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# Age-related differences in the temporal and spatial regulation of matrix metalloproteinases (MMPs) in normal skin and acute cutaneous wounds of healthy humans

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**Abstract.** Despite the association of increasing age with chronic wound-healing disorders and an impaired rate of healing of acute cutaneous wounds, the role of matrix metalloproteinases (MMPs) is unknown. To determine the spatial and temporal patterns and activities of MMP-1, -2, -3 and -9, 132 healthy humans aged between 19 and 96 years underwent 4-mm punch biopsies followed by wound excision between day 1 and day 180 post-wounding. Wounds showed an age-related increase in MMP-2 and MMP-9 immunostaining from day 3; this was associated with degradation of gelatin as shown by zymograms and with increased proteinase activity as shown by azocoll assays. Distinct spatial localisations for each MMP were observed: MMP-2 was found in epidermal structures; MMP-9 was observed in inflammatory cells up to day 21; MMP-1 was localised to keratinocytes at the wound margin. Normal old skin showed pro-MMP-2 bands on zymography and increased MMP-2 immunostaining. These results indicate that: (1) intrinsic ageing is associated with the up-regulation of MMPs previously associated with chronic wound healing; (2) wound-tissue proteinases are essentially active up to day 21 postwounding; and (3) intrinsic ageing may predispose to tissue breakdown disorders because of MMP-2 up-regulation in normal skin.

**Key words:** Ageing – Skin – Proteinase – Wound – Human

## Introduction

Wound healing is a highly regulated process, involving re-epithelialisation, matrix deposition and remodelling. The turnover of matrix components is an intrinsic component of the repair process necessary for initial wound debridement to remove necrotic cell and tissue debris. However, if tissue breakdown is excessive, new matrix is potentially degraded, resulting in an impaired rate of healing. The relative contribution of tissue breakdown to the delay in age-related wound healing is unknown.

The metalloproteinases are a family of at least 10 zinc-dependent endoproteinases thought to play a role in matrix remodelling (Woessner 1991; Mauviel 1993). Interstitial collagenase (MMP-1) cleaves native triple helical collagens, which are then susceptible to the gelatinases (MMP-2=72 kDa gelatinase and MMP-9=92 kDa gelatinase) that also cleave type IV and V collagens and elastin (Katsuda et al. 1994). Recently, MMP-2 has been shown to cleave native type I collagen (Aimes and Quigley 1995). A third group of proteinases are the stromelysins, of which MMP-3 is a member; these are not widely expressed but are readily induced by growth factors in cultured fibroblasts and act upon a variety of natural substrates, viz. proteoglycans, fibronectin, globular type IV collagen and laminin (Mauviel 1993).

Multiple levels of regulation in the expression and activation of MMPs are known and result in tight control during normal physiological processes. The accelerated breakdown of connective tissue in pathological states, such as periodontal disease and tumour invasion, appears to be a failure of the normal regulation of degradation processes (Murphy and Reynolds 1993). In addition, chronic wound-healing states are associated with raised wound-fluid levels of MMP-2 and MMP-9 (Wysocki et al. 1993; Bullen et al. 1995) and reduced levels of proteinase inhibitors (Bullen et al. 1995). Moreover, fluid from chronic wounds is capable of degrading matrix components such as fibronectin (Grinnell et al. 1992), the breakdown products of which can then act as proteinases for degrading laminin and gelatin (Planchenault et al. 1990).

Wound fluid does not necessarily reflect tissue levels of proteinases: its content may be correlated more closely with circulating levels or may vary significantly because of differential absorption/activation during the collection procedures. Thus, in vivo proteinase activity dur-

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ing wound healing is unknown. Moreover, wound fluid does not provide information regarding the spatial tissue localisation of proteinases, which has been reported to be differentially regulated for the various proteinase groups: MMP-2 has been localised to fibroblasts and MMP-9 to epidermal and endothelial cells (Oikarinen et al. 1993; Salo et al. 1994), whereas MMP-1 is associated with keratinocytes in healing cutaneous wounds (Porras-Reyes et al. 1991; Inoue et al. 1995). This is particularly significant as the composition of the extracellular matrix affects the proteinase profile. For example, MMP-1 synthesis by keratinocytes is enhanced by basement membrane type IV collagen, whereas MMP-9 is down-regulated (Peterson et al. 1990; Sarret et al. 1992). In this study, we have investigated (1) the temporal and spatial patterns of MMP-1, -2, -3 and -9 in normal human skin and in acute healing wounds of healthy subjects aged 19 to 96 years, by means of immunocytochemistry, and (2) the activities of these proteinases isolated from wound and normal tissue, by means of zymography and proteinase assays.

## 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 health-status-defined subjects of both sexes (screened by the SENIEUR protocol; Ligthart et al. 1984) aged between 19 years and 96 years of age were divided into three age-groups (19–39 years, young; 40–59 years, middle-aged; 60+ years, old) with equal numbers of males and females in each group. Subjects were non-smokers and receiving no medication, with no past medical history of note. Medical examination was in all cases normal and all subjects aged over 50 years had normal Chest X-Ray, electrocardiogram, 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% lignocaine was infiltrated into the skin. Two 4-mm punch biopsies (Steifel Labs, Bucks., UK) were taken from the anaesthetised area and haemostasis was maintained for 10 min. Each biopsy of normal skin was bisected; one-half was embedded in Optimal Cutting Temperature compound (OCT; Miles, Elkhart, Ind.), frozen over liquid nitrogen, and stored at –70°C, and one-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, UK) for 24 h and then left uncovered until re-biopsy.

At fixed time-points from day 1 to 6 months (days 1, 3, 7, 14, 21, 84 and 180) postwounding, the left upper inner arm was cleaned with isopropyl alcohol, elliptical excisions of the wounds were made following 1% lignocaine infiltration and two sutures were used to close the wound. Each 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 in the case of wound tissue, all excisions were made carefully to avoid any potential contamination with normal skin.

### *Immunocytochemistry*

Stepped sections (7 μm) were cut on a Leitz cryostat at –20°C, placed on poly-L-lysine-coated slides (Sigma, Poole, UK), fixed in acetone for 10 min, reacted with rabbit antihuman antibodies to active and pro-enzyme MMP-1, -2, -3 and -9 (Biogenesis, Poole, UK, used at dilutions of 1:100 (MMP-3 and -9), 1:10 (MMP-2) and 1:50 (MMP-1), for 18 h at 4°C, and then washed three times with phosphate-buffered saline (PBS). Swine anti-rabbit fluorescein isothiocyanate (FITC)-conjugated secondary antibody (DAKO, Bucks., UK) at a dilution of 1:40 in PBS was applied for 20 min, followed by three PBS washes. For dual immunostaining, primary antibody incubation was as described and then anti-rabbit tetramethylrhodamine isothiocyanate (TRITC)-conjugated antibody 1:40 (Sigma, Poole, UK) was applied for 20 min. Sections were washed three times with PBS, incubated for 1 h with mouse monoclonal anti-human LP9 (Serotec, Oxford, UK; monocytes/macrophages) or mouse monoclonal antihuman CD15 (Serotec; granulocytes), washed with PBS three times, incubated with goat anti-mouse IgM-FITC antibody 1:40 (Zymed, Cambs., UK) for 20 min, mounted in a non-fading medium containing DABCO (1,4-diazobicyclo-(2,2,2)-octane) and photographed by using a Leitz Aristoplan microscope under standard conditions. The intensity of staining, relative to normal, in each case was scored on the following scale by two observers "blinded" to the specimen identity: –5, no staining; –4, 0%–25%; –3, 26%–50%; –2, 51%–75%; –1, 76%–99%; 0, normal skin from each individual; 1, 101%–125%; 2, 126%–150%; 3, 151%–175%; 4, 176%–200%; 5, 200%+. This scoring system produced reproducible results in our hands. For each primary antibody, control sections were stained by using the above protocol but substituting PBS or 10% goat serum for the primary antibody; no background staining was produced. In all cases where regions devoid of immunoreactivity were found within wounds, adjacent areas of normal epidermis and dermis served as internal semi-quantitative controls for relative levels of staining intensity.

