Selective Pick-Up of Increased Iron by Deferoxamine-Coupled Cellulose Abrogates the Iron-Driven Induction of Matrix-Degrading Metalloproteinase 1 and Lipid Peroxidation in Human Dermal Fibroblasts *In Vitro*: A New Dressing Concept

Jutta Wenk,[1] Angelika Foitzik,*[1] Volker Achterberg,* Andrea Sabiwalsky, Joachim Dissemond, Christian Meewes, Andrea Reitz,* Peter Brenneisen, Meinhard Wlaschek, Wolfgang Meyer-Ingold,* and Karin Scharffetter-Kochanek

*Beiersdorf AG, Hamburg, Germany; Department of Dermatology, University of Cologne, Germany

Using atomic absorption spectrum analysis, we found iron levels in exudates from chronic wounds to be significantly increased (3.71 ± 1.56 μmol per g protein) compared to wound fluids from acute wounds derived from blister fluids (1.15 ± 0.62 μmol per g protein, $p < 0.02$), drainage fluids of acute wounds (0.87 ± 0.34 μmol per g protein, $p < 0.002$), and pooled human plasma of 50 volunteers (0.42 μmol per g protein). Increased free iron and an increase in reactive oxygen species released from neutrophils represent pathogenic key steps that – via the Fenton reaction – are thought to be responsible for the persistent inflammation, increased connective tissue degradation, and lipid peroxidation contributing to the prooxidant hostile microenvironment of chronic venous leg ulcers. We herein designed a selective pick-up dressing for iron ions by covalently binding deferoxamine to cellulose. No leakage occurred following gamma sterilization of the dressing and, more importantly, the deferoxamine-coupled cellulose dressing retained its iron complexing properties sufficient to reduce iron levels found in chronic venous ulcers to levels comparable to those found in acute wounds. In order to study the functionality of the dressing, human dermal fibroblasts were exposed to a Fenton reaction mimicking combination of 220 μM Fe(III) citrate and 1 mM ascorbate resulting in a 4-fold induction of matrix-degrading metalloproteinase 1 as determined by a matrix-degrading metalloproteinase 1 specific enzyme-linked immunosorbent assay. This induction was completely suppressed by dissolved deferoxamine at a concentration of 220 μM or by an equimolar amount of deferoxamine immobilized to cellulose. In addition, the Fe(III) citrate and ascorbate driven Fenton reaction resulted in an 8-fold increase in malondialdehyde, the major product of lipid peroxidation, as determined by high pressure liquid chromatography. This increase in malondialdehyde levels could be significantly reduced in the presence of the selective pick-up dressing coupled with deferoxamine suggesting that the deferoxamine dressing, in fact, prevents the development of a damaging prooxidant microenvironment and also protects from unfavorable consequences like matrix-degrading metalloproteinase 1 and lipid peroxide induction. ***Key words: chronic wounds/dressing/Fenton reaction/Haber–Weiss reaction/iron/reactive oxygen species.*** ***J Invest Dermatol 116:833–839, 2001***

Chronic venous leg ulcers represent the final outcome of lower extremity chronic venous insufficiency in most cases. It is a debilitating recurrent complication with an estimated cost of 1 billion dollars annually in the U.S.A. for its treatment. Moist wound healing providing dressings in combination with compression therapy represent the state-of-the-art ulcer treatment. Even though progress has been achieved, current efforts defining the highly aggressive chronic wound microenvironment promise further improvement of this treatment modality.

Chronic leg ulcers fail to progress through the normal pattern of wound repair involving inflammation, granulation tissue formation, and remodeling, but instead remain in a chronic inflammatory state with little signs of healing. There is increasing evidence that the persisting infiltration of neutrophils and macrophages (Rosner *et al*, 1995) in conjunction with elevated iron depositions in the ulcer tissue (Ackerman *et al*, 1988) play a major role in the generation of the prooxidant hostile microenvironment in chronic venous leg ulcers. Venous hypertension results in enhanced erythrocyte extravasation into the interstitium through widened interendothelial pores (Wenner *et al*, 1980). Iron is released from hemoglobin of degraded erythrocytes, ferritin, hemosiderin (Thomas *et al*, 1985;

Manuscript received August 21, 2000; revised January 10, 2001; accepted for publication February 1, 2001.

Reprint requests to: Dr. Wolfgang Meyer-Ingold, Beiersdorf AG, Unnastrasse 48, D-20245 Hamburg, Germany. Email: karin.scharffetter@uni-koeln.de

Abbreviations: CDI, 1,1′-carbonyldiimidazole; DFO, deferoxamine; LC/MS, liquid chromatography/mass spectrometry; MDA, malondialdehyde; MMP-1, matrix-degrading metalloproteinase 1.

[1]These authors contributed equally.

The authors declared no conflict of interest.

Biemond *et al*, 1988), aconitase, and other iron–sulfur proteins (Fridovich, 1995) via the attack of reactive oxygen species (ROS) and proteolytic enzymes occurring in nonhealing wounds. Subsequently, iron in combination with hydrogen peroxide released by activated neutrophils generates the highly toxic hydroxyl radical via the Fenton reaction (Halliwell and Gutteridge, 1989) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\cdot + OH^-$. The resulting ferric iron (Fe^{3+}) can be reduced to ferrous iron (Fe^{2+}) in the Haber–Weiss reaction involving superoxide anions: $Fe^{3+} + O_2^{-}\cdot \rightarrow Fe^{2+} + O_2$, thus perpetuating the Fenton reaction and tissue damage. The hydroxyl radical initiates local lipid peroxidation of polyunsaturated fatty acids in cellular membranes resulting in an inflammatory response with continuous recruitment of neutrophils (Gutteridge *et al*, 1979; Weiss, 1989; Cheatle, 1991; Schaich, 1992; Falanga and Eaglstein, 1993).

