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# Defective Extracellular Matrix Reorganization by Chronic Wound Fibroblasts is Associated with Alterations in TIMP-1, TIMP-2, and MMP-2 Activity

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**Chronic leg wounds are characterized by defective remodeling of the extracellular matrix, failure of reepithelialization, and prolonged inflammation. The hypothesis that this defective extracellular matrix remodeling is associated with phenotypic differences in the activity of the matrix metalloproteinases and tissue inhibitors of metalloproteinases was studied in chronic wound and patient-matched normal fibroblasts in three-dimensional collagen lattice systems. Chronic wound fibroblasts exhibited no differences in morphology or proliferation ($p > 0.1$) compared with patient-matched uninvolved dermal fibroblasts. The ability of chronic wound fibroblasts to reorganize extracellular matrix was significantly impaired, however, in comparison to the uninvolved dermal fibroblasts ($p < 0.01$). This difference in extracellular matrix reorganization was not related to differences in proliferation within the collagen lattices ($p > 0.05$) or attachment to type I collagen ($p > 0.1$). Marked differences were evident in matrix metalloproteinase-2 activity between chronic wound and patient-matched normal fibroblasts. Whereas levels of pro-matrix metalloproteinase-2 were similar between the two fibroblast populations ($p > 0.1$), the chronic wound fibroblasts exhibited significantly decreased levels of the 62 kDa active form of matrix metalloproteinase-2 ($p < 0.01$). Reverse zymography and enzyme-linked immunosorbent assay demonstrated that the decreased matrix metalloproteinase-2 activity was associated with increased production of tissue inhibitors of metalloproteinase-1 and -2 by the chronic wound fibroblasts ($p < 0.05$). Increased production of tissue inhibitors of metalloproteinases in chronic wound fibroblasts was also reflected in decreased levels of matrix metalloproteinase-1 ($p < 0.005$). These data suggest that the impaired ability of chronic wound fibroblasts to reorganize extracellular matrix *in vitro* is related to decreased levels of active matrix metalloproteinase-2 and matrix metalloproteinase-1 resulting from increased production of tissue inhibitors of metalloproteinase-1 and -2 by chronic wound fibroblasts. These findings provide a mechanism to explain the impaired cellular responses and extracellular matrix reorganization observed in chronic leg wounds *in vivo*.** ***Key words: chronic wound healing/extracellular matrix reorganization/fibroblast populated collagen lattice/matrix metalloproteinase. J Invest Dermatol 115:225–233, 2000***

Chronic wounds in the elderly are a major world health problem resulting in distress and disability and an increasing burden to health care providers (Ruckley, 1997). Chronic wounds encompass a spectrum of disease and exist in three principal forms (pressure sores, venous ulcers, and diabetic ulcers; Falanga, 1993). In normal wound healing, the regeneration of the epithelial and mesenchymal tissues of the skin is effected by keratinocytes and fibroblasts and is coordinated via complex cell-cell and cell-matrix interactions (Martin, 1997). These responses are altered in chronic wounds, however, with prolonged inflammation, a defective wound matrix, and a failure of reepithelialization (Herrick *et al*, 1992). Fibroblasts play a pivotal role in mediating wound healing responses, ranging from their synthesis and remodeling of the extracellular matrix (ECM) to the production of growth factors (Martin, 1997). The phenotype of fibroblasts repopulating experimental wounds *in vivo* has been shown to influence both wound healing responses and clinical outcome (Lorenz *et al*, 1992). Interestingly, it has been shown that fibroblasts from tissues that exhibit preferential wound healing *in vivo* (i.e., oral and fetal tissues) exhibit more rapid migration and an increased ECM reorganizational ability than do normal adult skin fibroblasts *in vitro* (Burd *et al*, 1991; Stephens *et al*, 1996; Ellis *et al*, 1997; al-Khateeb *et al*, 1997).

Workers have previously studied the phenotype of fibroblasts from elderly patients and chronic wounds themselves to determine if the impaired healing is related to alterations in fibroblast cellular phenotype within the wound (Hasan *et al*, 1997; Herrick *et al*, 1997; Stanley *et al*, 1997; Mendez *et al*, 1998; Vande Berg *et al*,

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Abbreviations: CM, conditioned medium; CWF, chronic wound fibroblast; FPCL, fibroblast-populated collagen lattice; F-SCM, fibroblast-serum-containing medium; MMP, matrix metalloproteinase; NBCS, newborn calf serum; NF, uninvolved dermal fibroblast; PDL, population doubling level; SA β-Gal, senescence-associated β-galactosidase; TIMP, tissue inhibitor of metalloproteinase.

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1998; Agren *et al*, 1999). Decreased population-doubling, proliferation, and increased fibroblast senescence have all been described in chronic wound fibroblasts (CWF) in monolayer culture (Stanley *et al*, 1997; Mendez *et al*, 1998; Vande Berg *et al*, 1998; Agren *et al*, 1999). It has also been suggested that this impaired proliferation explains the failure of refractory wounds to respond to treatment (Stanley *et al*, 1997; Vande Berg *et al*, 1998; Agren *et al*, 1999). These findings are not universal, however, and contrast with studies in three-dimensional collagen lattice systems (Herrick *et al*, 1996), the active cellular proliferation observed within these wounds, and clinical response to treatment (over 75% of these wounds heal with simple compressive therapy; Mayberry *et al*, 1991). Moreover, workers have shown that CWF synthesize comparable amounts of ECM to normal fibroblasts; suggesting that the defective wound matrix within chronic wound lesions may be due to differences in ECM remodeling within the wound site, possibly mediated by alterations in local matrix metalloproteinase (MMP) activity (Herrick *et al*, 1996).

MMPs play an important role in the regulation of cellular migration and ECM remodeling following injury (Tomasek *et al*, 1997; Deryugina *et al*, 1998; Shi *et al*, 1999). Interestingly, fibroblast MMP production and activation has been shown to be increased in tissues that exhibit increased rates of healing (i.e., fetal fibroblasts; Gould *et al*, 1997). In health, fibroblast MMP production and activation is regulated by a number of factors including expression of cytokines/growth factors (Ries and Petrides, 1995), mechanical properties of the ECM/actin cytoskeleton (Tomasek *et al*, 1997), and cell-matrix interactions (Azzam and Thompson, 1992). MMP activity is also partly regulated by the tissue inhibitors of metalloproteinases (TIMPs) and a number of proteinases including membrane-type MMPs (Nagase, 1997). The balance of MMPs and TIMPs following wounding appears crucial in directing successful wound repair (Reynolds, 1996).

ECM catabolism is known to be altered with fibroblast aging; increases in MMPs, plasminogen activator inhibitor 1, and TIMPs production have previously been demonstrated in normal aged fibroblasts (Zeng and Millis, 1994; West *et al*, 1996; Ashcroft *et al*, 1997a, b). Precisely how these age-related changes contribute to the pathogenesis of chronic wounds is currently unknown, as to date there have been no studies of patient-matched fibroblast/ECM reorganization in chronic leg ulcer patients. It is important to ascertain whether the previously described age-related changes in the skin contribute to the chronicity of these wounds and to determine whether a distinct (possibly reversible) phenotype of CWF can be identified.