### *Gelatin zymography*

Gelatin zymography (Herron et al. 1986) was used to assess the pattern of proteinase production in tissue biopsies, by means of 9% acrylamide gels containing 0.1% gelatin (bloom 300, Sigma, Poole, UK). In this study, protein was extracted from wound tissue and normal skin (Weeks et al. 1976), in contrast to previous reports assessing proteinase activity in wound fluid. Protein extraction was carried out on fresh-frozen samples from days 1, 3, 7, 14, 21, 84 and 180 post-wounding and normal skin, and homogenised by using 1 ml homogeniser buffer (containing 6 M urea, 100 mM Tris/HCl buffer pH 7.6, 15 mM $CaCl_2$, 0.25% Triton X-100). Samples were spun at 11 000 *g* for 20 min at 4°C and the supernatent protein content assessed (Bradford 1976). Samples were subjected to sodium dodecylsulphate/polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970) under non-reducing conditions. Each lane was loaded with a volume equivalent to 10 μg protein/mg wet weight (loading the equivalent of 100 μg wet weight of tissue gave similar results). Separate lanes were loaded with standards, viz. 5–50 ng pro-enzymes MMP-1, -2, -3, -9 (Biogenesis, Bucks., UK), or pro-enzymes activated with 2 mM p-aminophenylmercuric acetate (APMA, Sigma) according to the manufacturer's instructions. After electrophoresis (Bio-Rad Mini-Protean apparatus), gels were washed for 1 h in 2.5% Triton X-100 to remove SDS, rinsed in distilled water and incubated overnight at 37°C in reaction buffer. Duplicate gels were incubated in an identical manner with the addition of the metalloproteinase inhibitor 10 mM EDTA (BDH, Poole, UK) or the serine proteinase inhibitor 1.7 mM AEBSF (aminoethylbenzenesulphonylfluoride, Sigma, Poole, UK). The gels were stained with 0.5% Coomassie brilliant blue for 1 h and destained for 30 min in Gel fix (10% acetic acid, 40% methanol, in distilled water). Areas of proteinase activity appeared as clear zones against a dark blue background. Broad-range molecular weight standards (Bio-rad, Herts., UK) were used for molecular mass markers. This method detected both pro- and active enzyme, even if they had bound TIMP prior to the SDS-PAGE step.

### *Western blotting*

Protein samples (extracted as described for zymography) and MMP-9 antigen (Biogenesis) were dissolved in non-reducing sample buffer, heated at 100°C for 3 min and subjected to SDS-PAGE on 9% acrylamide gels at 20 mA until the tracking dye reached the bottom of the gel. Parallel zymograms were run incorporating 0.1% gelatin and were processed as described previously. Gels were incubated in transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol, pH 8.3) for 30 min prior to blotting. Polypeptides were transferred to nitrocellulose paper (Bio-Rad, pore size 0.2 μm) at 20 mA for 30 min (Bio-Rad Blot transfer semi-dry system). Efficiency of transfer was assessed by incubating the blotted gel in 0.1% Coomassie brilliant blue for 1 h followed by 30 min in Gel fix. Blots were incubated with blocking solution (4% Marvel low-fat milk in 10 mM Tris, 150 mM NaCl, 0.5% Tween-20, pH 7.5) for 18 h at 4°C before incubation with mouse IgG anti-human MMP-9 (3 μg/ml ; Oncogene Science, Cambs., UK) for 2 h. The blots were washed 5 times with blocking solution and incubated with the secondary antibody (horseradish-peroxidase-conjugated goat anti-mouse; DAKO) at 1:3000 dilution in blocking solution for 1 h. Signals were visualised by using the ECL system (Amersham International, Bucks., UK).

### *Azocoll proteinase assay*

The azocoll method of assessing proteinase activity detects both metalloproteinases and serine proteinases (Chavira et al. 1984). Azocoll (CalBiochem, Notts., UK) was washed in buffer (50 mM Tris/HCl, 1 mM $CaCl_2$, pH 7.8) for 2 h and suspended in the same buffer at a final concentration of 5 mg/ml. Triplicate samples of 40 μg protein/mg wet weight (or 200 μg wet weight) tissue extracts were made up to a volume of 50 μl with buffer, mixed with 100 μl azocoll suspension and incubated for 21 h at 37°C with continuous shaking at 100 *g*. Parallel samples were activated with 2 mM APMA in dimethyl sulphoxide for 3 h prior to incubation with azocoll. Duplicate samples were incubated with 1.7 mM AEBSF (Sigma) or 10 mM EDTA. The incubation was stopped by centrifugation at 11 000 *g* for 8 min at 4°C. Absorbance ($OD_{520}$) of the supernatants was measured by means of a Milton Roy spectronic 201 spectrophotometer. A standard curve was produced by means of collagenase (clostridiopeptidase A, Sigma) with an enzyme activity of 347 U/mg solid.

### *Statistical analysis*

All data are presented as means+SD. Differences between means were evaluated by a three-factor ANOVA (all data followed a normal distribution to allow the ANOVA to be performed). In all circumstances, $P<0.05$ was considered to be significant.

## Results

### *Temporal and spatial patterns of pro-enzyme and active MMP -1, -2, -3 and -9*

In normal skin, MMP-1 was localised faintly in all layers of the epidermis and hair follicles. MMP-2 was just detectable in the basal epidermal layers, most prominently in the old age-group, around hair follicles and blood vessels throughout the dermis. MMP-3 immunoreactivity (-ir) was present in all epidermal layers, hair follicles and blood vessels in the papillary dermis and had a band-like reticular pattern in the papillary dermis. MMP-9 was observed in hair follicles and papillary blood vessels. Thus, in normal skin, MMP-2 staining increased with increasing age, with no age- or sex-associated changes being observed for MMP-1, -3 or -9 staining.