In addition to direct damage of structural proteins of the extracellular matrix (Monboisse and Borel, 1992), the hydroxyl radical and other ROS enhance the synthesis and activity of matrix-degrading metalloproteinases (Wysocki *et al*, 1993; Weckroth *et al*, 1996; Saarialho-Kere, 1998) and serine proteases with concomitant inactivation of tissue inhibitors of matrix metalloproteinases (Mast and Schultz, 1996) and serine proteases (Grinnell *et al*, 1992; Rao *et al*, 1995; Grinnell and Zhu, 1996; Herrick *et al*, 1997) finally tilting the balance towards proteolysis. Apart from enhanced connective tissue degradation, serine proteases have been identified to degrade key growth factors in tissue repair such as platelet-derived growth factor (Wlaschek *et al*, 1997; Yager *et al*, 1997) and others.

The identification of factors delaying wound healing has been crucial for the development of a novel therapeutic concept that finally led to a new dressing concept called the selective pick-up principle.[2] According to this optimized concept, we focused on the strategy to remove or eliminate the deleterious substances from the wound exudate by selectively acting biomolecules that are bound to the surface of traditional wound dressing materials instead of applying potentially beneficial agents to the wounds (Meyer-Ingold *et al*, 1998; Edwards *et al*, 1999). This selective sequestration is likely to result in a positive influence on wound healing or even in a reversal of this process from chronicity to healing. Furthermore, a more general benefit of selective pick-up dressings includes their ability to adjust the composition of wound fluids locally compared to the systemic action of drugs.

In a first attempt to disrupt the detrimental sequence of events leading to a prooxidant hostile microenvironment and impaired healing – and based on the herein reported finding of increased iron concentrations in the exudate of chronic venous leg ulcers – we have designed and developed a first prototype of a selective pick-up dressing for iron ions based on the iron chelator deferoxamine (DFO) covalently immobilized to a cellulose dressing.

The *in vitro* efficacy and functionality of the iron pick-up dressing was demonstrated by its iron-complexing properties, and its protective activity concerning the iron-driven upregulation of matrix-degrading metalloproteinase 1 (MMP-1) and lipid peroxidation in human dermal fibroblasts when subjected to different *in vitro* settings simulating the hostile microenvironment of venous leg ulcers.

MATERIALS AND METHODS

Cell culture Dermal foreskin fibroblasts were established by outgrowth from biopsies of healthy human donors (Fleischmajer *et al*, 1981) at an age of 3–6 y and cultured in Dulbecco's modified Eagle's medium supplemented with glutamine (2 mM), penicillin (400 U per ml), streptomycin (50 mg per ml), and 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Cells were used at passages 5–15 corresponding to cumulative population doubling rates of 10–32 (Bayreuther *et al*, 1992).

[2]Meyer-Ingold W, Eichner W, Ettner N, Schink M: The composition of chronic wound fluid can be modulated by a dressing. *Wound Rep Reg* 7, A 281, 1999 (abstr.)

Generation of ROS and the Fenton reaction The Fenton reaction was simulated by exposure of confluent fibroblasts to concentrations of 220 μM Fe(III) citrate and 1 mM ascorbate in the presence or absence of H_2O_2 in serum-free medium. Non-toxic concentrations of dissolved DFO (220 μM), equimolar concentrations of DFO immobilized to cellulose, and cellulose dressings without DFO were used for the incubation. By means of the transwell chamber system (Costar, Bodenheim, Germany) we avoided mechanical stress induced by direct contact between cells and the dressing. Cytotoxicity was analyzed using the MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide] assay (Green *et al*, 1984). The viability of cells was more than 80% of nontreated controls at the used concentrations of Fe(III) citrate, ascorbate, dissolved DFO, and cellulose-coupled DFO. After a 4 h exposure of fibroblasts to different treatment modalities, which was performed in medium with or without serum, fibroblasts were washed with phosphate-buffered saline and either fresh medium without serum was added to determine the MMP-1 production after 24 h using a MMP-1 specific enzyme-linked immunosorbent assay (ELISA), or fibroblasts were directly lyzed in water for the high pressure liquid chromatography (HPLC) based measurement of malondialdehyde (MDA), the major product of lipid peroxidation.

ELISA An MMP-1 "sandwich" ELISA assay was performed according to the manufacturer's protocol (Amersham Pharmacia Biotech, Braunschweig, Germany) using precoated 96-well immunoplates, rabbit antihuman MMP-1 antibodies, and an antirabbit horseradish peroxidase conjugate. 3,3′,5,5′-Tetramethylbenzidine was used as peroxidase substrate. Optical densities were determined at 450 nm using a microtiter plate reader LP 400 (Sanofi Diagnostics Pasteur, Freiburg, Germany). MMP-1 concentrations in the samples were determined against standard curves using Graph Pad Software (San Diego, CA).

Measurement of thiobarbituric-acid-reactive substances Thiobarbituric-acid-reactive substances were measured as described previously (Yu *et al*, 1986) with minor modifications. Briefly, to 0.5 ml of cell lysate, 20 μl 5% butylated hydroxytoluene, 0.35 ml 20% trichloroacetic acid, and 0.5 ml 1.4% thiobarbituric acid were added. The mixtures were incubated at 95°C for 15 min. Following centrifugation, the samples were analyzed using HPLC on a 4 × 250 mm LiChrospher 100 RP-18 column with fluorescence detection. The mobile phase consisted of 55% potassium phosphate (50 mM, pH 5.5) and 45% methanol. Standard solutions of MDA (0.1–1 μM) were used for calibration. MDA concentrations were expressed per mg protein. Protein was determined using the Bradford assay (Biorad, Munich, Germany).