In this study, using CWF and patient-matched normal fibroblasts, we sought to determine the ability of CWF to interact with, and remodel, the ECM. The association between ECM reorganization by these cell populations and their production and activation of MMPs and their inhibitors TIMP-1 and TIMP-2 was studied. It was shown that whereas CWF exhibit a similar cellular phenotype to patient-matched normal fibroblasts *in vitro*, they exhibit a markedly impaired ECM reorganizational ability associated with decreased levels of active MMP-2 and MMP-1. These altered MMP levels were associated with increased production of TIMP-1 and TIMP-2 by the CWF.

## MATERIALS AND METHODS

**Source and culture of human dermal fibroblasts** Cultures of CWF and patient-matched uninvolved dermal fibroblasts (NFs) were obtained with informed consent from patients with established venous leg ulcers attending the Wound Healing Clinic at the University Hospital of Wales, Cardiff. Patients with diabetes, systemic immunosuppression, or evidence of local infection were excluded from the study. Local ethical committee approval was obtained for this work. A 6 mm biopsy was taken from the chronic wound bed and the uninvolved outer aspect of the ipsilateral thigh. Cultures were established by a single-cell suspension technique following enzymatic degradation of the specimens as previously described (Stephens *et al*, 1996). Briefly, tissue was incubated overnight with dispase (2 mg per ml; Boehringer Mannheim, Lewes, U.K.) to separate epidermal tissue from the dermal tissue. Wound-bed tissue, although lacking epidermis, was treated identically. Dermal tissue specimens were then disaggregated overnight utilizing bacterial *Clostridium histolyticum* A collagenase (1 mg per ml; Boehringer Mannheim). Fibroblast cultures were maintained in fibroblast-serum-containing medium (F-SCM) containing Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (2 mM), nonessential amino acids (1 ×), antibiotics (100 U per ml penicillin G, 100 mg per ml streptomycin sulfate, 0.25 mg per ml amphotericin B) and 5% (vol/vol) newborn calf serum (NBCS). Reagents, unless specified, were all obtained from Life Technologies, Paisley, U.K. The cultures were maintained at 37°C in a 5% $CO_2$ humidified atmosphere. At confluency, fibroblasts were split at a ratio of 1:3. Cells were utilized for all experiments between passages (P) 3 and 9.

**Assay of fibroblast proliferation in monolayers** Fibroblast proliferation in monolayers was assayed using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) dye-reduction assay (Mosmann, 1983). Fibroblasts were recovered from culture by trypsinization and placed in 96 well microtiter plates at a cell density of $5 \times 10^3$ cells per well in F-SCM. After 24 or 72 h 25 μl of MTT (5 mg per ml; Sigma, Poole, U.K.) was added to each well and the plates were incubated for 4 h at 37°C. A hundred microliters of extraction buffer [10% (wt/vol) sodium dodecyl sulfate (SDS)/0.5 M dimethylformamide; Sigma] was then added to each well and the plates were incubated overnight at 37°C. The absorbance of each well was then assessed using a Dynex MRX spectrophotometer (Dynex Technologies, Billinghurst, U.K.) equipped with a 550 nm filter. Statistical analysis was performed using Student's *t* test.

**Senescence-associated β-galactosidase (SA β-Gal) staining** SA β-Gal staining was performed as described previously (Dimri *et al*, 1995). Briefly, passage-matched (P 3–9) fibroblasts were cultured as monolayers or in collagen lattices. Fibroblasts that had been aged *in vitro* (> P 13) and grown to over-confluence were utilized in these experiments as positive controls. Cells were fixed in 4% (vol/vol) formaldehyde for 15 min and then incubated for 12–16 h at 37°C in SA β-Gal staining solution [1 mg per ml 5-bromo-4-chloro-3-indolyl β-D-galactosidase (X-Gal), 40 mM citric acid (pH 6.0), 40 mM sodium phosphate (pH 6.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM sodium chloride, 2 mM magnesium chloride; all from Sigma]. Senescent fibroblasts were identified as stained blue cells by standard light microscopy and the percentage of positive staining cells was quantified by direct counting.

**Fabrication of fibroblast-populated collagen lattices (FPCLs)** Fibroblasts derived from culture by trypsinization were utilized to construct FPCLs. The fibroblasts were resuspended in medium (F-SCM) prepared with NBCS that had been pretreated with Gelatin-A Sepharose (1 part Gelatin-A Sepharose to 5 parts NBCS; Amersham Pharmacia Biotech, St. Albans, U.K.) to remove endogenous MMP-2 and MMP-9 (Azzam and Thompson, 1992). Type I rat-tail collagen was extracted as described previously (Rowling *et al*, 1990). Fibroblasts ($1 \times 10^6$ in 0.75 ml of F-SCM) were added to 53 mm bacteriologic grade plates containing 3 ml of 2 × DMEM [40 parts 10 × DMEM, 10 parts $NaHCO_3$ (7.5% wt/vol), four parts L-glutamine (200 mM), four parts nonessential amino acids (100×), 140 parts $H_2O$, and five parts NaOH (1 M)], 0.75 ml of 0.1 M NaOH, 0.75 ml of Gelatin-A Sepharose pretreated NBCS, and 2.25 ml of 1.7 mg per ml rat-tail type I collagen (a total lattice volume of 7.5 ml). The FPCLs were incubated at 37°C for 60 min to allow the collagen to polymerize. They were then detached from the edge of the plate and 2 ml of F-SCM (Gelatin-A Sepharose pretreated) was added. FPCLs were maintained at 37°C in a 5% $CO_2$ humidified atmosphere.

**Measurement of FPCL contraction and collection of conditioned medium (CM)** As a circular shape is retained during contraction the diameter of the contracting FPCL was measured from day 0 to day 7. The degree of cell-mediated contraction was estimated from three separate lattice diameter measurements. For each sample, experiments were performed in triplicate. On days 1, 2, 3, and 7 after initial fabrication 400 μl of CM surrounding the lattices was collected from each individual FPCL, combined, and stored at −20°C for future analysis. Four hundred microliters of F-SCM (Gelatin-A Sepharose pretreated) was added to replace that collected. Statistical analysis was performed using Student's *t* test.

**Assay of fibroblast proliferation in FPCLs** Fibroblast proliferation in the collagen lattices was assessed using "scaled-down" FPCLs (containing

$2 \times 10^4$ cells in a total lattice volume of 200 μl) fabricated in 24 well plates coated (to render them nonadherent) with 1% (wt/vol) bovine serum albumin (Sigma). Fibroblasts were recovered from five combined FPCLs by enzymatic degradation as previously described (Stephens *et al*, 1996). Briefly lattices were solubilized by incubation for 60 min at 37°C with 200 μl of phosphate-buffered saline (PBS) containing 2 mg per ml collagenase. Following incubation for 20 min at 37°C with 100 μl of 0.05% (wt/vol) trypsin/0.53 mM ethylenediaminetetraacetic acid, cells were recovered by centrifugation and viable cell numbers were determined by direct counting in a Neubauer hemocytometer using a 0.02% (wt/vol) Trypan Blue Solution (Sigma). Statistical analysis was performed using Student's *t* test.