### *After wounding*

The age-related differences in the immunostaining data are shown in Table 1. At day 1, MMP-1-ir was prominent at the leading edge of the wound epidermis in all age-groups with faint diffuse staining in the clot. By

**Table 1.** Immunostaining of MMP-2 and 9 in wounded skin of males (*M*) and females (*F*) of various ages. Values represent means with *n*=3. Values stated as score+SD (SD=0 if not stated). No age-related changes in MMP-1 or -3 were observed. * Significant increase compared with young subjects,** significant increase compared with young and middle-aged, when using Tukey's critical range test to complement the three-way ANOVA (critical value 0.5 for MMP-2, 0.89 for MMP-9)

| MMP-2 | 20–39 years | | 40–59 years | | 60–96 years | |
|---|---|---|---|---|---|---|
| | F | M | F | M | F | M |
| Day1 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| Day 3 | 2 | 2 | 2.3+0.4* | 2.3+0.4* | 3+0.4** | 2.8+0.4** |
| Day 7 | 1 | 1 | 1 | 1 | 2.3+0.4* | 2* |
| Day 14 | 0.5 | 0.5 | 0.5 | 0.5 | 1.3+0.4* | 1* |
| Day 21 | 0.75+0.4 | 0.5 | 1 | 1 | 1 | 1 |
| Day 84 | 0 | 0 | 0 | 0 | 0.5* | 0.5+0.4* |
| Day 180 | 0 | 0 | 0 | 0 | 0.5* | 0.5* |

| MMP-9 | 20–39 years | | 40–59 years | | 60–96 years | |
|---|---|---|---|---|---|---|
| | F | M | F | M | F | M |
| Day1 | 0.5 | 0.5 | 0.5 | 0.5 | 1** | 1** |
| Day 3 | 2.5+0.7 | 3 | 2.8+0.4 | 3 | 4+0.7* | 4** |
| Day 7 | 2 | 2 | 2 | 2 | 3** | 3+0.4 |
| Day 14 | 2 | 2 | 2 | 2 | 3** | 3** |
| Day 21 | 0 | 0 | 0.3+0.4 | 0 | 1+0.4** | 0.75+0.4** |
| Day 84 | 0 | 0 | 0.3+0.4 | 0 | 1** | 0.5+0.4** |
| Day 180 | 0 | 0 | 0 | 0 | 0 | 0 |

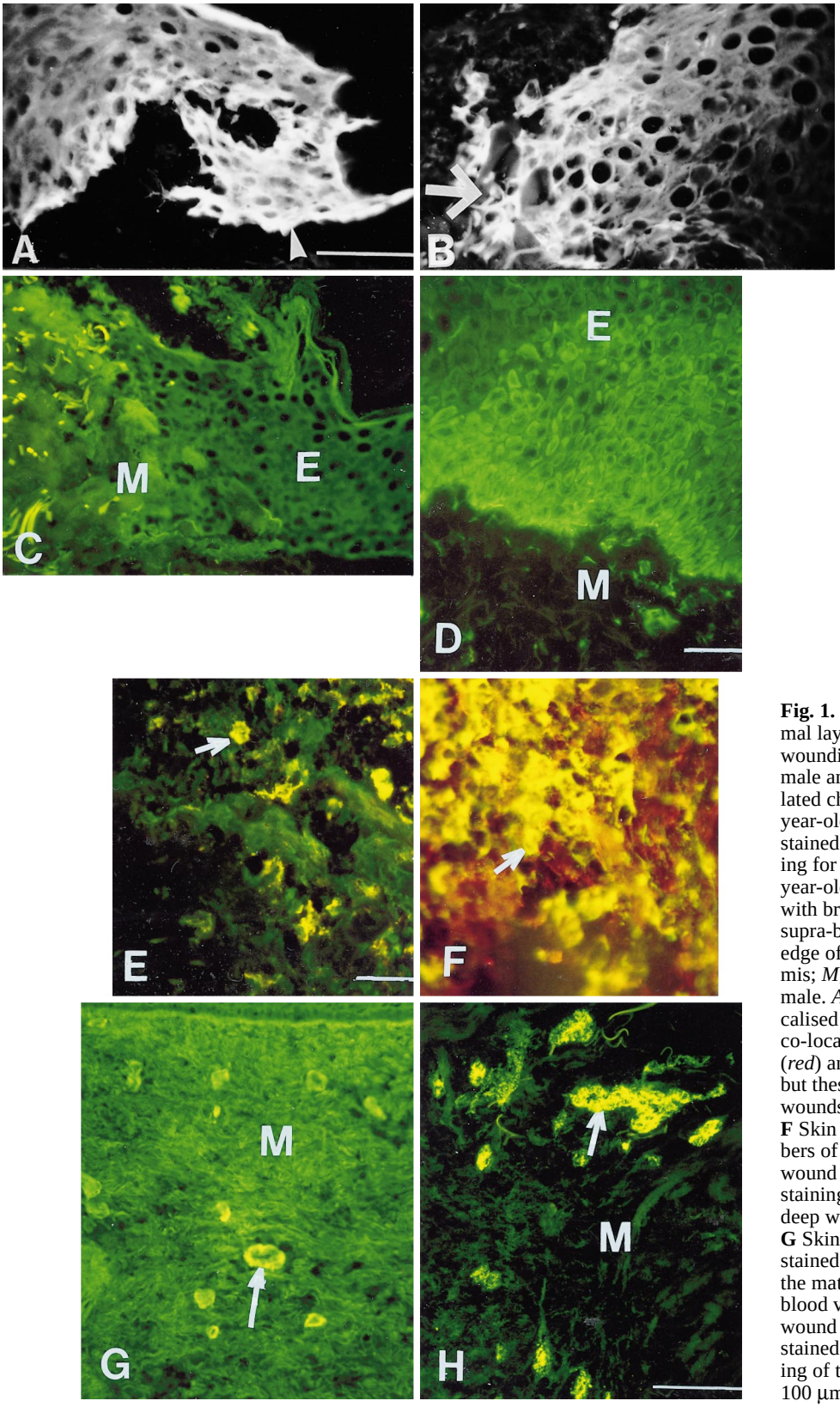


**Fig. 1.** Bright MMP-1 staining of all epidermal layers at the leading edge at day 3 post-wounding (*arrows*) in (**A**) an 82-year-old female and (**B**) a 25-year-old male. No age-related changes were noted. **C** Skin from a 24-year-old female 7 days post-wounding, stained for MMP-2 and with only faint staining for matrix MMP-2. **D** Skin from a 75-year-old female 7 days post-wounding and with bright staining for MMP-2 in basal and supra-basal epidermal cells at the leading edge of the migrating epidermis (*E*, epidermis; *M*, matrix). **E** Skin from a 25-year-old male. *Arrow,* CD15-positive cell with co-localised MMP-9 staining. Some cells exhibit co-localised (*yellow*) staining for MMP-9 (*red*) and CD15 cells (*green*) in the young but these are significantly fewer than in the wounds of the old at day 3 post-wounding. **F** Skin from a 92-year-old male. Large numbers of CD15-positive cells throughout the wound exhibit co-localisation with MMP-9 staining. Both sections were taken from the deep wound area above the subcutaneous fat. **G** Skin from a 72-year-old female at day 84, stained for MMP-3, illustrating staining of the matrix (*M*), and bright staining of the blood vessels (*arrow*). **H** Skin from the mid wound of a 72-year-old female (at day 84) stained for MMP-9, illustrating bright staining of the blood vessels (*arrow*). *Bars:* 100 μm