Collection of wound exudates and determination of total iron and protein content Wound fluids were collected from chronic venous leg ulcers of nine patients after coverage of the wounds with a transparent polyurethane semiocclusive film dressing (Cutifilm, Beiersdorf, Hamburg, Germany). After 30 min wound exudate was aspirated through the film dressing using a 30 G needle mounted on a 2 ml syringe. Acute wound exudate was obtained by drainage from four patients who had undergone mastectomy and one patient after excision of a myosarcoma, and from four patients by punctation of blisters from bullous pemphigoid. In addition, pooled human plasma derived from 50 donors was analyzed. The fluids were centrifuged at 10,000 × *g*, and supernatants were stored frozen at below –20°C before analysis. Only clear yellow exudates were analyzed for total iron levels using atomic absorption spectroscopy after microwave pressure disintegration with nitric acid. This method requires at least 200 μl of fluid with a detection limit of 1 ppm. The total protein content of each fluid was determined using established procedures (Bradford, 1976).

Covalent coupling of DFO to cellulose dressing DFO was covalently immobilized to a cellulosic support by using the 1,1′-carbonyldiimidazole (CDI) method (Hearn, 1987) yielding a stable urethane bond. Briefly, the procedure comprises two steps: (i) activation of the hydroxyl groups of cellulose support material (cotton gauze, viscose fabric, or cellulose film) was carried out with CDI in anhydrous acetone for 2 h according to an earlier published method (Feldhoff, 1992); (ii) reaction of the CDI-activated cellulose with DFO mesylate (Sigma, Deisenhofen, Germany) was performed as earlier described (Feldhoff, 1992) except that 25 mM sodium bicarbonate solution, pH 7.7, was used for 4 h. The extensively washed DFO–cellulose was air-dried at room temperature and sterilized by gamma irradiation at 30 kGy. Depending on the cellulose matrix used, between 20 and 100 μmol DFO could be bound to 1 g of cellulose fabric. For the production of placebo–cellulose all steps were carried out identically without adding CDI and DFO.

Leakage studies on gamma-irradiated immobilized DFO The stability of gamma-sterilized DFO–cellulose was examined by extracting 100 mg cellulose with 5 ml of either a methanol–water mixture (50:50 vol/vol) or physiologic solution (0.9% NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$) for 16 h at room temperature. The resulting extracts were analyzed directly, in the case of methanol, or after solvent exchange by solid phase extraction, in the case of physiologic extracts, by means of liquid chromatography/mass spectrometry (LC/MS) with a detection limit of 1 μg per ml for methanol extracts and 10 μg per ml for physiologic extracts.

In a parallel set of experiments, dry pulverized DFO was gamma irradiated (30 kGy) and subsequently analyzed for changes in its mass profile by means of LC/MS.

A third approach was used for the detection of volatile breakdown products of DFO– and placebo–cellulose before and after gamma sterilization applying headspace gas chromatography/mass spectrometry with a detection limit of 1–50 ppm. Three hundred and fifty milligrams of cellulose were equilibrated in gas-tight 20 ml vials at 80°C for 1 h. Thereafter analysis of 1 ml of the resulting headspace atmosphere was performed.

Determination of the amount of DFO coupled to cellulose (iron pick-up assay) and measurement of the kinetics of iron binding In order to determine the amount of functional DFO coupled to cellulose an indirect method was established. Accordingly, 15 mg of cellulose (placebo or verum) were incubated in 30 ml of a 150 μM $FeSO_4$ solution (pH 5.6) for 19 h at 25°C by mixing the content of tubes intensely on an overhead rotator (Heidolph REAX 2, Schwabach, Germany). The concentration of iron (Fe^{2+} and Fe^{3+}) in the supernatants before and after addition of cellulose samples was determined by means of the ready-to-use ferrozine-based assay MPR3 (Roche Diagnostics, Mannheim, Germany). This assay does not detect DFO-bound iron. The amount of DFO coupled to cellulose was calculated on the basis of the difference in iron concentration (Δ iron concentration, μM) as DFO chelates iron stoichiometrically. The resulting equation was: amount of immobilized DFO (μmol per g) = iron binding capacity (μmol per g) = Δ iron concentration (μM) × 2 ml per g sample. The results were corrected by subtracting the amount of unspecifically bound iron determined with placebo–cellulose. The results were expressed in μmol per cm^2 by multiplying the iron binding capacity with the area weight (g per cm^2).

The measurements of iron binding kinetics of gamma-sterilized DFO–cellulose were performed with slight modifications using an $FeSO_4$ solution at a concentration of about 50 μM and adding 40 ml iron solution per 15 mg cellulose sample (ratio DFO:Fe = 1.5:2.0). Samples were mixed intensely by overhead rotation at room temperature and iron concentrations were determined in the supernatants at defined time intervals.

In order to evaluate potential protein effects on the iron binding characteristics of DFO–cellulose a third setting was used: 50 mg (= 1.6 cm^2) of gamma-sterilized cellulose dressing (placebo or verum) were added to 1.33 ml of a 49.6 μM $FeSO_4$ solution supplemented with 30 mg per ml bovine serum albumin (BSA) (albumin fraction V, Merck, Darmstadt, Germany). After a 24 h period of intense mixing at 25°C, the iron concentration in the supernatants was determined as described above.

