To assess the differences in proteolytic activity of acute and chronic wound environments, wound fluids were collected from acute surgical wounds (22 samples) and chronic wounds (25 samples) of various etiologies, including mixed vessel disease ulcers, decubiti and diabetic foot ulcers. Matrix metalloproteinase (MMP) activity measured using the Azocoll assay was significantly elevated by 30 fold in chronic wounds (median 22.8 µg MMP Eq/ml) compared to acute wounds (median 0.76 µg MMP Eq/ml) (p < 0.001). The addition of the matrix metalloproteinase inhibitor Illomostat decreased the matrix metalloproteinase activity by approximately 90% in all samples, confirming that the majority of the activity measured was due to matrix metalloproteinases. Gelatin zymograms indicated predominantly elevated matrix metalloproteinase-9 with smaller elevations of matrix metalloproteinase-2. In addition tissue inhibitor of metalloproteinase-1 levels were analyzed in a small subset of acute and chronic wounds. When tissue inhibitor of metalloproteinase-1 levels were compared to protease levels there was an inverse correlation (p = 0.02, r = -0.78). In vitro degradation of epidermal growth factor was measured by addition of 125I labelled epidermal growth factor to acute and chronic wound fluid samples. There was significantly higher degradation of epidermal growth factor in chronic wound fluid samples (mean 28.1%) compared to acute samples (mean 0.6%). This also correlated to the epidermal growth factor activity of these wound fluid samples (p < 0.001, r = 0.64). Additionally, the levels of proteases were assayed in wound fluid collected from 15 venous leg ulcers during a nonhealing and healing phase using a unique model of chronic wound healing in humans. Patients with nonhealing venous leg ulcers were admitted to the hospital for bed rest and wound fluid samples were collected on admission (nonhealing phase) and after 2 weeks (healing phase) when the ulcers had begun to heal as evidenced by a reduction in size (median 12%). These data showed that the elevated levels of matrix metalloproteinase activity decreased significantly as healing occurs in chronic leg ulcers (p < 0.01). This parallels the processes observed in normally healing acute wounds. This data also supports the case for the addition of protease inhibitors in chronic wounds in conjunction with any treatments using growth factors. (WOUND REP REG 1999;7:442–452)

Wound healing is a dynamic biological process with many complex interactions that are only partially understood at the molecular level. Disruption of this molecular process leads to the formation of chronic wounds; however, the molecular pathophysiology of chronic wounds is not fully understood. Previous studies have documented that there are significant differences in key molecules found in the microenvironment of acute and chronic wounds. In other work from our laboratory, it was shown that the
levels of pro-inflammatory cytokines were significantly reduced when healing occurs in chronic leg ulcers. Moreover, the levels of growth factors in chronic leg ulcers do not change significantly when the ulcers are healing. It has also been shown that when the mitogenic activity of wound fluid was tested on cells in culture, wound fluid from chronic wounds inhibited the proliferation of cells while that from acute wounds stimulated growth. Our analysis of changes in the mitogenic activity of wound fluid from chronic leg ulcers showed that the proliferative activity increases during healing.

One mechanism through which the inflammatory cytokines may be exerting an effect within the chronic wound environment is by stimulating the production of the matrix metalloproteinases (MMPs). In vitro studies have shown that interleukin-1 and tumor necrosis factor-α directly stimulate the synthesis of MMPs and inhibit the synthesis of tissue inhibitors of metalloproteinases (TIMPs) in fibroblasts and endothelial cells. This suggests that prolonged exposure of cells within the wound to these pro-inflammatory cytokines may lead to an elevation in the ratio of MMPs to their natural inhibitors TIMPs.

The MMPs include a number of proteases responsible for the degradation of components of the extracellular matrix (ECM) such as collagen, fibronectin, laminin, proteoglycan and elastin. A key step in the degradation of the ECM is the extracellular secretion of MMPs. In vitro and animal studies have shown the important role of proteases in normal wound healing but the levels and duration of protease expression is limited and tightly controlled either by plasmin activation or by the presence of TIMPs.

