostaining. Other less likely explanations for this difference between mRNA and protein levels include a longer half-life of TIMP-2 protein in older subjects and/or less efficient translation of TIMP-2 mRNA in younger subjects. This is the first report to suggest that *in vivo* expression of TIMP-2 declines with age. This is of great interest, since we have observed that MMP-2 is up-regulated in normal ageing skin.[25] Thus, an imbalance may exist in proteolytic activity in normal aged skin, which could have profound effects on matrix turnover, not only influencing the observed dermal collagen fibre fragmentation,[26] but also the predisposition of ageing skin to matrix degradation following injury.

Following wounding, there was a marked increase in mRNA expression for both TIMP-1 and TIMP-2 by day 3 in the young, which had returned to basal levels by day 21. The increase in TIMP-1 expression was prolonged, and significantly greater from day 3 to day 21 post-wounding, compared with TIMP-2. This finding is consistent with observations in the chronic inflammatory disorder periodontitis, in which TIMP-1 is preferentially expressed.[27] The reason for this is at present unknown but presumably reflects mechanisms controlling the *ras* oncogene.[15] In the wounds of the aged subjects, basal mRNA levels were observed for TIMP-1 and -2 at all

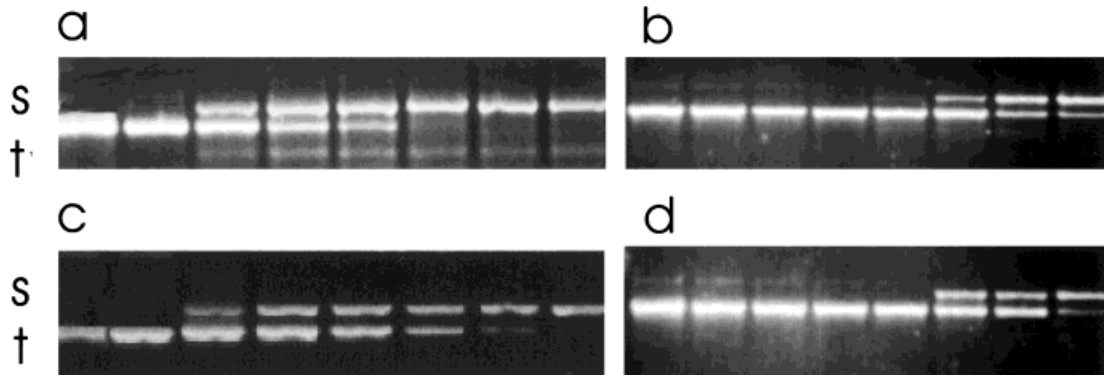

Fig. 7—Quantitative RT-PCR shows increased expression for TIMP-1 mRNA in normal young skin and acute wounds. The decreasing template dilutions run from left to right, with the amplicons of the sample cDNA of higher molecular weight than those of the template. (a) Normal young skin; (b) normal aged skin; (c) day 7 wounds, young subjects; (d) day 7 wounds, aged subjects. s = sample; t = template