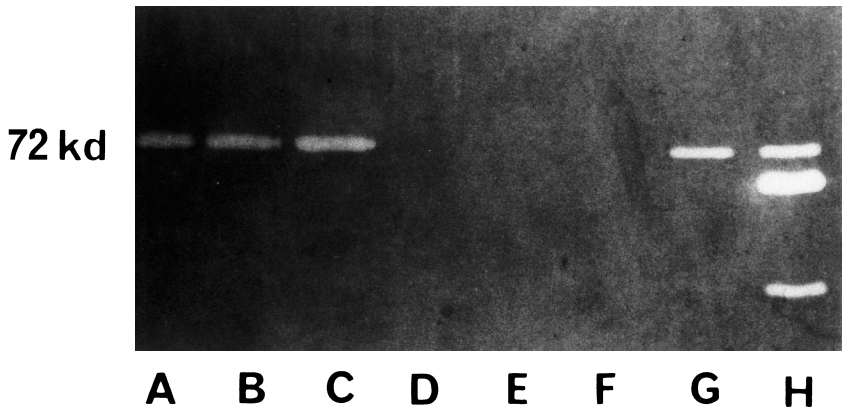


**Fig. 2.** Extract of normal old skin degrades gelatin revealing a 72-kDa band (*72 kd*); there is no activity in the skin of young subjects. Protein extracts from normal upper inner arm skin were run on gelatin zymograms. Proteinase activity was abolished by EDTA. *Lane A*, 96-year-old female; *lane B*, 82-year-old male; *lane C*, 83-year-old female; *lane D*, 25-year-old female; *lane E*, 50-year-old female; *lane F*, 23-year-old male; *lane G*, 65-year-old male; *lane H*, MMP-2 (pro-and active enzyme)

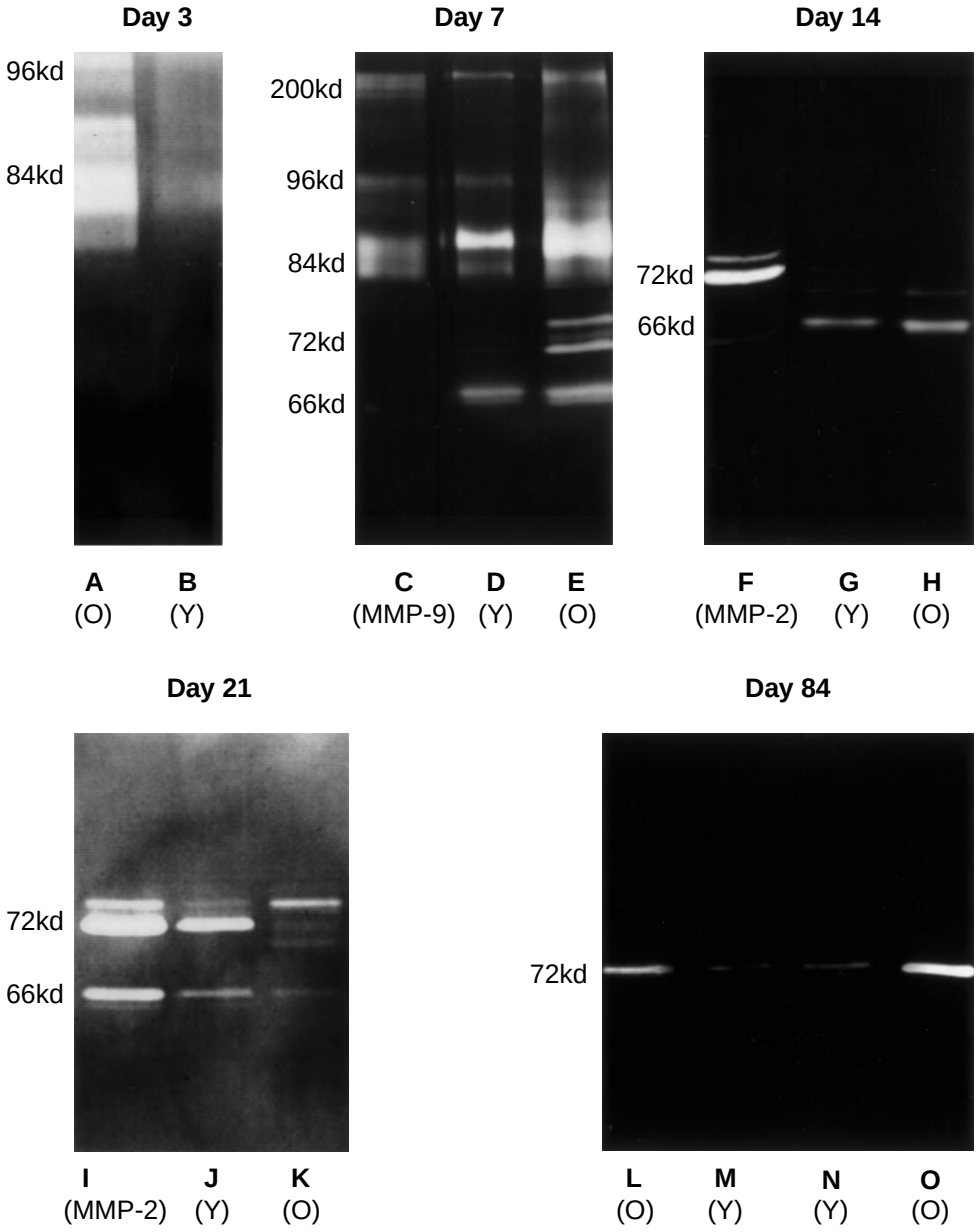


**Fig. 3.** Temporal pattern of proteinase activity obtained by using gelatin zymograms shows increased activity in the wounds of the old (*Y*, young subject; *O*, old subject; *M*, male; *F*, female). *Day 3*, Day 3 wound samples run on gelatin zymograms. *A*, 82-year-old female; *B*, 26-year-old female. Bands at 96 kDa (*96 kd*) and, most prominently, at 84 kDa (*84 kd*), corresponding to the MMP-9 active enzyme, were present with markedly increased intensity in the old. *Day 7*, Day 7 wounds. C, 5 ng MMP-9 antigen [pro-enzyme runs at 96 kDa (*96 kd*) and the active enzyme runs at 84 kDa (*84 kd*)]; *D*, 24-year-old female; *E*, 75-year-old female. Bands migrating with pro- and active MMP-9 (*96 kd*, *84 kd*) and MMP-2 (*72 kd*, *66 kd*) were most prominent in the wounds of the old. *Day 14*, Day 14 wounds. *F*, 5 ng MMP-2 antigen; *G*, 26-year-old female; *H*, 82-year-old female. Bands migrated with the active form of MMP-2 (*66 kd*). *Day 21*, Day 21 wounds. *I*, Pro- and active MMP-2; *J*, 25-year-old female; *K*, 78-year-old female with a transition to the pro-enzyme form of MMP-2 (*72 kd*). *Day 84*, Only the pro-enzyme band of MMP-2 was apparent (*72 kd*) with increased degradation by the wound tissue extracts from the old. *L*, 83-year-old female; *M*, 31-year-old female; *N*, 27-year-old male; *O*, 61-year-old male.

day 3, bright diffuse staining was apparent in the clot of all subjects and at the leading epidermal edge in all age-groups (Fig. 1A,B). At day 7, basal layers of the new epidermis stained brightly in all age-groups, with the clot staining in the old age-group only. From day 14 onwards, basal staining of the epidermis associated with complete re-epithelialisation was observed at all ages.