RESULTS

Iron is substantially increased in fluids derived from chronic venous leg ulcers compared to acute wounds or human plasma Total iron levels in nine exudates from patients suffering from chronic venous leg ulcers ranged between 56.2 and 182 μM with a mean of 110 μM and standard deviation of 42 μM. In order to compensate for different protein contents the values were related to grams of protein. Accordingly, iron levels in chronic wound fluids ranged between 1.86 and 5.7 μmol per g protein with a mean of 3.71 μmol per g protein and a standard deviation of 1.56 μmol per g protein (**Fig 1**). Substantially lower iron levels were found in pooled human plasma (21.8 μM Fe and 0.42 μmol Fe per g protein, respectively), in acute wound exudates from blisters of four patients (35.8 ± 0.7 μM Fe and 1.15 ± 0.62 μmol Fe per g protein, respectively), and in five drainage fluids (23.5 ± 9.3 μM Fe and 0.87 ± 0.34 μmol Fe per g protein, respectively).

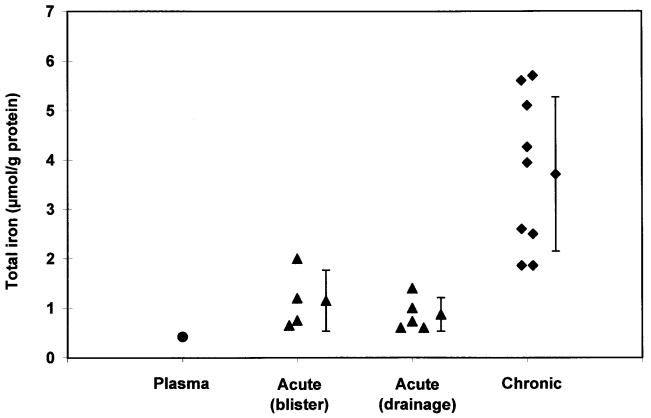

Figure 1. Total iron levels are significantly increased in chronic wound exudates compared to acute wound exudates and human plasma. Wound fluids were collected from chronic venous leg ulcers of nine patients, four patients who had undergone mastectomy, and one patient after surgical removal of a myosarcoma, and from blister fluids from four patients with bullous pemphigoid as detailed in *Materials and Methods*. Total iron content of each fluid and a sample of human plasma pooled from 50 donors was determined using atomic absorption spectrum analysis and expressed as μmol Fe per g protein. Data are shown as mean values with standard deviation. Significance was calculated using the Wilcoxon, Mann and Whitney *U* test ($p < 0.02$ – blister fluids; $p < 0.002$ – drainage fluids, compared to chronic wound fluids).

Effective reduction of iron ions from iron solutions by the gamma-sterilized DFO–cellulose dressing In order to assess the *in vitro* efficacy of the DFO–cellulose, the kinetics of iron uptake from a solution of 47 μM $FeSO_4$ by pieces of gamma-sterilized DFO–cellulose dressing was studied (DFO:Fe ratio 1.55:2.00) (**Fig 2**). The DFO dressing reduced the iron concentration in the BSA-free supernatant in a time-dependent manner by 50% after 6 h of incubation and 80% after 24 h. This corresponds to 93% of the total iron-binding capacity of the above specified cellulose. By contrast, no decrease in iron concentration was found up to 24 h for the same amount of control placebo dressing without DFO. When the DFO:Fe ratio was changed to 3.7:1, a 50% reduction was already achieved after 10 min, a 90% reduction after 30 min of incubation, and a 98% reduction of the initial iron concentration after 24 h of incubation. In order to demonstrate the ability of the iron pick-up dressing to remove iron ions also in the presence of protein, placebo or DFO-coupled cellulose was incubated in a BSA-containing $FeSO_4$ solution with a protein:cellulose ratio of 25 mg per cm^2. This test design mimics the conditions in chronic wound fluids concerning protein, total iron concentration, and the maximal volume of exudate produced in leg ulcers, which is 1.2 ml per cm^2 wound area in 24 h (Thomas *et al*, 1996). After a 24 h period of incubation, the initial iron concentration was reduced in the presence of BSA alone by 23%, in the placebo-containing sample by 25%, and in case of the DFO-coupled dressing by 99.5%.

DFO and DFO–cellulose withstand gamma irradiation Gamma-sterilized DFO–cellulose was subjected to two different extraction solvents and these extracts were analyzed using the highly sensitive method of LC/MS. Neither DFO nor its breakdown products could be detected. Furthermore, no change in the chemical structure of gamma-irradiated dry pulverized DFO was detectable, which would be identified by a different mass profile. In addition, no volatile breakdown products of DFO–cellulose before and after gamma sterilization were found. The iron-binding capacity of DFO–cellulose decreased negligibly after gamma irradiation as determined by means of the iron pick-up assay (data not shown).