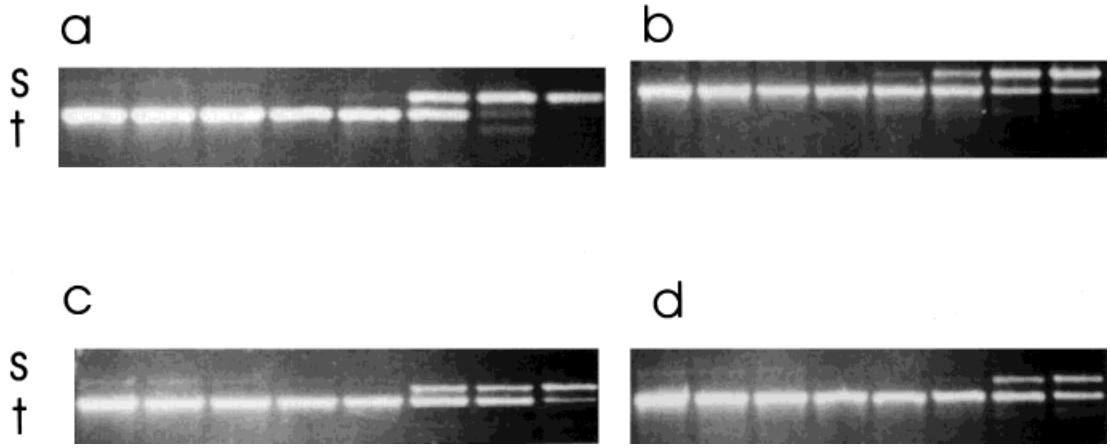

Fig. 8—Quantitative RT-PCR shows an increase in TIMP-2 mRNA expression in normal young skin and in day 7 wounds of young subjects. The decreasing template dilutions run from left to right, with the amplicons of the sample cDNA of higher molecular weight than those of the template. (a) Day 7 wounds, young subjects; (b) day 7 wounds, aged subjects; (c) normal young skin; (d) normal aged skin. s = sample; t = template. For details see the Materials and Methods section

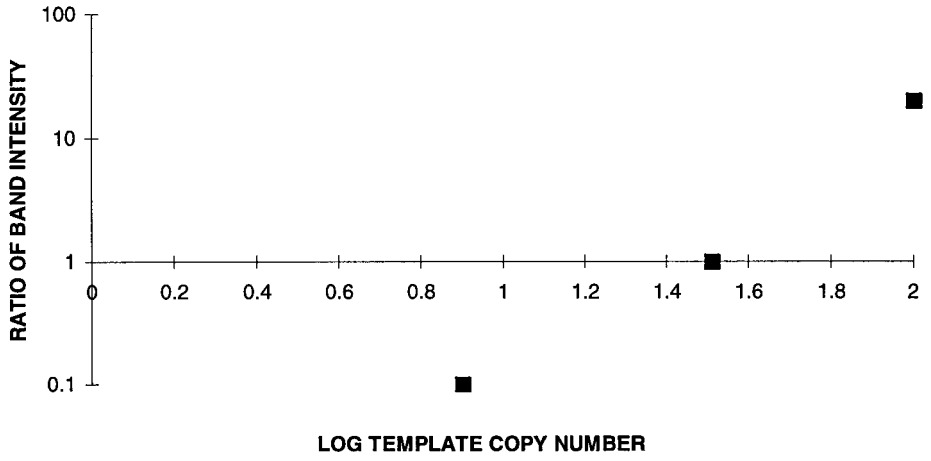

Fig. 9—Analysis of copy number for TIMP-1 mRNA in a day 21 wound from a young subject. Photographs of the ethidium bromide-stained gels were digitized and band intensities were determined as stated in the text. The log of the ratios of template:target were plotted versus the log of the input copy number of template for each lane. The linearity of the slope, $R^2 = 0{\cdot}97$, and the slope of the line, 0·98 (regression analysis), show that there is equal efficiency of annealing and polymerization

time-points post-wounding. Age-related changes in TIMP production appear to be associated with reduced steady-state levels of mRNA. Despite low levels of mRNA, of interest is the early increase in TIMP-1 and -2 protein observed in the wounds of the aged. This may represent preformed intracellular protein such as that present in invading inflammatory cells, a lack of specificity in the immunostaining technique, an altered half-life of the protein or mRNA, or, as speculated upon previously, post-transcriptional modifications in the young. Significantly reduced levels of TIMP-1 and -2 mRNAs were observed in the 6-month wounds of the young in comparison with normal skin. Hypertrophic scars are characterized by an increase in matrix turnover and collagenase activity[28] and thus of interest are the low TIMP levels in 6-month wounds in the young compared with normal young skin, suggesting a greater turnover in wound tissue, leading to worse scarring in terms of macroscopic quality (Ashcroft *et al.*, manuscript in preparation).