Controlled degradation of the ECM is required for the removal of damaged components and to allow cell migration and angiogenesis. The restructuring of the ECM is also required to allow cellular adherence and the laying down of the basement membrane. These functions are essential during the normal wound healing process, however, it is thought that excess production of MMPs over TIMPs may contribute to matrix destruction, including cartilage degradation in both osteoarthritis and rheumatoid arthritis and tissue damage in periodontal disease and pulmonary fibrosis. Studies investigating periodontal disease have shown that the inflammatory cell proteases, i.e., neutrophil elastase and MMPs, are causative factors in the disease process rather than the bacterial proteases as first suspected. Other serine proteases such as neutrophil elastase and cathepsin G contribute to the pathology of cystic fibrosis and one treatment developed recently has been the administration of aerosolized α1-antitrypsin.

The role of proteases in human chronic wounds has been increasingly investigated in recent years. Elevated levels of MMPs in the granulation tissue of chronic pressure ulcers suggest that a highly proteolytic environment contributes to the chronicity of these wounds. MMPs have also been shown to be present in elevated amounts in wound fluid from chronic leg ulcers, compared to that from acute wounds. More recently elevated MMP activity has been reported in a series of 5 decubitus patients by Yager et al. These authors showed that MMP-9 and to a lesser extent MMP-2 were elevated 10- to 25-fold in decubitus patients compared to mastectomy and TIMPs were reduced in chronic fluids. Bullen et al. have also reported that TIMPs are decreased and MMP-9 levels increased in chronic wounds. Others have reported that neutrophil elastase is responsible for the degradation of fibronectin (not MMPs) and that the levels of α1-antitrypsin, the natural inhibitor of neutrophil elastase were low in chronic wounds compared to acute wounds.

These data therefore suggest that proteases may play a role in the impaired healing of chronic cutaneous wounds. This implication has led us to formulate the hypothesis that a detrimental imbalance of the MMP/TIMPs ratio may not only lead to a degradation of the ECM, but also to degradation of growth factors and their respective receptors within the wound environment.

The purpose of this study was to examine the levels of MMPs, neutrophil elastase, and TIMPs in a large series of wound fluids from acute surgical wounds and chronic nonhealing cutaneous wounds. It was also designed to examine changes in the levels of these proteases in wound fluid from a group of nonhealing chronic venous ulcers that subsequently entered a healing phase. The effect of acute and chronic wound fluids on the degradation of peptide growth factors was also examined.

**MATERIALS AND METHODS**

Informed consent was obtained from patients for all sample collections. Acute wound fluid was obtained from patients undergoing radical mastectomy for primary breast disease were recruited as in-patients of the Shands Hospital, University of Florida, Gainesville, FL. Patients selected had primary breast...
carcinoma without evidence of metastatic spread and had not undergone chemotherapy, radiation, or hormonal manipulation prior to surgery. Wound fluid that had accumulated in the vacuum drain from the chest wall during an 8-hour period was drained and stored at –80 °C. Samples collected during a given 24-hour period were then pooled immediately prior to analysis. Wound fluid samples were collected for up to 7 days. Wound fluid collected during the 8 hours following the immediate postop period were discarded due to blood contamination.

**Chronic wound fluid collection**

Patients included in this part of the study all had chronic wounds of varying etiology that had been present for more than 4 weeks duration. Patients were recruited as in-patients from Shands Hospital, Gainesville and the Orlando Regional Medical Center, Orlando, FL. These wounds included diabetic foot ulcers, mixed vessel disease ulcers, and decubitus ulcers that were not clinically infected. Patients in this group had been admitted to the hospital for further treatment and the wounds were classified as chronic nonhealing wounds. Wound fluid was collected from the general chronic wounds by covering the wound with a transparent occlusive dressing (Opsite, Smith & Nephew, Hull, UK) for approximately 1 hour. Fluid that had accumulated was then aspirated and stored at –80 °C.