**Cell attachment assay** Cell attachment assays were performed as described previously (Aumailley *et al*, 1989). Wells of a 96 well microtiter plate were incubated overnight at 4°C with a 40 μg per ml solution of rat-tail type I collagen, and then nonspecific binding was blocked by incubation with 1% (wt/vol) bovine serum albumin for 4 h at 4°C. Fibroblasts were trypsinized and resuspended in complete growth medium lacking NBCS at a concentration of $1.5 \times 10^5$ cells per ml. A hundred microliters of cell suspension was added to individual wells and the plate was incubated for 60 min at 37°C in a 5% $CO_2$ humidified atmosphere. Nonadherent cells were removed by aspiration and the remaining adherent cells were washed (PBS), fixed with ethanol (70% vol/vol; 15 min), and stained with crystal violet (0.1% wt/vol; 25 min; Sigma). Excess dye was removed by washing (× 5) with distilled water and the stain was solubilized using 25 μl of 0.2% (vol/vol) Triton X-100 (Sigma). The absorbance was read using a Dynex MRX spectrophotometer equipped with a 550 nm filter. Statistical analysis was performed using Student's *t* test.

**Total cellular RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR)** On days 1, 2, 3, and 7 FPCLs were removed from culture, blotted on 3 mm filter paper (5 min), snap-frozen in liquid nitrogen, and stored at –70°C. Total cellular RNA was isolated by a technique based on the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). FPCLs were disaggregated mechanically in the presence of Ultraspec RNA solution (Biotecx Laboratories, Houston, TX). Total RNA was then extracted with chloroform (Sigma) and precipitated using isopropanol (Sigma). RNA concentrations were determined by spectrophotometry and samples were stored at –70°C for future analysis.

cDNA was synthesized in 25 μl volumes containing ≈1 μg of total RNA (preheated at 95°C for 4 min), 1 × PCR buffer, 1.5 mM dNTPs, 2 U per μl RNasin, 0.12 μg per μl random hexamer primers, 4 mM dithiothreitol and 12 U per μl Moloney murine leukemia virus reverse transcriptase (all reagents were purchased from Promega, Uppsala, Sweden). The reaction was incubated at 25°C for 10 min, 42°C for 60 min, and 95°C for 5 min.

DNA amplification was carried out in 25 μl volumes containing 2 μl of the reverse transcriptase reaction, 1 pM 3′ and 5′ PCR primers [specific for MMP-1, MMP-2, TIMP-1, TIMP-2 (Tarnuzzer *et al*, 1996) or MMP-13 (Ravanti *et al*, 1999)], 1 × PCR buffer, 0.5 mM dNTPs, and 0.05 U per μl reaction of *Taq* DNA polymerase. Amplification of the DNA was carried out by incubation for one cycle at 94°C for 2 min followed by 40 cycles of denaturation (94°C, 30 s for MMP-1, MMP-2, TIMP-1, or TIMP-2; 95°C, 90 s for MMP-13), annealing (55°C, 30 s for MMP-1, MMP-2, TIMP-1, or TIMP-2; 61°C, 90 s for MMP-13), and extension (68°C, 75 s for MMP-1, MMP-2, TIMP-1, or TIMP-2; 72°C, 2 min for MMP-13), and finally one cycle at 68°C for 15 min. Negative controls included the substitution of $H_2O$ in place of the RNA and the omission of the reverse transcriptase step prior to PCR amplification. PCR products were separated on 1.5% (wt/vol) agarose gels and visualized by ethidium bromide staining and ultraviolet illumination; images of the stained gels were captured with a Bio-Rad gel documentation system (Bio-Rad, Hemel Hempstead, U.K.).

**Zymographic analysis of CM** To determine the relative amounts of pro-enzyme and active MMP species produced by the fibroblasts in the FPCL systems gelatin zymography was employed. Equal volumes (15 μl) of CM were electrophoresed through gelatin (2 mg per ml; Sigma) containing 12% (vol/vol) SDS-polyacrylamide gels. SDS was removed by soaking the gels in 2.5% (vol/vol) Triton X-100 (60 min at room temperature), and MMPs were activated by incubation in activation buffer [25 mM Tris (pH 7.6), 5 mM $CaCl_2$, 25 mM NaCl, 5% wt/vol Brij 35] overnight at 37°C. Gels were stained with Coomassie Blue (0.05% wt/vol Coomassie Blue, 12% vol/vol acetic acid, 54% vol/vol methanol), and destained (5% vol/vol methanol, 7.5% vol/vol acetic acid), and images of the gels were captured with a Bio-Rad gel documentation system. MMPs were identified by the appearance of clear bands and by calculation of relative molecular weights. Quantitation of the MMP bands was undertaken using the Bio-Rad Image Analyst software. Experiments involving activation of the MMPs with the organomercurial agent aminophenylmercuric acid were undertaken by incubation of the CM sample with aminophenylmercuric acid (2 mM; Sigma) for 2 h at 37°C prior to electrophoresis. Controls involved running normal, nonconditioned F-SCM to establish that any observed gelatinolytic activity was not due to the presence of endogenous MMPs within the F-SCM.

**MMP and TIMP enzyme-linked immunosorbent assays (ELISAs)** MMP-1, MMP-13, TIMP-1, and TIMP-2 Biotrak™ ELISAs (Amersham Pharmacia Biotech) were performed according to the manufacturers' instructions. Briefly, MMP or TIMP standards and CM samples (appropriately diluted) were added to the ELISA plates and incubated at 20°C–27°C for 1–2 h (dependent upon the specific ELISA). Wells were then aspirated and washed (×4) with wash buffer; horseradish peroxidase conjugate was added and the plates were incubated as before. Following aspiration and washing (×4), 3,3′,5,5′-tetrabenzidine substrate was added to each well and the plates were re-incubated for 30 min. The reaction was stopped by the addition of 1 M sulfuric acid and absorbances were read, within 30 min, using a Dynex MRX spectrophotometer equipped with a 450 nm filter. Calculation of the relative amounts of MMPs (-1 and -13) and TIMPs (-1 and -2) in the CM samples was made by comparison with the respective standard curves.

**Reverse zymography of fibroblast CM** Reverse zymography was employed to determine the relative amounts of the TIMP species produced by the fibroblasts in the FPCL systems. Reverse zymography was performed in a similar manner to zymography except that purified MMP-2 (30 μg per ml; Coughlan *et al*, 1998) was incorporated into a 14% (vol/vol) SDS-polyacrylamide gel along with 1 mg per ml gelatin. TIMPs were identified by the appearance of dark bands and by calculation of relative molecular weights. Quantitation of the TIMP bands was undertaken using the Bio-Rad Image Analyst software. Controls involved running normal, nonconditioned F-SCM.