Our observations regarding MMP levels, combined with the data presented here, suggest that an age-related proteolytic imbalance exists in both normal skin and acute wounds, with chronic non-healing wounds possibly forming the extreme of the ageing spectrum. Manipulation of the proteolytic balance has been achieved *in vitro* using a variety of factors.[4,29] Therapeutic manipulation of proteinase levels in the wounds of the elderly, either by stimulating TIMPs, for example by TGF-β or retinoic acid, or by inhibiting proteinases, for example with synthetic MMP inhibitors, would appear to be a vital strategy for accelerating the healing of such wounds. It may also be a useful strategy for reducing normal dermal ageing.

ACKNOWLEDGEMENTS

Gillian Ashcroft is a Wellcome Clinician Scientist Fellow. Statistical advice was kindly given by Julie Morris, University Statistician, University of Manchester. The dressings used in this study were kindly donated by Smith & Nephew, York, U.K.

REFERENCES

1. Wysocki AB, Staiano-Coico L, Grinnell F. Wound fluid from chronic leg ulcers contains elevated levels of metalloproteinases MMP-2 and MMP-9. *J Invest Dermatol* 1993; **101:** 64–68.
2. Murphy G. Reynolds JJ. Extracellular matrix degradation. In: Royce PM, Steinmann B, eds. Connective Tissue and its Heritable Disorders. New York: Wiley, 1993; 287–316.
3. West MD, Pereira-Smith OM, Smith JR. Replicative senescence of human skin fibroblasts correlates with a loss of regulation and overexpression of collagenase activity. *Exp Cell Res* 1989; **184:** 138–147.
4. Bizot-Foulon V, Bouchard B, Horneback W, Dubertret L, Bertaux B. Uncoordinate expression of type I and III collagens, collagenase and tissue inhibitor of matrix metalloproteinase 1 along *in vitro* proliferative life span of human skin fibroblasts. Regulation by all-*trans* retinoic acid. *Cell Biol Int* 1995; **19:** 129–134.
5. Woessner JF Jr. Matrix metalloproteinases and their inhibitors in connective tissue remodelling. *Faseb J* 1991; **5:** 2145–2154.
6. Howard EW, Bullen EC, Banda MJ. Regulation of the autoactivation of human 72 kDa progelatinase by tissue inhibitor of metalloproteinases-2. *J Biol Chem* 1991; **266:** 13064–13069.
7. Hanemaaijer R, Koolwijk P, le Clercq L, de Vree WJA, van Hinsbergh VWM. Regulation of matrix metalloproteinase expression in human vein and microvascular endothelial cells. *J Biochem* 1993; **296:** 803–809.
8. Clark IM, Powell LK, Cawston TE. Tissue inhibitor of metalloproteinases (TIMP-1) stimulates the secretion of collagenase from human skin fibroblasts. *Biochem Biophys Res Commun* 1994; **203:** 874–880.
9. Moses MA, Langer R. A metalloproteinase inhibitor is an inhibitor of neovascularization. *J Cell Biochem* 1991; **47:** 230–235.
10. Murphy AN, Unsworth EJ, Stetler-Stevenson WG. Tissue inhibitor of metalloproteinases-2 inhibits bFGF-induced human microvascular endothelial cell proliferation. *J Cell Physiol* 1993; **157:** 351–358.
11. Hayakawa T, Yamashita K, Tanzawa K, Uchijama E, Iwata K. Growth-promoting activity of tissue inhibitor of the metalloproteinases (TIMP-1) for a wide range of cells. A possible new growth factor in serum. *FEBS Lett* 1992; **298:** 29–32.
12. Hayakawa T, Yamashita K, Shinagawa A. Cell growth-promoting activity of tissue inhibitor of metalloproteinases-2 (TIMP-2). *J Cell Sci* 1994; **107:** 2372–2379.