Dissolved and cellulose-coupled DFO reduced the iron-driven induction of interstitial collagenase (MMP-1) We here established an *in vitro* model closely simulating the Fenton and Haber–Weiss reactions occurring in chronic venous leg ulcers. For

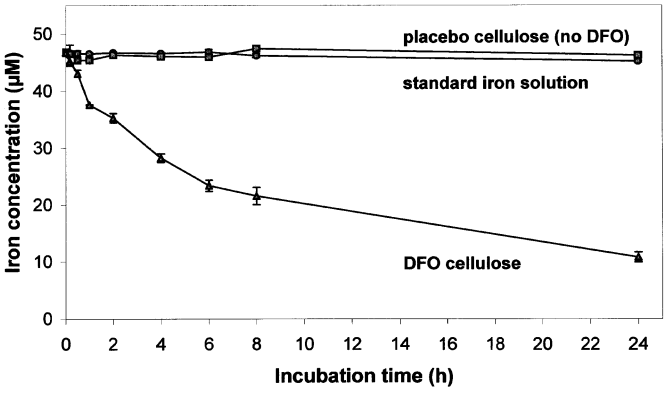

Figure 2. Effective reduction of iron ions in iron solution by gamma-sterilized DFO–cellulose. In order to determine the iron binding capacity of active functional DFO-coupled cellulose and placebo–cellulose, we have used the iron pick-up assay as detailed in *Materials and Methods*. Briefly, 15 mg of cellulose sample (placebo or DFO verum) were added to 40 ml $FeSO_4$ solution at an initial concentration of 47 µM (ratio DFO:Fe = 1.5:2.0). The concentration of iron was determined in the supernatants before and after addition of cellulose samples as detailed in *Materials and Methods*. The results were expressed as µmol per l. Three independent experiments were performed in triplicate. Each point represents the mean value with standard deviation.

this purpose human dermal fibroblasts were incubated with 220 µM Fe(III) citrate and 1 mM ascorbate in the presence of DFO (220 µM) dissolved in PBS, an equimolar amount of DFO coupled to cellulose, and control cellulose without DFO as detailed in *Materials and Methods*. We have used Fe(III) citrate concentrations in this *in vitro* model that relate to the upper range of Fe concentrations found in fluid from chronic wounds. Exposure of human dermal fibroblast monolayer cultures to Fe(III) citrate and ascorbate resulted in a 3-fold induction of the MMP-1 protein concentration compared to the mock-treated control. Exogenous addition of H_2O_2 did not further enhance this induction, most probably indicating that intracellular H_2O_2 levels are sufficient to drive the Fenton reaction (data not shown). This induction of MMP-1 could be suppressed by 75% in the presence of dissolved DFO at a concentration of 220 µM (**Fig 3*A***). Similar results were observed in the presence of 10%-serum-containing medium during the incubation with Fe(III) citrate (data not shown) indicating that the effect of Fe(III) citrate on MMP-1 induction is independent of serum. DFO-coupled cellulose suppressed the MMP-1 induction in human dermal fibroblasts by 80% of the control cellulose. Interestingly, addition of DFO–cellulose even at 2 h after initiating the Fenton reaction with Fe(III) citrate and ascorbate resulted in an almost complete inhibition of the MMP-1 induction indicating that iron-driven effects are at least in part responsible for the observed effects (**Fig 3*B***).

Dissolved and cellulose-coupled DFO reduced the iron-driven increase in lipid peroxidation Using the *in vitro* setting with Fe(III) citrate and ascorbate to simulate conditions of chronic venous leg ulcers, we have studied the effect of dissolved and cellulose-coupled DFO on lipid peroxidation. The Fe(III) citrate, ascorbate driven Fenton reaction resulted in a 5-fold increase in MDA, the major product of lipid peroxidation, as determined by HPLC based methods. This increase in MDA levels could be significantly reduced in the presence of dissolved DFO (**Fig 4*A***) or the pick-up dressing coupled with DFO (**Fig 4*B***) clearly indicating the functionality of dissolved and cellulose-coupled DFO.

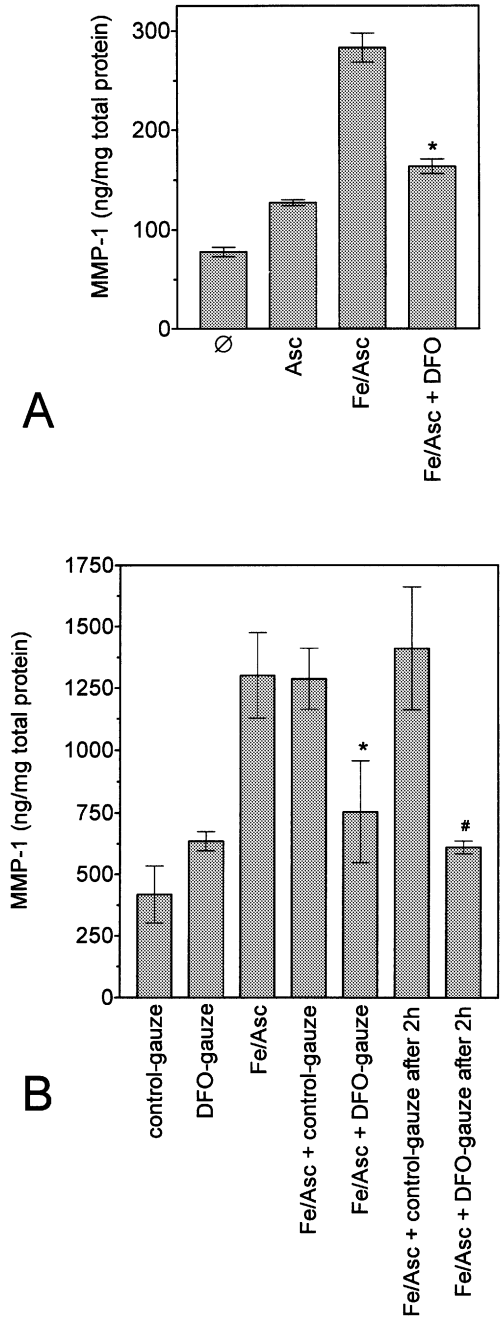

Figure 3. Dissolved and cellulose-coupled DFO suppresses the iron-driven induction of interstitial collagenase (MMP-1). Confluent monolayer cultures of human dermal fibroblasts were exposed for 4 h to a combination of Fe(III) citrate at a concentration of 220 µM and ascorbate at a concentration of 1 mM in the absence and presence of dissolved DFO (220 µM) (*A*), an equimolar amount of DFO immobilized to cellulose, or control cellulose without DFO (*B*) as detailed in *Materials and Methods*. Thereafter, cells were washed and after an incubation period of 24 h supernatants were collected and subjected to an MMP-1 specific ELISA. Three independent experiments have been performed in triplicate. Each bar represents the mean of a representative experiment with standard deviation. *p = 0.0002 (*A*); *p = 0.0247, #p = 0.0026 (*B*) compared with cells exposed to Fe(III) citrate/ascorbate (Student's *t* test).