## RESULTS

**CWF do not exhibit morphologic or histochemical evidence of increased senescence** Fibroblasts were successfully established from all of the biopsies (n = 4) taken from the wound base of chronic venous leg ulcers and from patient-matched uninvolved skin. At low passage [≤ P 9 or population doubling level (PDL) ≤ 25] CWF were morphologically similar to the NFs, in both monolayer and three-dimensional collagen lattice culture, having a typical elongated, bipolar appearance with little evidence of prominent stress fibers or cells stained positive for SA β-Gal (**Fig 1*a*, *b***). At these passages/PDLs there were no differences in the number of cells exhibiting the typical perinuclear blue SA β-Gal staining between normal and patient-matched CWF in monolayer culture (mean positive staining, 1.6% of cells). These findings contrasted with the morphologic appearance and staining pattern of the high-passage, over-confluent fibroblasts (**Fig 1*c***; mean positive staining, 19.6% of cells). Moreover, using the MTT assay system, it was shown that, whereas all fibroblast populations studied exhibited proliferation *in vitro*, there were no significant differences in proliferation between CWF and NFs (**Fig 2**; $p > 0.1$).

**CWF exhibit a decreased ECM reorganizational ability compared with patient-matched NFs, independent of proliferation or cellular attachment to type I collagen** CWF and NFs were seeded into type I collagen lattices and contraction was observed over 7 d. Reorganization of the lattice was characterized by an initial rapid phase of lattice reorganization followed by a plateau phase when maximal reorganization had been achieved (**Fig 3**). CWF demonstrated a significantly decreased initial rate of ECM reorganization and maximal contraction of the FPCL compared with NFs (n = 3; $p < 0.01$). Such differences in ECM reorganization may, in part, be explained by a difference in the observed ability of fibroblasts to proliferate within the FPCL or to attach to the ECM substrate. There were no significant differences in cell number, however, between CWF and NFs within collagen lattices at any time point (**Fig 4**; $p > 0.05$). In keeping with previous studies using these

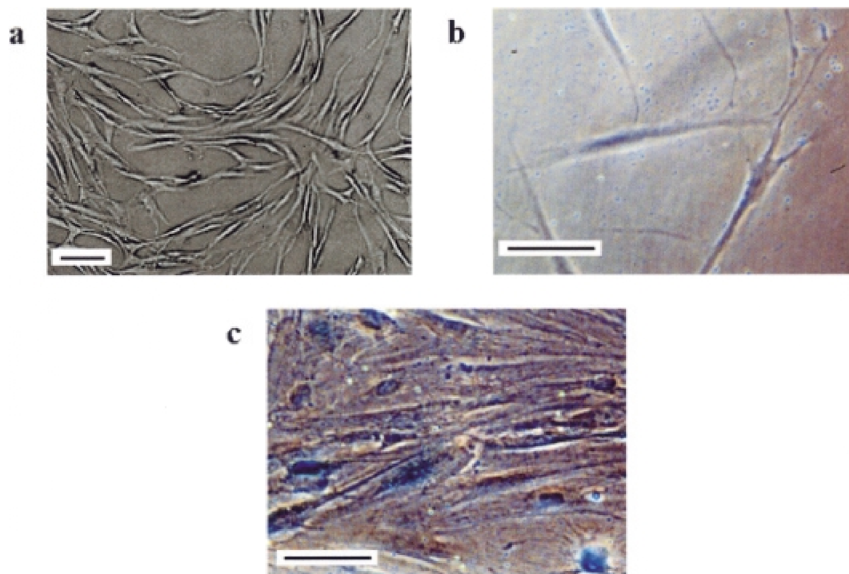


**Figure 1. CWF were not senescent and were similar in morphology to normal dermal fibroblasts at low passage.** (*a*), (*b*) CWF (P 5) demonstrate normal fibroblast morphology. (*b*) CWF showed a typical lack of SA β-Gal staining in contrast to (*c*) high-passage, over-confluent normal dermal fibroblasts (positive control). *Scale bars*: 50 μm.

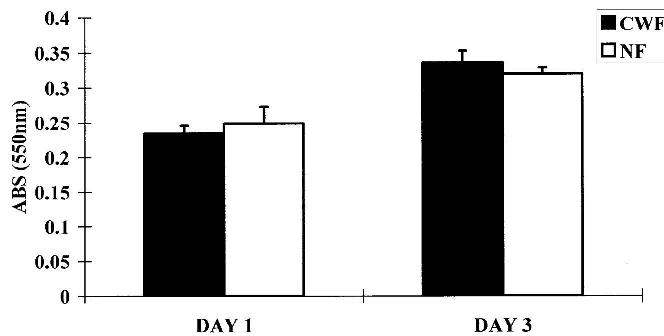


**Figure 2. CWF showed no difference in proliferation compared with patient-matched NFs.** CWF and patient-matched NFs were placed in 96 well microtiter plates at a density of $5 \times 10^3$ cells per well and incubated for 24 or 72 h. Proliferation was assessed by the MTT dye-reduction assay. Values represent the mean ± SEM (n = 3).

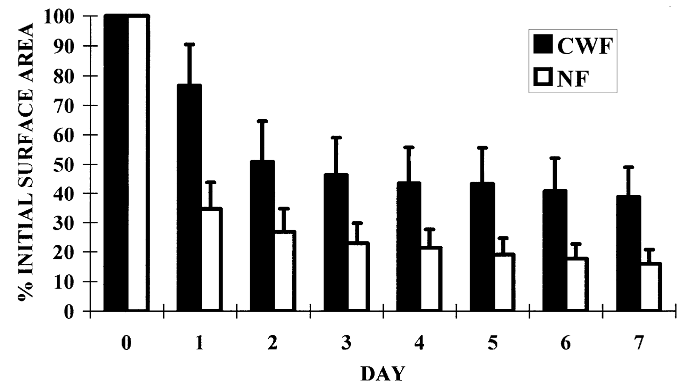


**Figure 3. Phenotypic variation in type I collagen lattice contraction by CWF and patient-matched NFs.** Cultured CWF and patient-matched NFs were seeded into type I collagen lattices (starting collagen concentration 1.7 mg per ml) at a cell density of $1 \times 10^6$ cells per 53 mm bacteriologic grade plate. Diametric contraction was measured over a period of 7 d. Values represent the mean ± SEM (n = 3).

floating collagen lattice systems, an initial decrease in cell number (0–72 h) was followed by a plateau period to the end of the experiment at day 7 (Nakagawa *et al*, 1989; Fluck *et al*, 1998). There were similarly no differences in the ability of CWF and NFs to attach to type I collagen, the principal ECM component of the FPCL (data not shown; $p > 0.1$).

**CM from chronic wound FPCLs contains less active MMP-2 than that from patient-matched uninvolved dermal FPCLs** MMP-2 plays an important role in the turnover of the ECM and has been demonstrated to play a role in *in vitro* collagen lattice reorganization (Tomasek *et al*, 1997; Deryugina *et al*, 1998). RNA was isolated from FPCLs seeded with CWF and NFs at days 1, 2, 3, and 7 and analyzed for the expression of MMP-2 by RT-PCR. Expression of MMP-2 by both CWF and NFs was evident at 24 h (**Fig 5*a***) and continued to the end of the investigation (data not shown).