13. Bertaux B, Hornebeck W, Eisen AZ, Debertret L. Growth stimulation of human keratinocytes by tissue inhibitor of metalloproteinases. *J Invest Dermatol* 1991; **97:** 679–685.
14. Saarialho-Kere UK, Chang ES, Welgus HG, Parks WC. Distinct localization of collagenase and tissue inhibitor of metalloproteinases expression in wound healing associated with ulcerative pyogenic granuloma. *J Clin Invest* 1992; **90:** 1952–1957.
15. Leco KJ, Hayden LJ, Sharma RR, Rocheleau H, Grennberg AH, Edwards DR. Differential regulation of TIMP-1 and TIMP-2 mRNA expression in normal and Ha-*ras*-transformed murine fibroblasts. *Gene* 1992; **117:** 209–217.
16. Bullen EC, Longaker MT, Updike DL, *et al.* Tissue inhibitor of metalloproteinases-1 is decreased and activated gelatinases are increased in chronic wounds. *J Invest Dermatol* 1995; **104:** 236–240.
17. Stricklin G, Li L, Nanney LB. Localisation of mRNAs representing interstitial collagenase, 72-kDa gelatinase, and TIMP in healing porcine burn wounds. *J Invest Dermatol* 1994; **103:** 352–358.
18. Ligthart GJ, Corberand JX, Fournier C, *et al.* Admission criteria for immunogerontological studies in man: the SENIEUR protocol. *Mech Ageing Dev* 1984; **28:** 47–55.
19. Meikle MC, Hembry RM, Holley J, Horton C, McFarlane CG, Reynolds JJ. Immunolocalisation of matrix metalloproteinases and TIMP-1 (tissue inhibitor of metalloproteinases) in human gingival tissues from periodontitis patients. *J Periodont Res* 1994; **29:** 118–126.
20. Okada Y, Gonoji Y, Nakanishi I, Nagase H, Hayakawa T. Immunohistochemical demonstration of collagenase and tissue inhibitor of metalloproteinases (TIMP) in synovial lining cells of rheumatoid synovium. *Virchows Arch B [Cell Pathol Incl Mol Pathol]* 1990; **59:** 305–312.
21. Hoyhtya M, Fridman R, Komarek D, *et al.* Immunohistochemical localization of matrix metalloproteinase 2 and its specific inhibitor TIMP-2 in neoplastic tissues with monoclonal antibodies. *Int J Cancer* 1994; **56:** 500–505.
22. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* 1987; **162:** 156–159.
23. Tarnuzzer RW, Macauley SP, Farmerie WG, *et al.* Competitive RNA templates for detection and quantitation of growth factors, cytokines, extracellular matrix components and matrix metalloproteinases by RT-PCR. *Biotechniques* 1996; **20:** 670–674.
24. Peterson MJ, Woodley DT, Stricklin GP, O'Keefe EJ. Enhanced synthesis of collagenase by human keratinocytes cultured on type I or type IV collagen. *J Invest Dermatol* 1990; **94:** 341–346.
25. Ashcroft GS, Horan MA, Herrick SE, Tarnuzzer R, Schultz GS, Ferguson MWJ. Age-related differences in the temporal and spatial regulation of matrix metalloproteinases (MMPs) in normal skin and acute cutaneous wounds of healthy humans. *Cell Tissue Res* (in press).
26. Fenske NA, Lober CW. Structural and functional changes of normal aging skin. *J Am Acad Dermatol* 1986; **15:** 571–585.
27. Nomura T, Takahashi T, Hara K. Expression of TIMP-1, TIMP-2 and collagenase mRNA in periodontitis-affected human gingival tissue. *J Periodont Res* 1993; **28:** 354–362.
28. Craig P. Collagenase activity in cutaneous scars. *Hand* 1983; **5:** 239–241.
29. Nomura S, Hogan BLM, Wills AJ, Heath JK, Edwards DR. Developmental expression of tissue inhibitor of metalloproteinase (TIMP) RNA. *Development* 1989; **105:** 575–583.