**Non-healing/healing phase chronic venous leg ulcer wound fluid collection**

Patients examined in this study had chronic leg ulcers in the gaiter region excluding the foot. Patients were recruited as in-patients at Fremantle Hospital, Western Australia. All patients had the cause of their ulcers assessed by careful history, clinical examination and evaluation by a series of diagnostic studies. Venous photoplethysmography (PPG), was performed to determine the presence of venous disease. Venous disease was considered present when the refilling time was less than 25 seconds as previously established for this laboratory. Arterial disease was determined by measuring the arterial Doppler pressures and was defined as an ankle/brachial ratio less than 0.9. Blood tests were also carried out to complete the diagnosis, these included a full blood panel, urea and electrolytes, liver function tests, blood sugar levels and rheumatoid serology.

All patients included in this study had venous disease and had been treated as outpatients with compression therapy to improve venous function. In all cases the patients’ ulcers had failed to respond to outpatient treatment as defined by no reduction in the size of the ulcer in more than 3 months or a continued increase in the size of the ulcer. Patients were admitted to the hospital for bed rest, 6 hourly saline compresses and eventual skin grafting. Following at least 2 weeks of bed rest in the hospital, the ulcers showed clinical signs of healing, such as reduction of slough, increased granulation tissue formation, and epithelialization. This healing was more objectively measurable as a reduction in the size of the ulcer surface area. This model provides the opportunity of studying the healing process of individual chronic leg ulcers directly and allows the comparison of each patient’s responses to themselves. Each individual patient provides their own control. The admission did not include intervention for other potential causes of delayed healing.

Wound fluid samples were collected from patients within 24 hours of admission when the ulcers were in a nonhealing phase and after 2 weeks of regular dressings and bed rest when the ulcers had entered a healing phase. Wound fluid was collected from each patient in a standardized manner as previously described. The patient was fasted from midnight, and a transparent occlusive film (Opsite, Smith & Nephew) was placed over the wound at 08:00 hours. The patient’s leg was placed in a dependent position and the patient was encouraged to drink one liter of water, which aided in standardizing the patient’s state of hydration. The fluid was aspirated from beneath the dressing after a period of one hour, transferred into plain collection tubes, centrifuged at 14,000 × g for 5 minutes to pellet any particulate matter, and stored in aliquots at –80 °C.

**Ulcer assessment**

All ulcers were photographed on admission and after 2 weeks to provide a visual record of any changes in appearance of the ulcer, such as reduction of slough, granulation tissue formation and epithelialization of the edge. The surface area of all ulcers was measured on admission and at 2 weeks by tracing around the edge of the ulcer onto clear plastic and then photocopying the tracing onto paper. The surface area of the ulcer was then calculated using a Kent digital planimeter (Jayco, Westride, Australia). From these measurements the percentage reduction in surface area of the ulcer after 2 weeks could be calculated as an indication that healing was occurring.
Azocoll assay for MMP activity
The azocoll assay is a preferred method for analysis of gelatinases, MMP-2 and MMP-9. Azocoll (Sigma Chemical Co., St Louis, MO) was measured using a modification of the method developed by Chavira et al. Azocoll was washed and suspended in 0.05 M Tris-HCl, 1 mM CaCl$_2$, pH 7.8, at a final concentration of 5 mg/ml. Briefly, 50 µl of the MMP assays described above were carried out. Richard Galardy). Samples were preincubated with an inhibitor IIomostat (Galardin, gift provided by Dr. Richard Galardy). The azocoll suspension in a 1.5-ml reaction tube. The reaction tubes were placed at 37 °C in a shaker that inverted the tubes 30 times per minute. After 6 hours of incubation, the reactions were stopped by centrifuging the samples at 10,000 × g (Beckman Microfuge II Beckman, Coulter Inc., CA) for 8 minutes at 4 °C. The absorbance of the supernatant solution was measured at 520 nm with a Milton-Roy spectrophotometer (Milton Roy, PA). Readings were compared to a standard curve constructed using crude collagenase from Clostridium histoliticum (Worthington Biochemicals, NJ) as the reference protease standard. The protease levels were expressed as net µg of protease equivalents per ml of wound fluid.