MMP-2 was localised to the clot and leading epidermal edge at day 1, at greater staining intensities than in normal skin, at all ages. At day 3, brighter staining of the clot was apparent in the old compared with the other age-groups; in addition, scattered macrophages stained brightly in the deep aspect of the wounds of young subjects. By day 7, the basal and suprabasal layers of the epidermal edge stained brightly in the old, with faint neo-epidermal staining in the young and middle-aged groups (Fig. 1C,D). At days 14 and 21, all layers of the new epidermis stained brightly in the old, with faint epidermal staining in the young and middle-aged, and with staining of dermal macrophages in the old subjects. By days 84 and 180 postwounding, basal and suprabasal epidermal layers stained at the wound site with faint perivascular staining increasing with subject age. In general, there was a significant increase in staining intensity with age (three-way ANOVA $P<0.0001$) and a greater decrease with time after wounding in young persons ($P<0.001$).

MMP-3-ir at the wound edge had a diffuse pattern in subjects of all ages at day 1, with faint cellular staining below the clot in the young. An increase of diffuse staining below the neoepidermis was observed at day 3, most prominently at the wound edge. A similar pattern was present at day 7 but with some cellular staining in all ages. By days 14 and 21, all age-groups displayed diffuse staining throughout the wound; this distinguished the latter from normal dermis. At day 84, diffuse staining was again present throughout the wound with blood vessels staining brightly at all ages (Fig. 1G) and faint cellular staining remaining only in the young group. By day 180, the cellular staining in the young subjects had disappeared.

MMP-9 was present in the clot at day 1 with more intense staining in the old subjects. By day 3, intense but diffuse staining of the clot for MMP-9 was apparent; this was far greater in the old subjects and co-localised with CD15 (granulocyte marker). The wounds of the young and middle-aged resembled those of the old at day 1. In the old subjects at day 3, neutrophils within the wound and at the wound edge stained brightly for MMP-9 (Fig. 1E,F), as did the clot and perivascular areas at the wound edge. By days 7 and 14, MMP-9-ir was diffuse throughout the clot in subjects of all ages but remained brighter than normal dermal staining, with cellular staining predominantly in macrophages, the latter peaking at day 21. At days 21 and 84, MMP-9-ir of blood vessels in the granulation tissue was apparent in subjects of all ages, with increased vessel staining in the old both at the wound edge and within the granulation tissue (Fig. 1H). The latter staining was most obvious in old females. By day 180, blood vessel staining was still present in all age-groups, and had a staining intensity similar to that of normal skin. Overall, a significant increase in MMP-9-ir occurred with subject age ($P<0.001$).

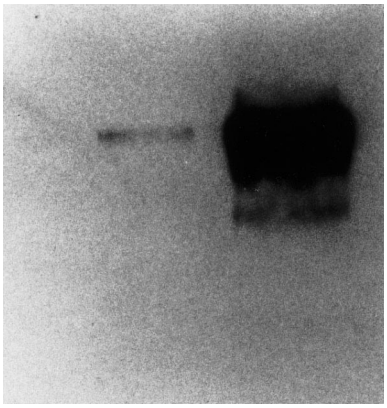


**Fig. 4.** Western blotting confirms MMP-9 as the proteinase degrading gelatin in the day 7 wounds of the old. The 84 kDa active form of the enzyme was detected only in the old wounds. Since this technique detected over 50 ng MMP-9 antigen (and not 5 ng), it implies that the tissue extracts from old subjects at day 7 contained over 50 ng MMP-9 and those from young subjects less than 50 ng. *A*, 25-year-old male; *B*, 92-year-old male; *C*, 500 ng MMP-9 antigen

*Increased MMP-2 activity in normal old skin with an increase in the activity of MMP-2 and -9 in acute wounds of old subjects*

Gelatin zymograms (Fig. 2) were degraded by protein extracts from normal skin in all 12 old subjects over 60 years (both sexes), in 2 (out of 12) middle-aged subjects (1 male and 1 female) and in 1 (out of 12) young subject aged 22 years (female). The band of lysis corresponded to the pro-enzyme form of MMP-2 (72 kDa gelatinase) and its activity was inhibited by EDTA but not AEBSF (data not shown). Tissue extracts from wounds at days 3 and 7 degraded gelatin with bands at 96 and 84 kDa migrating with the standard pro- and active MMP-9 antigen (5 ng) and large molecular weight complexes at 200 kDa (see Fig. 3). Activity was greater in the old subjects than in the young and middle-aged, with peak activity at day 7 post-wounding (Fig. 3), and was abolished by EDTA but not AEBSF. Immunoblotting (of the day 7 wounds only; Fig. 4) confirmed the bands as representing MMP-9. This technique could detect 50 ng and 500 ng, but not 5 ng, of MMP-9 antigen, indicating that more than 50 ng MMP-9 was present (per 10 μg/protein) in the old wounds at day 7. From day 7 post-wounding, gelatin was also degraded by 72 and 66 kDa bands that migrated with pro- and active MMP-2 standards (Fig. 3). Again, activity was greatest in old subjects. From day 14 to day 21, a transition occurred from the active to the pro-enzyme form of MMP-2. At days 84 and 180, gelatin was degraded in all subjects by a 72 kDa band migrating with pro-enzyme MMP-2 (Fig. 3) and was inhib-

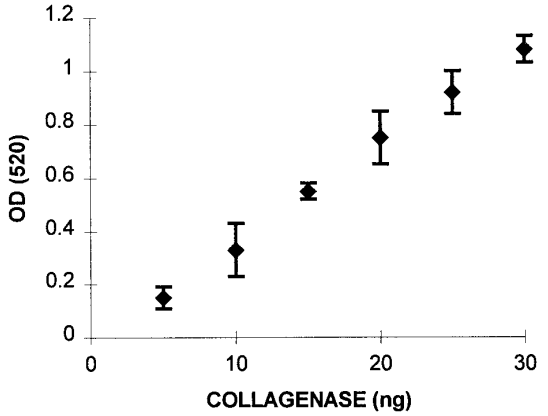


**Fig. 5.** Collagenase standard curve. Collagenase (Sigma) had an enzyme activity of 347 U/mg solid. Each OD unit represents 0.01 U of collagenase activity. Regression analysis $r$=0.92. Data shown are mean values +SD from triplicate samples

ited by EDTA but not AEBSF, confirming it as an MMP (data not shown). Identification of MMP-2 antigen by using immunoblotting with a 1:10 dilution of rabbit anti-human MMP-2 (Biogenesis) was not sensitive enough to localise tissue MMP-2: only 500 ng MMP-2 antigen could be detected by immunoblotting.