DISCUSSION

Venous leg ulcers are common and cause considerable morbidity in the adult population. As healing may be slow or may never be achieved, ulcers create persistent and substantial demands on resources. A wide variety of dressings have been used, but to date there is no dressing on the market that can claim to significantly enhance tissue repair. This may at least partly be due to the limited knowledge on the microenvironment of chronic wounds. In fact,

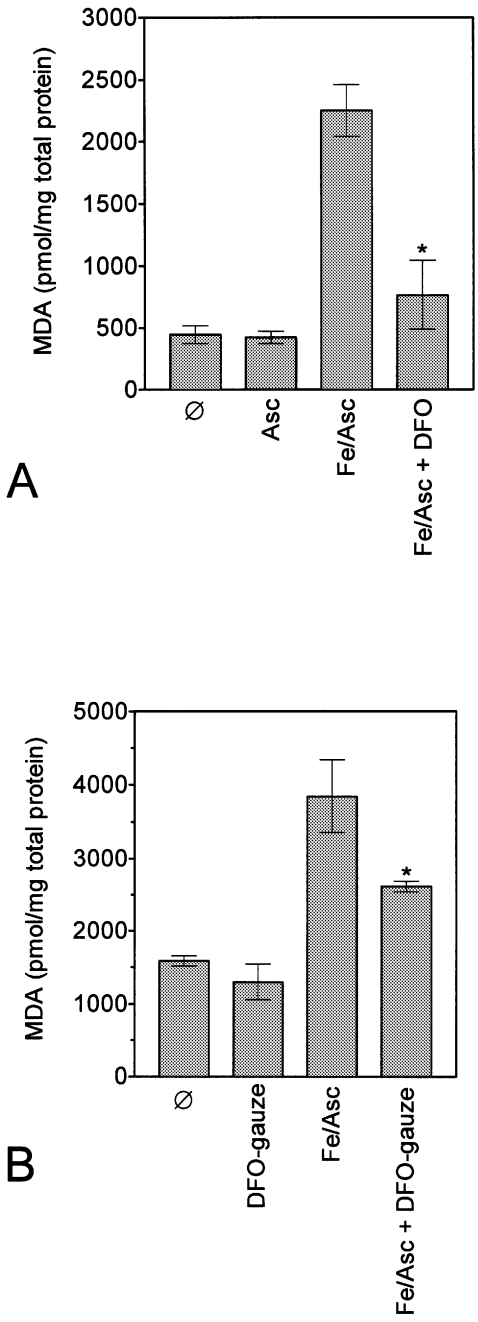

Figure 4. Dissolved and cellulose-coupled DFO reduces the iron-driven increase in lipid peroxidation. Confluent monolayer cultures of human dermal fibroblasts were exposed to a combination of Fe(III) citrate at a concentration of 220 μM and ascorbate at a concentration of 1 mM in the absence and presence of dissolved DFO (220 μM) (*A*), an equimolar amount of DFO immobilized to cellulose, and control cellulose without DFO (*B*) as detailed in *Materials and Methods*. The major product of lipid peroxidation, MDA, was measured in cell lysates using established procedures as detailed in *Materials and Methods*. MDA concentrations are expressed per mg protein. Three independent experiments in triplicate were performed to determine MDA concentrations. Each bar represents the mean of a representative experiment with standard deviation. *p = 0.0019 (*A*); *p = 0.0135 (*B*) compared to Fe(III) citrate/ascorbate treated cells (Student's *t* test).

the tremendous impact of the microenvironmental conditions on the outcome of wound healing has increasingly become apparent. Several comprehensive studies demonstrate that the proteolytic activity of matrix metalloproteinases and serine proteases is significantly upregulated in the exudate of chronic wounds (Grinnell *et al*, 1992; Wysocki *et al*, 1993; Rao *et al*, 1995; Grinnell and Zhu, 1996; Mast and Schultz, 1996; Weckroth *et al*, 1996; Herrick *et al*, 1997; Saarialho-Kere, 1998), thus contributing to the degradation of the extracellular matrix (Grinnell *et al*, 1992), growth factors (Wlaschek *et al*, 1997; Yager *et al*, 1997; Lauer *et al*, 2000) and potentially their receptors.

In this study we have first focused on the identification of potential deleterious factors that contribute to the prooxidant hostile microenvironment of chronic leg ulcers. Based on our findings with enhanced iron levels in the exudates of chronic wounds, we have designed and developed a novel iron chelating and reactive oxygen scavenging dressing with DFO-coupled cellulose and have subsequently evaluated this iron-selective pick-up dressing for its efficacy and functionality in several *in vitro* assays closely simulating prooxidant conditions in chronic venous leg ulcers.