Azocasein assay for non-specific protease activity
Protease activity was assayed using azocasein (Sigma Chemical Co., St. Louis, MO) as a substrate. The azocasein method detects a variety of enzymes including metalloproteinases and serine proteinases. The reaction solution consisted of 750 µl of azocasein solution (4 mg/ml in 0.05 M Tris-HCl, pH 7.5), 220 µl of 0.05 M Tris-HCl, pH 7.5, and 30 µl of either sample or protease standard soy trypsin (Sigma Chemical Co.). This solution was incubated at 37 °C for 24 hours, and the reaction stopped by adding 250 µl of 20% trichloroacetic acid. The precipitate formed after standing on ice for 10 minutes was removed by centrifugation at 16,000 × g for 2 minutes, and the absorbance of the supernatant measured at 405 nm using a Milton-Roy single beam spectrophotometer. Protease concentrations in samples were calculated from the constructed standard curve using trypsin (Sigma Chemical Co.) as the reference protease standard (range 1 µg–251 µg). Protease levels were expressed as µg trypsin equivalents per ml of fluid.29

Inhibition of MMPs in wound fluid
The specificity of the protease activity was tested by inhibition of MMP activity using the specific MMP inhibitor IIomostat (Galardin, gift provided by Dr. Richard Galardy). Samples were preincubated with an excess of IIomostat for 30 minutes at 37 °C before each of the MMP assays described above were carried out.

Gelatin and casein zymography methods
Gelatin zymography of wound samples was performed using Tris-glycine 10% polyacrylamide gels containing 0.1% gelatin (Novex, San Diego, CA) using methods previously described.19,30 Briefly, wound fluid samples were incubated with an equal volume of sample buffer (63 mM Tris, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 0.0025% bromophenol blue) for 10 minutes at room temperature. Equal volumes of sample (20 µl) were applied to gels and resolved by slab gel electrophoresis (125 V constant for 90 minutes); gels were placed in zymogram renaturing buffer (2.5% Triton X-100 in water) and the gel was washed for 90 minutes at 37 °C with 2 changes. The gel was then placed in zymogram developing buffer (50 mM Tris, pH 7.8, 5 mM CaCl$_2$, 200 mM NaCl$_2$, 0.02% Brij-35) overnight at 37 °C with gentle agitation. After incubation, the gel was then stained with RAPID Coomassie Stain (Diversified Biotech, Boston, MA). Low molecular weight standards (BioRad Laboratories, Hercules, CA) and MMP-9 and MMP-2 standards (Glycomed Inc, Alameda, CA) were run concurrently with samples. Casein zymography was performed using the procedure as described above, using Tris-glycine 10% polyacrylamide gels containing 0.1% casein (Sigma).

Neutrophil elastase activity assay
Wound fluid samples (5–10 µl) or 5–5000 ng purified human neutrophil elastase (Calbiochem-Novabiochem Corporation, San Diego, CA) were incubated for 1 hour at 37 °C in a final volume of 100 µl of 0.1 M HEPES buffer, pH 7.4, containing 0.5 M NaCl, 10% DMSO and 1 mM elastase substrate (methoxyssuccinyl-alal-ala-pro-val-p-nitroanilide, Sigma Chemical Co.). Substrate degradation was continuously monitored by measuring OD$_{405}$. The maximum linear rate obtained for each sample was determined and a standard curve prepared from the elastase data. Wound fluid activity was converted to µg of elastase per ml of fluid.

Cathepsin G activity determination
Wound fluid samples (5–10 µl) or 5–5000 ng purified human neutrophil cathepsin G (Calbiochem-Novabiochem) were incubated for 1 hours at 37 °C in a final volume of 100 µl of 0.1 M HEPES buffer, pH 7.4, containing 0.5 M NaCl, 10% DMSO and 3 mM cathepsin G substrate (succinyl-alal-ala-pro-phe-p-nitroanilide, Sigma Chemical Co.). Substrate degradation was continuously monitored by measuring OD$_{405}$. The maximum linear rate obtained for each sample was determined and a standard curve prepared from the human neutrophil cathepsin G data. Wound fluid ac-
Activity was converted to µg of cathepsin G per ml of fluid.