Having established that MMP-2 was expressed by the fibroblasts within the lattice systems, MMP-2 protein levels were studied. Zymographic analysis of CM from FPCLs seeded with CWF and NFs at days 1, 2, 3, and 7 demonstrated that the 72 kDa pro-enzyme form of MMP-2 was evident at 24 h and increased in both CWF and NF CM over the 7 d period of study (**Fig 5*b***). Interestingly, the 62 kDa active form of MMP-2 was typically not evident in either CM until 72 h. The pro-enzyme form of MMP-9 (92 kDa) was an inconsistent finding in both the CWF and NF

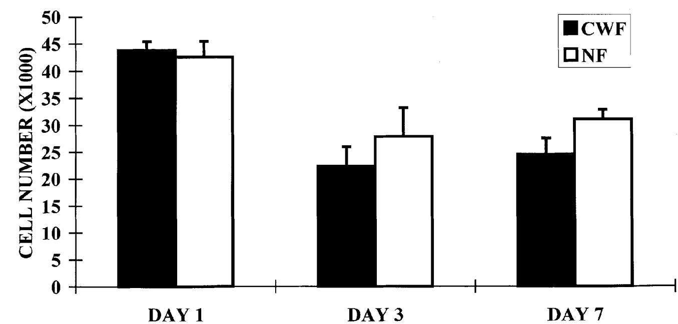


**Figure 4. CWF cultured in a three-dimensional environment showed no difference in cell number compared with patient-matched NFs.** Cultured CWF and patient-matched NFs were seeded into "scaled down" type I collagen lattices at a cell density of $2 \times 10^4$ cells per well of a 24 well plate. At 24, 72, and 168 h five lattices were pooled and subjected to collagenase digestion, and viable cell counts were performed. Values represent the mean ± SEM (n = 3).

CM. Analysis of CM at day 7 showed that, whereas there were no apparent differences in the production of the pro-enzyme form of

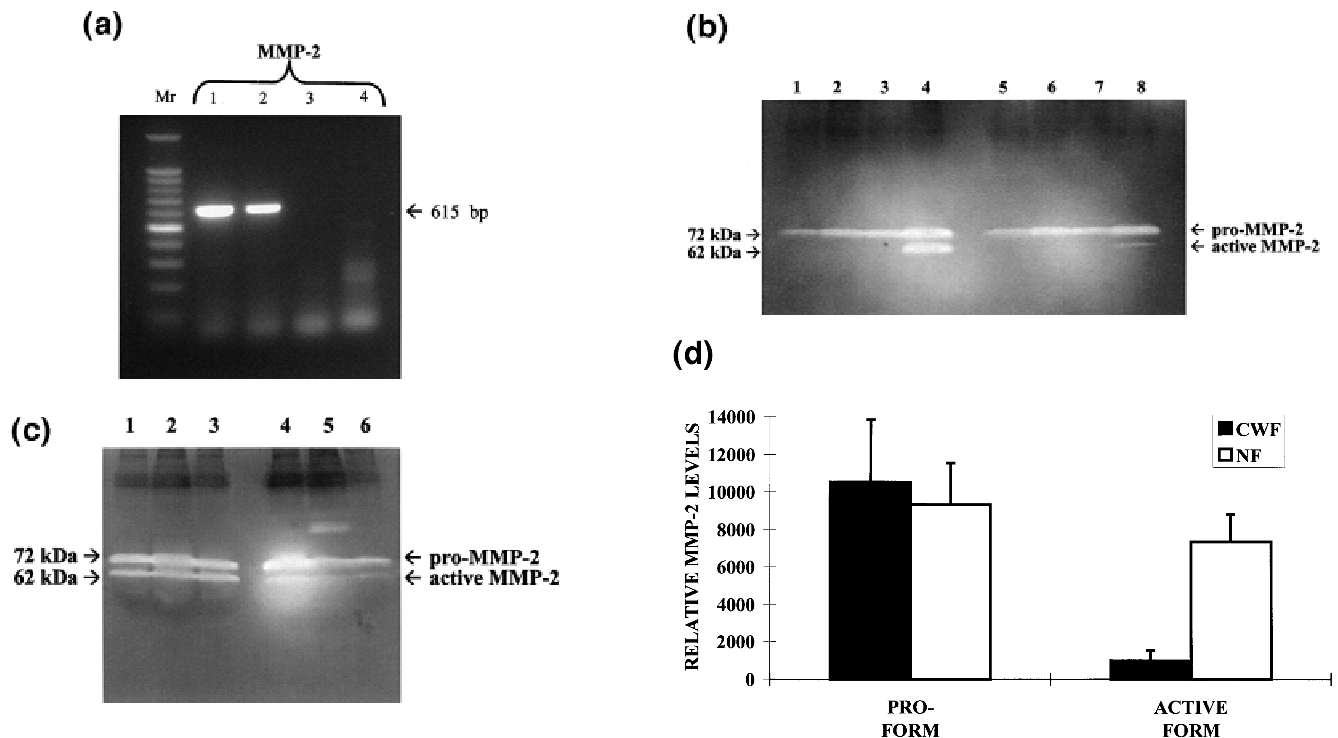


**Figure 5. CWF demonstrate lower active MMP-2 levels than patient-matched NFs.** (*a*) Expression of the MMP-2 gene within FPCLs assessed by RT-PCR. Both CWF and NFs expressed the MMP-2 gene in type I collagen lattices: *lane 1*, NFs; *lane 2*, patient-matched CWF (CWF); *lanes 3–4*; negative controls for NFs and CWF. (*b*) Zymography of fibroblast CM: *lanes 1–4*, CM from NFs (days 1, 2, 3, and 7, respectively); *lanes 5–8*, CM from patient-matched CWF at the same time points. The time-dependent increase in the levels of active 62 kDa MMP-2 was greater in NF CM than that observed in patient-matched CWF CM. (*c*) Zymography of day 7 fibroblast CM from different (n = 3) matched samples: *lanes 1–3*, CM from the NFs; *lanes 4–6*, CM from patient-matched CWF. Levels of the 72 kDa pro-form of MMP-2 were similar in CWF and NF CM. The 62 kDa active form of MMP-2 was clearly decreased in the CWF CM. (*d*) Densitometric analysis of relative pro-enzyme and active MMP-2 levels in CM from CWF and patient-matched NFs after 7 d in culture in a type I collagen lattice. Values represent the mean ± SEM (n = 3).

MMP-2 between CWF and NFs, there were significant differences in the amount of the activated form of this MMP (**Fig 5*c***). Although semiquantitative densitometric analysis of the zymograms showed no differences in pro-MMP-2 levels between NFs and CWF CM ($p > 0.1$), CWF exhibited significantly lower levels of active MMP-2 ($p < 0.01$) than their patient-matched uninvolved dermal counterparts (**Fig 5*d***).

**Differences in CWF and patient-matched NF active MMP-2 are related to differences in active TIMP-1 and TIMP-2 levels** TIMPs play an important role in the turnover of the ECM in that they regulate the activities of the MMPs. RNA was isolated from FPCLs seeded with CWF and NFs at days 1, 2, 3, and 7 and analyzed for the expression of TIMP-1 and TIMP-2 by RT-PCR. Expression of TIMP-1 and TIMP-2 by both CWF and NFs was evident at day 1 (**Fig 6*a*, *b***) and continued to the end of the investigation (data not shown).