*Azocoll degradation: greatest in normal old skin and in the acute wounds of old females*

The azocoll assay produced a linear result with increasing concentrations of collagenase up to an $OD_{520}$ reading of 2, under the experimental conditions employed (Fig. 5). Proteinase activity of protein extracted from normal tissue and from tissue at day 3, 7, 14, 21, 84, and 180 post-wounding was determined by using the azocoll degradation method. This method is important when as-

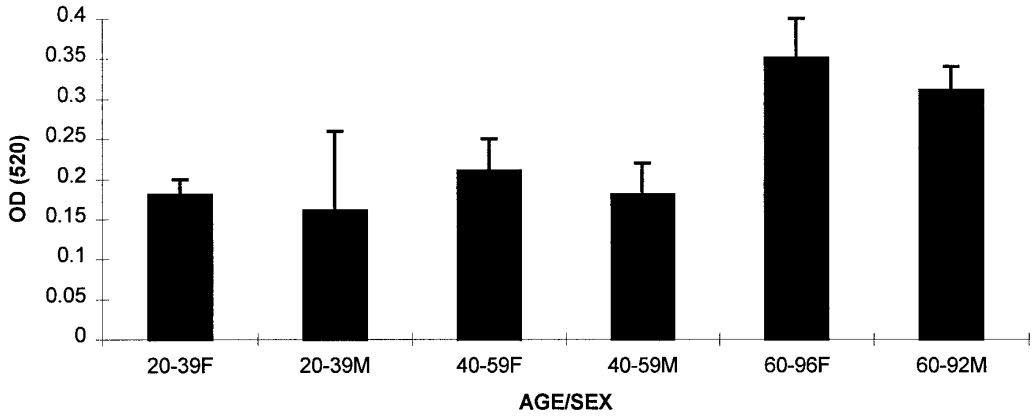


**Fig. 6.** Azocoll proteinase assay showing significantly increased activity in old non-sun-exposed skin following APMA activation. Proteinase activity of normal tissue following protein extraction was assessed by azocoll degradation. Data were corrected for background by using the buffer as a blank. Increasing age (combined for males and females) was associated with significantly increased proteinase activity ($P$=0.005). Prior to APMA activation, all samples gave optical density readings of less than 0.11 with no significant age or sex changes ($P$>0.5; data not shown). All activity was inhibited by EDTA. Data represent mean+SD. *M*, Male; *F*, female; *number*, age

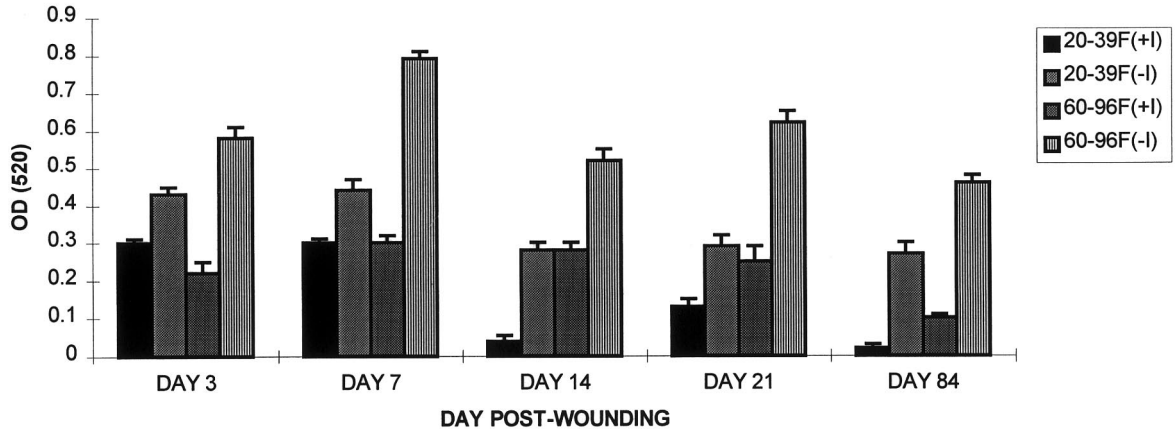


**Fig. 7.** Azocoll proteinase activity of acute wound extracts from female subjects on days 3 to 84 post-wounding activated with APMA, with and without MMP inhibitors, shows increased activity with subject age. Samples were incubated with azocoll, with (*+I*) and without (*–I*) the MMP inhibitor EDTA. Up to day 21 post-wounding, no significant increase in activity occurred following APMA activation. However, from day 21, no significant activity was present (<0.1 OD) in any sample prior to activation, indicating that, at day 21 post-wounding proteinases were in the pro-enzyme form. Data represent mean+SD

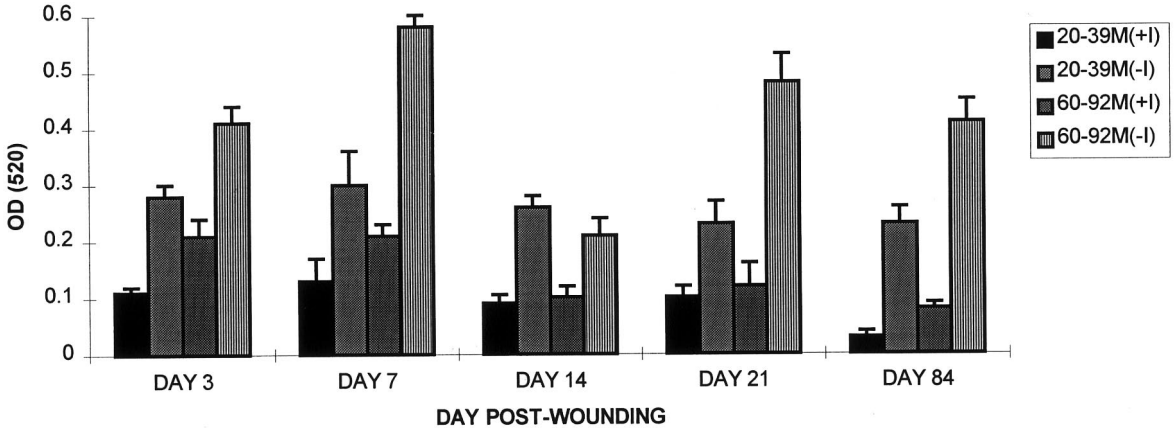


**Fig. 8.** Azocoll proteinase activity of acute wound extracts from male subjects on days 3 to 84 post-wounding activated with APMA, with and without MMP inhibitors, shows increased activity with subject age. Two protein samples from each age/wound-age group were activated with APMA, incubated with (*+I*) and without (*–I*) EDTA and analysed for proteinase activity. Up to day 21 post-wounding, no significant increase in activity occurred following APMA activation, but from day 21, no significant activity was present (<0.1 OD) in any sample prior to activation, indicating that, at day 21 post-wounding, proteinases were in the pro-enzyme form. In combination with the data from Fig. 7, MANOVA analysis of the combined results for both sexes showed that increasing age was associated with significantly increased activity ($P<0.0001$), with females having higher scores than males ($P=0.006$), and that EDTA had a highly significant effect ($P<0.0001$). Data represent mean+SD