**TIMP-1 determination by ELISA**
The levels of TIMP-1 were determined using an ELISA as described previously. This ELISA detects both free TIMP-1 and TIMP-1/MMP complexes although not with the same efficiency as free TIMP-1.

**Degradation of cytokines in wound fluid**
The effect of wound fluid on the degradation of cytokines was tested by adding 50 µL of wound fluid to 20 µl of 125I-epidermal growth factor (Chiron Corp., CA) containing 50,000 dpm (specific activity = 82 µCi [3.03 MBq]/µg). The 125I-EGF solution contained 165 pg of EGF in a buffer containing 10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mg bovine serum albumin/ml. The samples were incubated for 24 hours before 100 µl of human IgG (20 mg/ml) were added as carrier protein and 4 ml of 10% trichloroacetic acid were added and the precipitate pelleted by centrifugation at 7000 × g for 20 minutes at 4 °C. The supernatant solution was removed and the radioactivity in the pellets measured with a gamma scintillation counter. The data were then expressed as a percentage of EGF degraded per ml of wound fluid per 24 hours. Controls included incubation of 125I-EGF with buffer to measure spontaneous degradation of the substrate. Levels of protease activity measured were expressed as the net ng of EGF degraded per ml of wound fluid. In separate experiments EGF degradation was tested on a number of wound fluid samples in the presence of the MMP inhibitor Illomostat. Percent degradation were analyzed for statistical significance using the nonparametric Mann Whitney U-test.

**RESULTS**
There were 8 patients from which acute wound fluid was collected from mastectomy wound drains. The median age of the patients was 49 years (range 31–67 years). Samples were collected over a 7-day period with a total of 22 samples collected.

A total of 25 patients with chronic wounds were included in this part of the study. The median age of the patients was 63 years (range 23–83 years). There were 12 females and 13 males. There were 17 patients with decubiti, 5 patients with mixed vessel disease ulcers and 3 patients with diabetic foot ulcers.

There were 15 patients included from which paired wound fluid samples were collected from each patient on admission (nonhealing phase) and then again after 2 weeks of hospitalization (healing phase). The median age of the patients was 77 years (range 55–91 years). There were 5 females and 10 males, and 7 patients had purely venous ulcers and 8 patients had venous ulcers with some minor arterial disease also present (dopplers > 0.6 and < 0.9). Median size reduction was 12%.

**Azocoll protease levels in wound fluid**
The levels of protease activity as measured by the azocoll assay varied greatly between the acute and chronic wounds. There was no significant difference between acute wound fluid samples collected on different days therefore this data was collated and these samples were analyzed and presented as one group. The mean protease level in acute wound fluid was 0.75 µg MMP Eq/ml ± 0.3 (median 0.76 µg MMP Eq/ml) and the mean level in all of the chronic wounds was 59.9 µg MMP Eq/ml ± 70.9 (median 22.8 µg MMP Eq/ml). This difference was statistically significant, p < 0.001 (Mann Whitney U-test). The chronic wound group was further divided into wounds of different etiologies, nonhealing venous leg ulcers; median 23.3 µg MMP Eq/ml (n = 15), mixed vessel disease ulcers; median 30.64 µg MMP Eq/ml (n = 5), diabetic ulcers; median 93.9 µg MMP Eq/ml (n = 3) and decubiti; median 20.36 µg MMP Eq/ml (n = 17) as shown in Figure 1. There was no significant difference between the two groups of chronic wounds with large numbers, i.e., chronic leg ulcers and decubiti, p = 0.82, however, due to small numbers the other groups could not be compared statistically. (Figure 1)

Figure 2 shows a comparison of the protease analysis of the wound fluid samples collected from chronic leg ulcers in a nonhealing and healing phase. When the venous leg ulcer samples were analyzed for dif-
ferences between the nonhealing and healing samples
the mean level of MMP activity in the nonhealing
samples was 38 ± 10 µg MMP Eq/ml (median 23.3 µg
MMP Eq/ml, range 1.3–140.4 µg MMP Eq/ml). After
two weeks treatment, the protease levels decreased in
12 of the 15 patients to a mean of 11 ± 4 µg MMP Eq/ml
(the mean percentage reduction was 49%) (median
4.8 µg MMP Eq/ml, range 0.3–64.9 µg MMP Eq/ml).
This decrease in MMP activity during healing was
found to be statistically significant when assessed us-
ing the Wilcoxon matched pairs signed ranks test
\( p < 0.01 \) (Figure 2A).