Having established that TIMPs were expressed by the fibroblasts within the lattice systems, TIMP protein levels were also studied. Reverse zymographic analysis of CM from FPCLs seeded with NFs at days 1, 2, 3, and 7 demonstrated time-dependent increases in the levels of TIMP-1 and TIMP-2 (which were particularly evident in the initial 48 h; **Fig 6*c***). Contrastingly, in the CWF, whereas the levels of TIMP-1 increased, those of TIMP-2 remained relatively constant throughout the 7 d. This was due to the increased levels of both TIMP-1 and TIMP-2 activity evident in the CM from CWF at 24 h (**Fig 6*d*, *e***; $p < 0.05$). These findings were confirmed using commercial, specific ELISAs that showed increased levels of TIMP-1 and TIMP-2 protein in CWF CM at day 1 (**Fig 6*f***; $p < 0.05$) and confirmed the time-dependent increase in TIMP production by normal fibroblasts observed by reverse zymography ($p < 0.05$; data not shown).

**Increased TIMP-1 and TIMP-2 levels in CWF are associated with decreased MMP-1 production** The demonstration of differences in TIMP-1 and TIMP-2 activity led to the investigation of the expression and production of the fibroblast collagenases MMP-1 and MMP-13 by the CWF and NFs in these systems. Both CWF and NFs expressed MMP-1 and MMP-13 in the lattice systems (**Fig 7*a*, *b***). Interestingly, the increased levels of TIMP-1 and TIMP-2 by CWF were mirrored by decreased MMP-1 production by these cells. ELISA demonstrated that at day 7 MMP-1 protein production was significantly decreased in medium conditioned by CWF compared with NFs (**Fig 7*c***; $p < 0.005$). Whereas MMP-13 was expressed by fibroblasts in these systems, the protein levels of MMP-13 were not detectable by ELISA in either the CWF or NF CM at any time point over the 7 d period of the investigation (data not shown).

## DISCUSSION

Distinct phenotypic differences exist between fibroblasts derived from chronic wounds and normal skin fibroblasts in the same patients. The use of patient-matched fibroblasts was deemed important as age- and patient-specific variations in fibroblast cellular and biosynthetic responses have been extensively demonstrated (Ashcroft *et al*, 1997a; al-Khateeb *et al*, 1997). These studies failed to demonstrate any evidence to support the concept that CWF are senescent. Whereas there is a generalized increase in cellular senescent changes in the normal skin of aged individuals (Faragher and Kipling, 1998), at low passage/PDL the growth and morphologic characteristics of the CWF and patient-matched normal fibroblasts were similar. No differences were evident between CWF and the normal fibroblasts with respect to proliferation, cell morphology, or cellular senescence (in monolayer or collagen lattices). These findings contrast with some (Stanley *et al*,