We report here that total iron concentrations in exudates from chronic venous ulcers are significantly elevated compared to iron concentrations in acute wound fluids. This is not unexpected, as increased iron levels in ulcer tissue have been reported earlier (Ackerman *et al*, 1988). Moreover, it is known that ROS and proteases are able to release iron from different iron-containing proteins (Thomas *et al*, 1985; Biemond *et al*, 1988; Fridovich, 1995) most probably enhancing the transfer of iron from the tissue into the exudate. Due to low available amounts of ulcer fluid, only total iron contents have been measured. Using potentiometric stripping analysis that allows a distinction between different species of iron complexes, Liyanage *et al* (1996) found that in not further specified wounds almost no free iron ions can be detected. According to these authors, a considerable amount, 18%, of iron binds to citrate naturally occurring in plasma and presumably in wound fluid, too. Because the ferric iron binding constant of DFO (10^{31}) is much higher than that of citrate ($10^{10.5}$) (Königsberger *et al*, 2000), DFO should be able to preferentially extract ferric iron from citrate-coupled complexes. Furthermore, it is well documented that DFO is able to extract and subsequently bind iron ions from ferritin and in part also from transferrin (Keberle, 1964).

Increased iron levels in the exudate of chronic wounds and its iron-catalyzed toxicity with the generation of the highly aggressive hydroxyl radical may causally contribute to severe tissue damage most extensively observed in superficial layers of the ulcer base. The causal role of the iron-driven generation of hydroxyl radicals in venous ulceration and its persistence is substantiated by a clinical study with 133 enrolled patients that convincingly demonstrates that topical application of powder containing hydroxyl radical scavenging dimethylsulfoxide or the xanthine oxidase inhibitor allopurinol significantly stimulates the healing rate of venous leg ulcers (Salim, 1991). This author did not identify increased iron levels to be involved in the pathogenic sequence underlying impaired tissue repair, however.

In fact, the detrimental effects of increased free iron and its damaging effects have clearly been shown in primary and secondary hemochromatosis, thalassemia, and congenital hemolytic anemia, particularly in the heart. A significant positive correlation between the extent of cardiac iron deposits and cardial dysfunction and failure has been documented (Buja and Roberts, 1971; Zeimer *et al*, 1978; Motulsky, 1985). Furthermore, increased iron levels are thought to play a causal role in the progression of osteoarthritis (Okazaki *et al*, 1981; Blake *et al*, 1984), injury of the spinal cord (Zhang *et al*, 1996), and extrinsic skin aging (Bissett *et al*, 1990; Brenneisen *et al*, 1998). Interestingly, experimental and clinical studies demonstrate that iron chelation by DFO substantially protects the myocardial fibers against iron-dependent damage (Grisaru *et al*, 1986) and decreases necrosis in dorsally localized pig skin flaps (Weinstein *et al*, 1989). As iron in conjunction with H_2O_2 and $O_2^{-}\cdot$ generated by activated neutrophils and – under hypoxic conditions – by xanthine oxidase most probably drives the toxicity underlying persistent ulceration, we have set out to design an iron chelating dressing with antioxidant properties potentially disrupting this detrimental sequence of events. Among several iron

chelators, DFO is the most specific and potent, with a stability constant of 10^{31} (Keberle, 1964). Furthermore, DFO has been extensively studied in a variety of experimental and clinical settings with well-established efficacy and toxicity profiles (BeDell and Hulbert, 1999; Dollery, 1999).

We report here on the activation of functional groups of the cellulose dressing and successful covalent coupling of DFO to a cellulose dressing. As a result, approximately 2 μmol DFO were covalently bound per square centimeter of cellulose dressing, providing an iron complexing capacity sufficient to cope with the iron levels found in chronic venous leg ulcers. Furthermore, it could be shown that DFO–cellulose is able to bind iron out of a BSA-containing iron solution as an artificial chronic wound fluid. As stability after gamma sterilization represents an important prerequisite for a dressing, leakage of gamma-sterilized DFO-coupled cellulose was analyzed. No leakage of DFO or its breakdown products from the dressing was detected. This is in line with data from the literature on the extraordinary stability of urethane bonds (Bethell and Ayers, 1981).

As connective tissue breakdown and lipid peroxidation represent pathogenic hallmarks preventing healing of chronic venous leg ulcers, we established a model that closely simulates the iron-driven Fenton reaction. Using this model system, we have screened dissolved and cellulose-bound DFO for their protecting properties from Fe(III) citrate/ascorbate driven upregulation of MMP-1, the major metalloproteinase in connective tissue breakdown, and enhanced lipid peroxidation. We found that exposure of fibroblasts to the Fenton reaction mimicking combination of 220 μM Fe(III) citrate and 1 mM ascorbate resulted in a 4-fold induction of MMP-1 levels. This induction was almost completely suppressed by dissolved DFO at a concentration of 220 μM or the equimolar amount of DFO covalently coupled to cellulose.

In addition, the Fe(III) citrate/ascorbate driven Fenton reaction resulted in a 5-fold increase in MDA, the major product of lipid peroxidation. The increase in MDA levels could be significantly reduced in the presence of dissolved DFO or DFO-coupled cellulose. Collectively, these data indicate that DFO in its dissolved or bound form effectively prevents the development of a prooxidant microenvironnment and, furthermore, protects from unfavorable consequences like MMP-1 and lipid peroxide induction. We anticipate that the protective effect of DFO by the above mentioned mechanisms limits tissue injury and the perpetuated recruitment of activated neutrophils characteristic for chronic venous leg ulcers. In fact, in a TPA (12-O-tetradecanoyl-phorbol-13-acetate) induced inflammation model, topical application of DFO prior to TPA treatment could almost completely inhibit the neutrophil influx into the interstitial tissue of mouse ears (Soybir *et al*, 1996).