The addition of the MMP inhibitor Illomostat
(Galardin) significantly decreased the protease levels
in all samples to a mean of 0.31 ± 0.04 µg MMP Eq/ml
(median 0.26 µg MMP Eq/ml, range 0.06–1.01 µg MMP
Eq/ml) (Figure 2B). This was an overall mean decrease
of approximately 90%, suggesting that the majority of
the activity being measured was due to MMPs.

**Azocasein protease levels in wound fluid**
The sequential wound fluid samples collected during
the nonhealing and healing phase of chronic venous
leg ulcers were further analyzed using the azocasein
assay which is a less specific substrate for MMPs as it
may also be degraded by serine proteases. As shown
in Figure 2C, azocasein protease levels decreased in 9
of the 15 patients during the healing phase. Although

![Figure 2](image-url)

**Figure 2.** Protease activity in wound fluid samples from nonhealing and healing chronic venous leg ulcer patients. (A) Azocoll
assay showing MMP activity. (B) Azocoll assay following the addition of the MMP inhibitor Illomostat to samples (note change in
scale). (C) Azocasein assay indicating nonspecific protease activity. (D) Azocasein assay following the addition of the MMP
inhibitor Illomostat to samples (note change in scale), the residual activity most likely represents serine protease activity. ■, Non-
healing wound fluid; □, Healing wound fluid.
there was a decrease in the majority of patients—mean levels decreased from 322 ± 94 µg trypsin Eq/ml to 190 ± 35 µg trypsin Eq/ml (medians 226.7 µg trypsin Eq/ml and 189.5 µg trypsin Eq/ml, respectively)—this decrease was not statistically significant (p > 0.05). Again, the addition of the MMP inhibitor Ilomostat significantly decreased the protease levels in all samples, but the mean reduction in this case was 66% (Figure 2D). The remaining activity detected in the azocasein assay most likely reflects the serine protease activity remaining following MMP inhibition. Again, there was no statistical difference between the nonhealing and healing samples.

**Gelatin and casein zymography of wound fluid**

The proteases in wound fluid were separated and identified by electrophoresis in gelatin and casein gels in order to identify specific MMP activity. The gelatin zymography of the nonhealing samples consistently showed six intense bands of > 200, 150, 100, 85, 65 and 45 kDa. These decreased in intensity in the paired healing samples from the 12 patients that showed a decrease in azocoll protease levels during healing. A representative sample of three such patients is shown in Figure 3. Incubation of the gelatin zymograms in buffer containing Ilomostat totally blocked band formation, indicating that all the bands detected in the gelatin zymograms were MMPs (not shown). The casein zymograms showed a different pattern of hydrolysis, with band formation at 150, 100, 65 and 29 kDa, without any significant difference between the nonhealing and healing phase (data not shown). Ilomostat had minimal effect on the pattern of bands, but the addition of the serine protease inhibitor phenylmethyl sulfinyl fluoride reduced the intensity of most bands, confirming the presence of serine proteases.

**Neutrophil elastase activity**

Neutrophil elastase activity was compared between 14 acute wound fluid samples and 13 general chronic wound fluid samples. The levels of neutrophil elastase in acute wounds was < 1 µg/ml (below the level of detection) in all patients, whereas in the chronic wounds there were elevated levels of activity in only 6 patients and 7 patients were < 1 µg/ml (data not shown). The median of the 6 chronic samples with elevated levels was 99.5 µg/ml (range, 5.9–344 µg/ml).