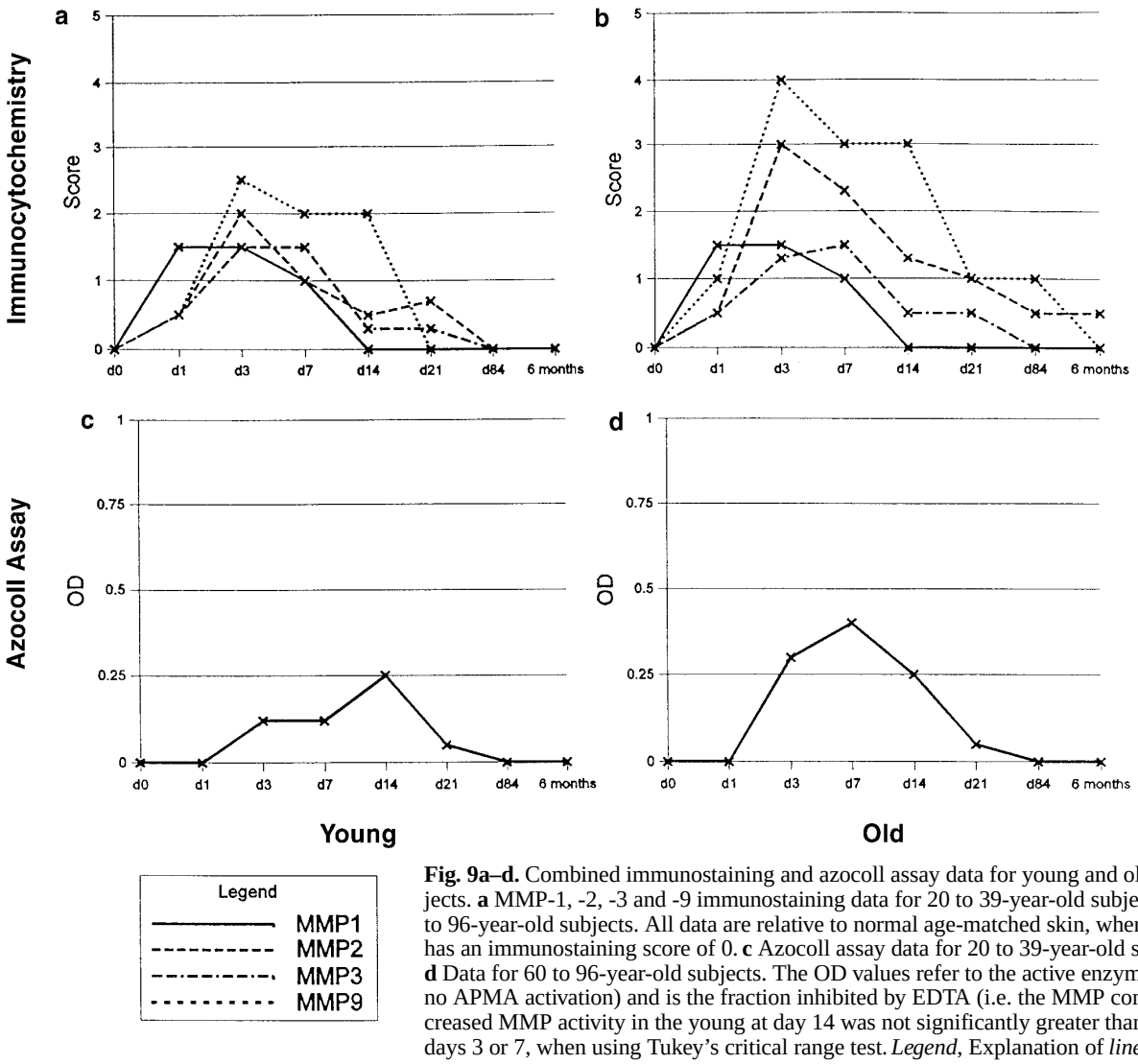


**Fig. 9a–d.** Combined immunostaining and azocoll assay data for young and old female subjects. **a** MMP-1, -2, -3 and -9 immunostaining data for 20 to 39-year-old subjects. **b** Data for 60 to 96-year-old subjects. All data are relative to normal age-matched skin, where normal skin has an immunostaining score of 0. **c** Azocoll assay data for 20 to 39-year-old subjects. **d** Data for 60 to 96-year-old subjects. The OD values refer to the active enzyme component (i.e. no APMA activation) and is the fraction inhibited by EDTA (i.e. the MMP component). The increased MMP activity in the young at day 14 was not significantly greater than the activity at days 3 or 7, when using Tukey's critical range test. *Legend*, Explanation of *lines* in **a–b**

sessing total in vivo proteinase activity, since zymography techniques may reveal degradation when the proteinase is bound in vivo to Tissue Inhibitor of Metalloproteinases. For normal skin, increasing age was associated with an increase in azocoll degradation only following APMA activation (Fig. 6). This indicated that proteinases were present in the pro-enzyme form in normal skin. Proteinase activity at all ages was inhibited completely by the metalloproteinase inhibitor EDTA but not by the general serine proteinase inhibitor AEBSF (data not shown).

Proteinase activity of wound samples increased with subject age at all time-points from day 3 post-wounding, with the exception of day 14 for old males (Figs. 7, 8). At day 1 post-wounding, activity was minimal for all samples (data not shown). Thereafter, the metalloproteinase inhibitor EDTA produced highly significant changes in proteinase activity (Figs. 7, 8) with marked proteinase activity remaining in the old age-group up to day 21. Proteinase activity remained even after EDTA treatment from day 3 to day 21, indicating that other classes of proteinases contributed significantly to wound proteinase activity. APMA activation had no significant increased effect on proteinase activity from day 3 to day 21 post-wounding in any age-group, implying that most proteinases were in the active form. From day 21 post-wounding, activity was only present after APMA activation, indicating that a shift to the pro-enzyme form of MMPs had occurred. At day 180 post-wounding, activity was similar to that of normal skin. The absolute MMP activity (the EDTA-inhibited fraction, prior to APMA activation) is shown in Fig. 9).