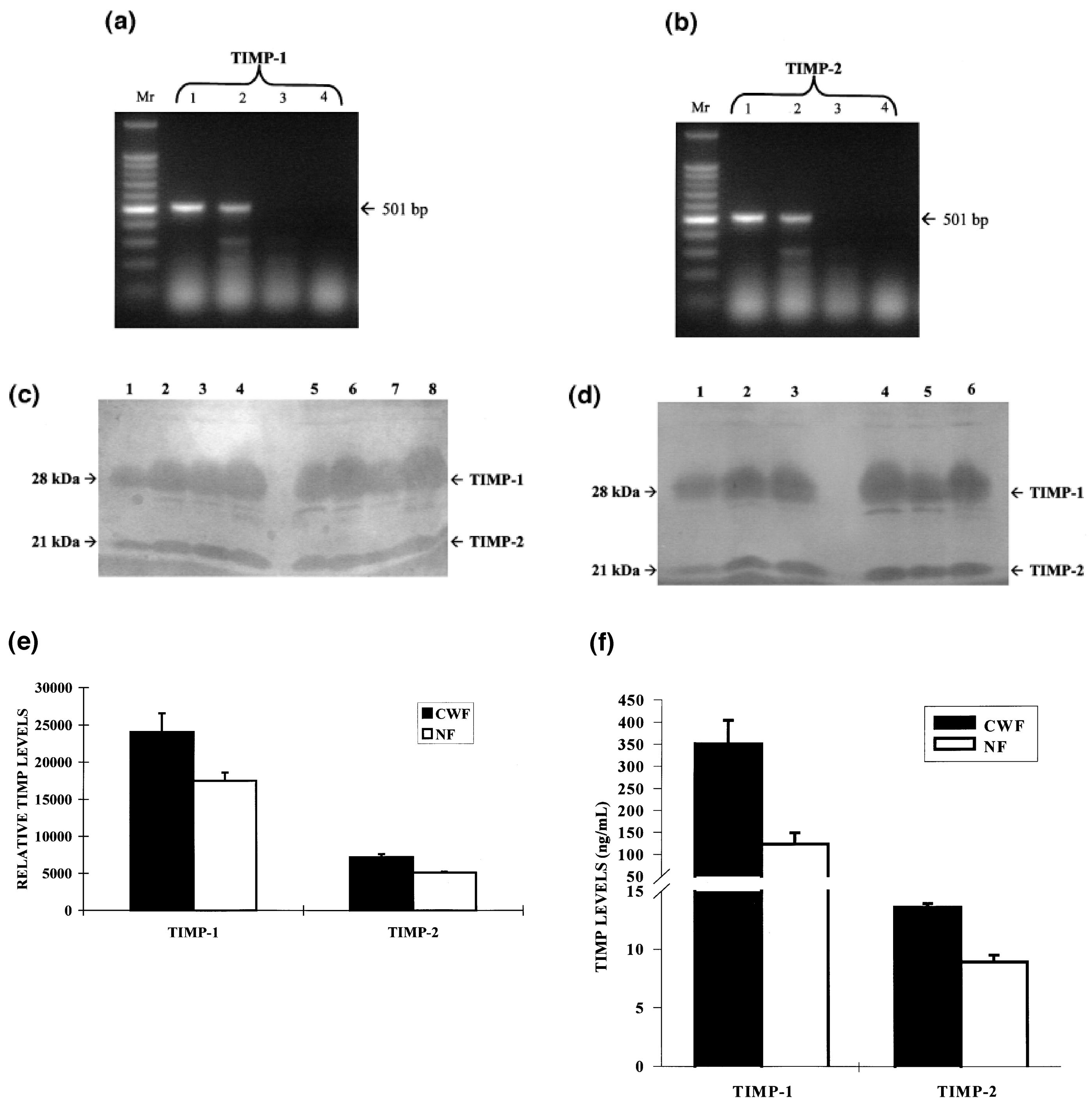


**Figure 6. Expression, activity, and protein production of TIMP-1 and TIMP-2 within FPCLs assessed by RT-PCR reverse zymography and ELISA.** (*a*) TIMP-1; (*b*) TIMP-2. Both CWF and normal fibroblasts, when cultured in type I collagen lattices, expressed the TIMP-1 and the TIMP-2 genes: *lane 1*, NFs; *lane 2*; patient-matched CWF (CWF); *lanes 3–4*, negative controls for NFs and CWF. (*c*) Reverse zymography of fibroblast CM: *lanes 1–4*, CM from NFs (days 1, 2, 3, and 7, respectively); *lanes 5–8*, CM from patient-matched CWF at the same time points. Levels of NF TIMP-1 (28 kDa) and TIMP-2 (21 kDa) showed a time-dependent increase over the 7 d. In comparison with patient-matched normal fibroblasts, CWF demonstrated increased TIMP-1 and TIMP-2 levels at 24 h. (*d*) Reverse zymography of day 1 fibroblast CM from different (n = 3) matched samples: *lanes 1–3*, CM from the NFs; *lanes 4–6*, CM from patient-matched CWF. Increased levels of both TIMP-1 (28 kDa) and TIMP-2 (21 kDa) were evident in CWF CM compared with NF CM at day 1. (*e*) Densitometric analysis of relative TIMP-1 and TIMP-2 levels in CM from CWF and patient-matched NFs after 1 d in culture in a type I collagen lattice. (*f*) Relative TIMP-1 and TIMP-2 protein levels assessed by ELISA. Equal volumes of day 1 CM from CWF and patient-matched normal fibroblasts were analyzed using Biotrak™ ELISAs. Values represent the mean ± SEM (n = 3).

1997; Mendez *et al*, 1998; Agren *et al*, 1999) but not all previous studies (Herrick *et al*, 1997; Phillips *et al*, 1998). The differences may relate, in part, to the cell culture techniques employed. In these studies enzymatic dissociation of the biopsies was employed whereas previous studies have used explant culture to isolate fibroblasts from the biopsies (Stanley *et al*, 1997; Mendez *et al*, 1998; Vande Berg *et al*, 1998; Agren *et al*, 1999). Although the passage number of the cells utilized in our experiments is similar to that in previous studies, the lower cell yield in explant culture will have resulted in cell populations of a considerably higher PDL than in this study. Furthermore, Vande Berg *et al* (1998) showed that fibroblast senescence was not uniform and varied with biopsy site

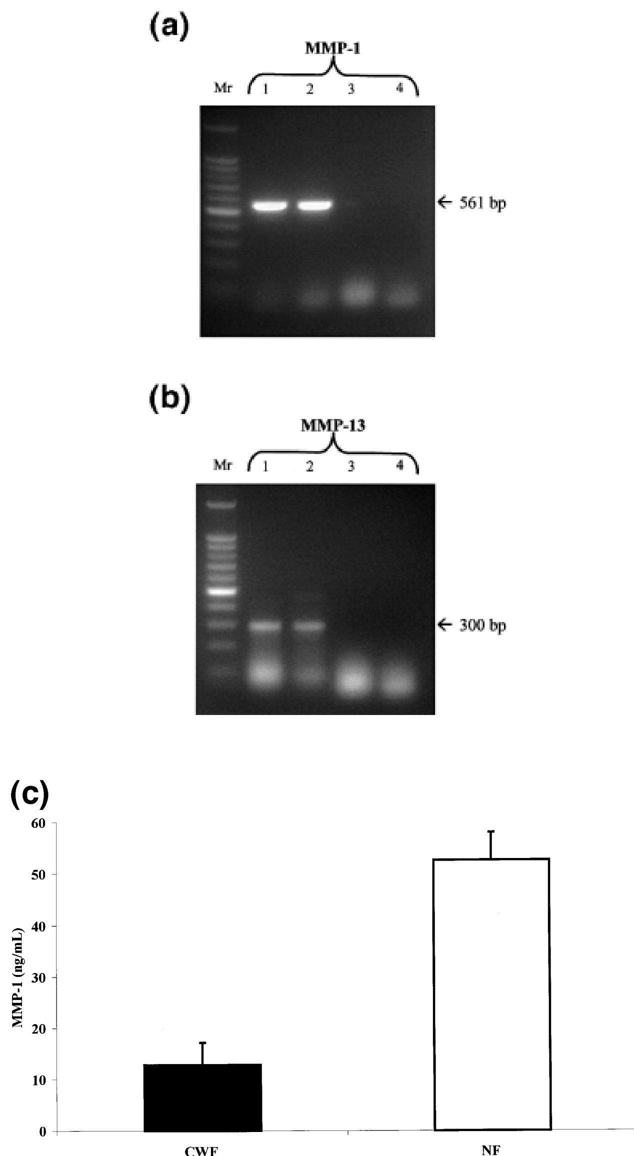


**Figure 7. Expression and protein production of MMP-1 and MMP-13 within FPCLs assessed by RT-PCR and ELISA.** (*a*) MMP-1 and (*b*) MMP-13. Both CWF and normal fibroblasts, when cultured in type I collagen lattices, expressed the MMP-1 and the MMP-13 genes: *lane 1*, NFs; *lane 2*, patient-matched CWF (CWF); *lanes 3–4*, negative controls for NFs and CWF. (*c*) Relative MMP-1 protein levels assessed by ELISA. Equal volumes of day 7 CM from CWF and patient-matched normal fibroblasts were analyzed using a Biotrak MMP-1 ELISA. Values represent the mean ± SEM (n = 4).

within the ulcer, but all of the specimens here were derived from the same site in the ulcer base.

Workers have suggested that this nonproliferative senescent cellular phenotype is important in mediating failure of response to treatment (Mendez *et al*, 1998; Vande Berg *et al*, 1998). It should be remembered that over 75% of these wounds heal with conventional therapy (Mayberry *et al*, 1991) and that the fibroblasts in these patients are obviously capable of effecting matrix synthesis, remodeling, and ultimately wound healing. Moreover, the notion that the chronic wound bed is an inert site of biologic unresponsiveness is difficult to attribute to fibroblast senescence as the number of senescent fibroblasts in wound biopsies is low (Mendez *et al*, 1998).

Although the CWF showed no difference in cellular growth or morphologic phenotype, the ability of CWF to reorganize an ECM was markedly impaired compared with patient-matched normal fibroblasts. Previously published work on FPCL systems suggests that fibroblast ability to reorganize/remodel the ECM *in vitro* is directly related to the clinical pattern of wound healing observed *in vivo*. Fibroblasts derived from sites that exhibit preferential healing (e.g., fetal skin, oral mucosa) demonstrate increased abilities to reorganize ECM and contract the FPCL systems (Burd *et al*, 1991; Stephens *et al*, 1996). This decreased ECM reorganization by CWF is in keeping with the defective healing and remodeling processes observed *in vivo* in chronic wounds. In support of this notion, it has been shown that the preferential ability of oral mucosal fibroblasts to reorganize FPCLs is reflected in their increased ability to migrate through ECM and to repopulate experimental *in vitro* wounds (Stephens *et al*, 1996; al-Khateeb *et al*, 1997).