The sequential wound fluid samples collected from the nonhealing and healing chronic venous leg ulcers were also analyzed for levels of neutrophil elastase. The levels of neutrophil elastase varied considerably between patients and decreased in 6 patients during the healing phase, increased in 5 patients and in 4 patients there were no detectable levels present in either the nonhealing or healing phase. Although the median levels decreased from 1.4 µg/ml (range, < 1–11.9 µg/ml) in the nonhealing samples to 0.6 µg/ml (range, < 1–8.6 µg/ml) in the healing samples this was not statistically significant (p > 0.05).

**Cathepsin G analysis of wound fluid**

A comparison of cathepsin G activity was performed on the 14 acute wound fluid samples and 13 general chronic wound fluid samples. The levels of cathepsin G activity in acute wounds was < 1 µg/ml (below the level of detection) in all patients, and in the chronic wounds there were only elevated levels in 4 patients and 9 patients were < 1 µg/ml (data not shown). The levels of cathepsin G activity in the elevated samples ranged from 5.2–49 µg/ml.

**TIMP levels in wound fluid**

TIMP-1 levels were analyzed in wound fluid samples from 3 acute wounds and 5 chronic wounds. The median TIMP-1 level in acute wounds was 44.0 µg/ml and in the chronic wounds 0.8 µg/ml.

When the levels of TIMP-1 are compared to the corresponding levels of protease in the same samples there is a negative correlation, so that when TIMP-1 levels are elevated the MMP levels are low and con-
versely when MMP levels are high TIMP-1 levels are low \( (p = 0.02, r = -0.78, \text{Spearman correlation}) \) (Figure 4).

**Cytokine degradation**

Wound fluid samples from 19 acute wounds and 8 chronic wounds were analyzed for the ability to degrade EGF over a 24-hour period. The median percentage of degradation of EGF by acute wound fluid was 0.6%, range, 0.2%-1.2% (mean: 0.6%) which was significantly lower than the median degradation observed in chronic wound fluid which was 8.5%, range, 1.2%-100% (mean 28.1%) \( (p < 0.001) \).

The protease levels of all wound fluid samples were correlated with the percentage degradation of EGF and there was a moderate correlation between protease levels and EGF degradation, which was statistically significant \( (p < 0.001, r = 0.64) \) (Figure 5).

EGF degradation by wound fluid samples was completely inhibited by the addition of the MMP inhibitor Illomostat, suggesting that the proteolytic degradation of EGF was largely due to the activity of MMPs (data not shown).

**DISCUSSION**

We have previously reported that pro-inflammatory cytokines including TNF-\(\alpha\), IL-1, IL-6, and IL-8, are elevated in wound fluid from chronic wounds of various etiologies and that TNF-\(\alpha\), IL-1, and IL-6 decrease as healing occurs in chronic venous ulcers.\(^{33}\) This study continues these previous studies and has examined the presence of proteases within acute and chronic wound fluid.

MMPs are a necessary component of the wound healing process and as such play an important role in cell migration and modification of the ECM. However, if the regulation of these molecules is disrupted they may be produced in excessive amounts and may lead to degradation of the ECM, preventing cellular migration and attachment, and may ultimately cause tissue destruction.\(^{8}\)

The results presented in this paper show that levels of MMP activity are elevated in a high percentage of chronic wounds when compared to acute wounds. The fact that the level of MMP activity in chronic wound fluid is 30 times that found in acute wounds, suggests that in chronic wounds there is disruption of the usual mechanisms controlling the levels of these enzymes. These results support the findings of previous investigators in which elevated levels of MMPs have been reported in the tissue and wound fluid of chronic wounds when compared to acute wounds.\(^{17-19}\) Also the results from this study support previous findings that there is a limited change in the MMP/TIMP ratio after 24 hours in acute wounds.\(^{20}\)

In addition to the comparison between acute and chronic wounds, we have shown for the first time that the activity of these proteases decreases consistently in a large number of patients with venous ulcers that progress from a nonhealing to a healing phase. This confirms and expands on data presented on a single patient by Wysocki et al. that indicates that proteases change during the healing of a chronic wound.\(^{34}\) This suggests that proteases within the extracellular fluid environment change considerably during healing and implies that a reduction in the activity levels of MMPs may be required for healing to occur in these chronic wounds.
The production of MMPs is stimulated by IL-1 and TNF-α. As presented in another article these pro-inflammatory cytokines are extremely elevated in wound fluid obtained from nonhealing chronic leg ulcers and decreases significantly during healing. The reduction observed in MMP activity may be directly related to these findings.33