## Discussion

This study demonstrates that the metalloproteinases MMP-1, -2, -3 and -9 are all up-regulated at the protein level in acute healing of cutaneous wounds of healthy subjects, with MMP-9 being the most prominent. MMP-2 and -9 are both detected in terms of proteolytic activity from day 3 in acute wounds with up-regulation of MMP-9 protein from day 3 to day 14 in the young, and a greater and more prolonged increase in the aged from day 1 to day 84. Acute wound fluid has previously been reported to contain higher activity of MMP-9 than of MMP-2 (Wysocki et al. 1993; Salo et al. 1994), consistent with our data. Our study shows a more prolonged response than that seen in acute wound fluid from mastectomy patients, in whom MMP-9 is maximal at 48 h post-wounding (Wysocki et al. 1993). This discrepancy may represent the problems of using wound fluid as an indicator of in vivo tissue proteinase levels, in addition to site differences; wound-blister fluid levels of MMP-9 do not differ with age up to 86 years but the levels in lower limb blisters are only 19% of those found in abdominal blisters (Kylmaniemi et al. 1995).

Zymography reveals latent and active proteinase forms, even if they are bound to TIMP prior to SDS-PAGE and therefore, despite indicating whether the enzyme has undergone an activation step in vivo, the amount of activity such a form will possess in vivo when bound to TIMP is uncertain (Woessner 1995). Thus, the azocoll assay provides further information regarding the activity of the proteinase in vivo. Azocoll proteinase assays have confirmed an increase in proteinase activity from day 3 to day 21 post-wounding, with a significantly decreased activity as a result of MMP inhibition by EDTA at all time-points. The remaining activity is abolished by the serine proteinase inhibitor AEBSF, which has its greatest effect at days 3 and 7 post-wounding, indicating that serine proteinases are also important during early wound repair. The zymogram data clearly shows that both pro- and active-enzyme components are present; however, the pro-enzyme band is far weaker from day 3 to day 14 post-wounding and no substantial additional activity is observed when activation of the wound tissue extractions is performed. This contrasts with assays of acute and chronic wound-fluid proteinase activity in which only pro-enzyme forms of MMP-2 and -9 have been observed. Activation of pro-enzyme MMPs is thought to depend upon cleavage enzymes or β1-integrin receptors (Seltzer et al. 1994). MMP-1 and -3 and not MMP-2 or -9 (Brown et al. 1990) are activated by plasmin in vitro (Nagase et al. 1991), although MMP-2 can be activated by MT-MMP (membrane-type matrix metalloproteinase), neutrophil elastase and cathepsin G (Strongin et al. 1995; Okada and Nakanishi 1989). Increased early neutrophil infiltration observed in healthy old wounds (G.S. Ashcroft et al., unpublished data) may be important not only as a source of MMP-9, but also as a mechanism for activating MMP-2 in vivo. The shift from active to pro-enzyme seen at day 21 post-wounding in both the zymography and azocoll data suggests a down-regulatory mechanism heralding the switch from matrix degradation to deposition.

Connective tissue cells, macrophages and neutrophils store MMP-9 in granules (Kjeldson et al. 1994). Interestingly, MMP-9 is co-localised with CD15 cells in the wounds from old subjects, with a number of neutrophils not staining for MMP-9 in the young. At later time-points, macrophages stain for MMP-9 in the wounds of the old. Despite a greater number of macrophages in the wounds of the young from days 3 to 14 post-wounding, very few are co-localised with MMP-9. This suggests that inflammatory cells in old subjects contain different quantities of proteinases or that their activation states differ with an amplified response to proteinase production signals. This phenomenon warrants further investigation, as it may play a fundamental role in the enhanced levels of proteinases in chronic wounds.

MMP-9 is also present in blood vessels from day 21 post-wounding, as are MMP-3 and MMP-2. The process of angiogenesis is dependent upon the degradation of the basement membrane surrounding intact capillaries and MMPs have been localised to artificially stimulated or wound endothelium in a number of studies: rat brain capillary endothelial cells produce pro- MMP-1 and -3 on stimulation with phorbol ester (Herron et al. 1986) and MMP-9 is localised to endothelial cells in human blisters at 24 h post-wounding (Oikarinen et al. 1993). The increase in endothelial proteinase staining may be

important in age-related differences previously observed in angiogenesis, with a significant increase in angiogenesis in the wounds of the old, especially females, compared with the young (G.S. Ashcroft et al., unpublished observations). The increase in MMP-9 observed in old females is interesting as hormonal control of proteinases has been postulated: for example, oestrogen suppresses uterine collagenase levels in vivo (Woessner 1991), so that a reduction of oestrogen in post-menopausal women may contribute to the raised proteinase levels.

The increased staining for MMP-3 from day 3 post-wounding in all subjects can be correlated with the findings in human burn-wound fluid from day 4 post-burn (Young and Grinnell 1994). MMP-2 is localised to keratinocytes from initial re-epithelialisation onwards, with more intense staining in the old subjects. A peak of MMP-2 has previously been observed in fibroblasts following the induction of human suction blisters (Oikarinen et al. 1993). In our study, macrophages are the predominant cell-type staining for MMP-2 within the wound, possibly reflecting differences in specific wound models. Localisation of collagenase concurs with recent reports in human wounds, with a down-regulation in keratinocytes on complete re-epithelialisation (Inoue et al. 1995). Collagenase has also been localised to fibroblasts (Inoue et al. 1995) from days 5 to 7 post-wounding, to macrophages at day 3 post-wounding in rats (Porras-Reyes et al. 1991), and to endothelial cells during porcine burn-wound healing (Stricklin et al. 1994). The most probable explanations for these variations in distribution include differences in species, wound type or antibody specificity.

In this study, the extraction of proteinases from skin depends upon detergent (Triton) and urea (Weeks et al. 1976). For the extraction of collagenase, the heating of the pellet following Triton extraction has been reported to enhance the recovery of enzyme compared with the method used in this study, since collagenase is insoluble in Triton (Woessner 1995). However, gelatinases are often found complexed to TIMP, the latter being soluble in Triton, and thus extraction of gelatinases may be more efficient than that of collagenase. Moreover, the immunostaining results in this study closely reflect the zymography and proteinase assay data, suggesting that the extraction technique used in this study is useful for the assessment of skin proteinase activity.

Similarities can be seen in the proteinase and cellular profiles between the acute wounds of healthy old subjects and chronic wound fluid (Wysocki et al. 1993). The impaired rate of wound healing observed in normal ageing and in chronic wounds may be secondary to the breakdown of newly formed matrix. Moreover, not only is the age-related increase in pro-MMP-2 potentially important in wound repair, because it suggests that old skin is primed for tissue breakdown, but the up-regulation at the protein level may also be important in the changes observed in dermal collagen and elastin with age in sun-protected skin. Such changes include the disappearance of the dermo-epidermal arcades of elastin fibres (Lavker et al. 1989), a decrease in collagen content and an increase of insoluble collagen in thin, loosely woven bundles (Lavker et al. 1989; Maekawa et al. 1970). Interestingly, we have also shown that TIMP levels are down-regulated with age (G.S. Ashcroft et al., in preparation), further compounding the elevated proteinase activity. Therefore, age-related alterations in the control and activity of dermal proteinases may be important in the structural changes observed with age in the skin and in rendering old skin susceptible to a variety of pathologies resulting in impaired or chronic wound healing. Therapeutic or prophylactic manipulation of dermal proteinase activity is an attractive proposition for controlling or preventing these disorders.

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