The precise mechanism of ECM reorganization in FPCL systems is unclear and a number of theories exist (Grinnell, 1994; Deryugina *et al*, 1998). In keeping with our previous studies (Stephens *et al*, 1996), we were able to clearly show that phenotypic differences in ECM reorganization were not related to differences in either proliferation or attachment to the type I collagen substrate of the lattice. The role of MMPs in mediating FPCL contraction has attracted considerable attention (Seltzer *et al*, 1994; Tomasek *et al*, 1997; Deryugina *et al*, 1998; Haas *et al*, 1998). It has been shown that MMPs are upregulated (in the three-dimensional mechanical environment of the lattice) via occupancy of β1 integrins (Tomasek *et al*, 1997) and that FPCL reorganization is, in part, dependent upon both membrane type 1 MMP (MT1-MMP) mediated activation of MMP-2 and cell–surface association of activated MMP-2 (Deryugina *et al*, 1998). Moreover, FPCL contraction can be effectively inhibited by the addition of recombinant TIMP-2 (Deryugina *et al*, 1998) and tetracycline-based MMP inhibitors (Myers and Wolowacz, 1998). MMP-2 production and activation by the CWF and patient-matched fibroblasts was, therefore, studied.

Gelatin zymography demonstrated that CWF exhibited significantly decreased levels of the active form of MMP-2 compared with normal fibroblasts. This compliments studies in which it has been shown that the rapid wound repopulation and repair by fetal fibroblasts (observed both *in vivo* and *in vitro*) is associated with increased levels of active MMP-2 production by the fetal fibroblasts (Gould *et al*, 1997). Whereas alterations in MMPs, TIMPs, and ECM catabolism have been extensively demonstrated in aged fibroblasts (Bell *et al*, 1979; Millis *et al*, 1992; Zeng *et al*, 1994; West *et al*, 1996; Ashcroft *et al*, 1997a, b) we demonstrate here that the CWF exhibit a phenotype distinct from their patient- and, therefore, age-matched normal fibroblasts. The concept that altered fibroblast MMP-2 activation in chronic wounds is important clinically is supported by studies in the (C57BL/KSJ-db/db) mouse, in which it has been recently shown that reduced fibroblast MMP-2 production is associated with impaired dermal healing *in vivo*.[1]

Potential mechanisms of the observed differential MMP activity in the CWF were investigated. MMP-2 activation is complex and occurs via a multistage process involving synthesis and the subsequent formation of a trimeric complex composed of activated MT1-MMP, TIMP-2, and the pro-MMP-2 molecule itself (Strongin *et al*, 1995). As the pro-form of MMP-2 produced by the CWF could be activated *in vitro* using aminophenylmercuric acid (data not shown) the decreased amounts of the active form of the enzyme were not due to structural mutations in the protein that may prevent activation. There are a number of mechanisms by which the observed differences in MMP-2 activation could be mediated, including alterations in TIMP-2 (Strongin *et al*, 1995) and MT1-MMP (Sato *et al*, 1994; Strongin *et al*, 1995). TIMP expression was studied in detail in the FPCL systems as the decreased MMP-2 activation observed could be related to either increased (resulting in MMP-2 inhibition and/or blockage of MT1-MMP) or decreased (resulting in decreased pro-MMP-2 activation) TIMP-2 activity. Whereas fibroblasts from both sources

[1]Wall and Murphy, *Int J Exp Pathol*, abstr., in press.

were shown to express TIMP-1 and TIMP-2 RNA (at all time points in the experiment) using RT-PCR, marked differences in TIMP activity were evident between CWF and patient-matched normal fibroblasts by reverse zymography and ELISA.

The demonstration of a marked difference in TIMP activity during differential collagen lattice reorganization led to the study of potential differences in the fibroblast collagenases. In agreement with the work of others (Unemori and Werb, 1986; Grinnell, 1994; Riikonen *et al*, 1995; Vaalamo *et al*, 1997) we showed that fibroblasts within collagen lattices expressed MMP-1 and MMP-13. In keeping with the findings of increased TIMP-1 and TIMP-2 production by CWF, we showed that MMP-1 protein levels were correspondingly decreased by these fibroblasts. MMP-13 was expressed by CWF in these systems, a finding in keeping with previous studies on leg ulcers (Vaalamo *et al*, 1997) and periodontal disease (Uitto *et al*, 1998). MMP-13 is not expressed, however, in monolayer culture (Vaalamo *et al*, 1997; Ravanti *et al*, 1999) but this was induced by culture of CWF and NFs within collagen lattices. Despite this apparent induced expression of MMP-13 in these systems, the levels of MMP-13 protein were so low as to be undetectable by the commercial ELISA we employed (with a lower detection limit of 0.1 ng per ml).

In the CWF TIMP-1 and TIMP-2 activity was clearly elevated at early time points. These increased levels of TIMP-1 and TIMP-2 may mediate the decreased levels of active MMP-2 and MMP-1 observed in the lattice systems and result in the decreased ability of the CWF to reorganize the ECM. Notwithstanding previously described alterations (both increases and decreases) in aged fibroblast TIMP production (Zeng *et al*, 1994; Ashcroft *et al*, 1997b), these data clearly demonstrate that the TIMP activity of CWF is increased in comparison with normal, aged fibroblasts from the same patient. It must be borne in mind that in addition to TIMP levels fibroblast MMP activity *in vivo* is modulated by local levels of plasminogen activator and growth factors (e.g., platelet-derived growth factor and transforming growth factor-β within the wound; Cullen *et al*, 1997; O'Kane and Ferguson, 1997). MT1-MMP expression was also studied in the FPCL systems using RT-PCR, and expression at all time points by both chronic and patient-matched fibroblasts was observed (data not shown). Enzymatic isolation of the cells from the FPCLs, however, effectively cleaves the extracellular portion of MT1-MMP from the fibroblast cell surface, making studies of protein levels problematic (Ellerbroek *et al*, 1999). In support of our findings, recent studies in melanoma have demonstrated that phenotypic variation in the control of MMP-2 activity is mediated principally by alterations in TIMP-2 levels rather than altered MT1-MMP levels (Kurschat *et al*, 1999).

Previous studies showing generalized increases in MMP activity within chronic wound fluid (Wysocki *et al*, 1993; Bullen *et al*, 1995; Weckroth *et al*, 1996) have led to the concept that inhibition of MMPs may stimulate chronic wound healing (Myers and Wolowacz, 1998). It must be noted, however, that MMP activity in chronic wound fluid reflects MMP production by a variety of cell populations within the wound environment (i.e., inflammatory cells and keratinocytes in addition to fibroblasts). Furthermore, the validity of utilizing chronic wound fluid to sample protease activity within the chronic wound environment has, itself, been questioned as wound fluid does not necessarily reflect tissue levels of proteinases (Ashcroft *et al*, 1997a). These data suggest that local production and activation of MMPs within the tissues may be more relevant to our understanding of the pathogenesis of chronic wounds.

These data provide further evidence for the role of fibroblast phenotype in mediating differential wound healing responses *in vivo* (Schor *et al*, 1996). Local alterations of cellular MMP activity in chronic wounds may play an important role in mediating the impaired cellular responses and ECM reorganization observed *in vivo*. Moreover, these data show that the generalized age-related damage (e.g., oxidative damage, genomic instability, and cell death) that accumulates in the dermis of elderly patients is likely to be influenced by environmental factors at the wound site. These data give further theoretical support to the clinical use of therapeutic strategies such as bio-active dressings, which effectively change the fibroblast phenotype within the chronic wound and have been demonstrated to improve chronic healing in humans (Falanga *et al*, 1998).

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