The results of the gelatin and casein zymography supports the results obtained using the azocoll and azocasein assays for individual samples. Zymography also showed that the 92 kDa band is the major form. This MMP is produced predominantly by inflammatory cells and keratinocytes35 and these data also support previous reports.19

In 1995, Rao et al. reported that 9 out of 10 samples of wound fluid collected from chronic wounds showed degradation of fibronectin and that α1 antitrypsin (a protease inhibitor) was also degraded and nonfunctional.22 These two molecules were found to be intact in wound fluid from acute wounds.22 It was shown that serine proteases were responsible for this degradation and that these molecules were chiefly responsible for the poor healing of these wounds. In the data presented in this paper, elevated levels of the serine protease, neutrophil elastase, were detected in 50% of chronic wounds when compared to acute wounds, however, there was no significant change in this protease during the healing of venous leg ulcers. Also when the protease activities were examined following the addition of Illomostat in the azocoll and azocasein assays there was no significant change in the serine protease levels during the healing of venous leg ulcers. This would suggest that it may not be necessary for a dramatic alteration in the levels of this class of protease during the healing process. Analysis of cathepsin G activity levels in wound fluid showed that this protease was not consistently elevated in chronic wounds compared to acute wounds and therefore may not be having a significant impact on healing.

The analysis of TIMP-1 levels in a small subset of patients indicated that those wounds with high MMP levels had reduced TIMP-1 levels possibly further exacerbating the effect of the MMPs. Chronic wounds had lower TIMP-1 levels and higher MMP levels than acute wounds, thus contrasting the differences in MMP activity between these types of wounds.

It is known that proteases in wound fluid from chronic wounds leads to increased degradation of the ECM, however, elevated protease levels in chronic wounds may also be exerting an effect on the wound environment by causing degradation of key functional molecules such as growth factors. The results of this study document that when the growth factor EGF was added to chronic wound fluid significantly more degradation occurs than when EGF was added to acute wound fluid. The rate of degradation of EGF in all wound fluid samples tested correlated directly with the protease activity present in these samples. Further confirming this result was the finding that when an MMP inhibitor was added to the wound fluid there was no degradation of EGF.

These results may help to explain the differential effects of growth factor treatment of chronic wounds reported in many clinical studies.36–38 Chronic wound studies in which there has been success in stimulating the healing rate with growth factors may be directly related to how well the wounds are debrided, presumably converting a chronic wound into one of an acute nature and consequently reducing the levels of proteases present in the wound environment.39 This may also be the case in studies testing platelet-derived growth factor on chronic wounds which have shown the most promising results in stimulating healing.40–42

Failure of treatment with topical EGF to achieve statistically significant healing may have been due to elevated MMPs.37 It may be speculated that if a protease inhibitor had been added to the wounds prior to the addition of the growth factor a far more dramatic effect may have been observed. If this hypothesis is correct then the addition of cytokines alone is not the optimal treatment for chronic wounds in which the protease levels are unknown.

It is not possible to say whether the changes in the levels of MMPs are responsible for healing or symptomatic of other changes occurring within the wound. However, the results of this study show that these molecules are present in excessive amounts in chronic leg ulcers and that a decrease in the levels occurs during healing. These findings suggest that these proteases play an important role in the pathophysiology of chronic wounds. Also, the elevated levels of the pro-inflammatory cytokines in chronic wound fluid, suggest that perhaps these cytokines may be exempt or protected from proteolytic degradation, further contributing to the proteolytic imbalances within the wound. Further work is required to confirm this hypothesis.

When taken together these studies suggest that a possible treatment option to be considered in the future would involve a combination of protease in-
inhibitors and anti-inflammatory agents, followed by the subsequent application of growth factors.

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