Based on our promising results a double blind placebo controlled clinical study has been started to prove the principle and assess the therapeutic value for patients suffering from nonhealing wounds.

We gratefully acknowledge the stimulating discussion with Cecilia Welling.

REFERENCES

Ackerman Z, Seidenbaum M, Loewenthal E, Rubinow A: Overload of iron in the skin of patients with varicose ulcers. Possible contributing role of iron accumulation in progression of the disease. *Arch Dermatol* 124:1376–1378, 1988

Bayreuther K, Francz PI, Rodemann HP: Fibroblasts in normal and pathological terminal differentiation. *Aging, Apoptosis Transformation Arch Geront Geriatr* (Suppl.3):47–74, 1992

BeDell LS, Hulbert MK, eds: *Physicians GenRx – Mosby's Complete Drug Reference*, 9th edn. St. Louis, MO: Mosby-Year Book, 1999

Bethell GS, Ayers JS: Investigation of the activation of various insoluble polysaccharides with 1,1′-carbonyldiimidazole and of the properties of the activated matrices. *J Chromat* 219:361–372, 1981

Biemond P, Swaak AJG, van Eijk HG, Koster JF: Superoxide dependent iron release from ferritin in inflammatory diseases. *Free Radic Biol Med* 4:185–198, 1988

Bissett DL, Chatterjee R, Hannon DP: Photoprotection effect of superoxide scavenging antioxidants against ultraviolet radiation-induced chronic skin damage in the hairless mouse. *Photodermatol Photoimmunol Photomed* 7:56–62, 1990

Blake DR, Gallagher PH, Potter AR: The effect of synovial iron on the progression of rheumatoid disease. *Arthritis Rheum* 27:495–501, 1984

Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254, 1976

Brenneisen P, Wenk J, Klotz LO, et al: Central role of ferrous/ferric iron in the ultraviolet B irradiation-mediated signaling pathway leading to increased interstitial collagenase (matrix-degrading metalloprotease (MMP-1) and stromelysin-1 (MMP-3) mRNA levels in cultured human dermal fibroblasts. *J Biol Chem* 273:5279–5287, 1998

Buja LM, Roberts WC: Iron in the heart: ethiology and clinical significance. *Am J Med* 51:209–221, 1971

Cheatle T: Venous ulceration and free radicals. *Br J Dermatol* 124:508, 1991

Dollery C: ed. *Therapeutic Drugs CD-ROM Database Release 1.0*. London: Churchill Livingstone – Harcourt Brace Ltd, 1999

Edwards JV, Batiste SL, Gibbins EM, Goheen SC: Synthesis and activity of NH_2- and COOH-terminal elastase recognition sequences on cotton. *J Peptide Res* 54:536–543, 1999

Falanga V, Eaglstein WH: The "trap" hypothesis of venous ulceration. *Lancet* 341:1006–1008, 1993

Feldhoff PW: Comparison of coupling procedures for development of affinity membranes: opimization of the CDI method. *Techniques in Protein Chemistry III*. London: Academic Press, 1992, pp 151–160

Fleischmajer R, Perlish JS, Krieg T, Timpl R: Variability in collagen and fibronectin synthesis by scleroderma fibroblasts in primary culture. *J Invest Dermatol* 76:400–403, 1981

Fridovich I: Superoxide radical and superoxide dismutases. *Annu Rev Biochem* 64:97–112, 1995

Green LM, Reade JL, Ware CF: Rapid colorimetric assay for cell viability: application to the quantitation of cytotoxic and growth inhibitory lymphokines. *J Immunol Methods* 70:257–268, 1984

Grinnell F, Zhu M: Fibronectin degradation in chronic wounds depends on the relative levels of elastase, α1-proteinase inhibitor, and α2-macroglobulin. *J Invest Dermatol* 106:335–341, 1996

Grinnell F, Ho CH, Wysocki A: Degradation of fibronectin and vitronectin in chronic wound fluid: analysis by cell blotting, immunoblotting, and cell adhesion assays. *J Invest Dermatol* 98:410–416, 1992

Grisaru D, Goldfarb AW, Gotsman MS: Deferoxamine improves left ventricular function in beta-thalassemia. *Arch Intern Med* 146:2344–2349, 1986

Gutteridge JMC, Richmond R, Halliwell B: Inhibition of the iron-catalysed formation of hydroxyl radicals from superoxide and of lipid peroxidation by desferrioxamine. *Biochem J* 184:469–472, 1979

Halliwell B, Gutteridge JMC: *Free Radicals in Biology and Medicine*, 2nd edn. Oxford: Clarendon Press, 1989

Hearn MTW: 1,1′-Carbonyldiimidazole-mediated immobilization of enzymes and affinity ligands. *Meth Enzymol* 135:102–117, 1987

Herrick S, Ashcroft G, Ireland G, Horan M, McCollum C, Ferguson M: Up-regulation of elastase in acute wounds of healthy aged humans and chronic venous leg ulcers are associated with matrix degradation. *Lab Invest* 77:281–288, 1997

Keberle H: The biochemistry of desferrioxamine and its relation to iron metabolism. *Ann N Y Acad Sci* 119:758–768, 1964

Königsberger LC, Königsberger E, May PM, Hefter GT: Complexation of iron(III) and iron(II) by citrate. Implications for iron speciation in blood plasma. *J Inorg Biochem* 78:175–184, 2000

Lauer G, Sollberg S, Cole M, et al: Expression and proteolysis of vascular endothelial growth factor is increased in chronic wounds. *J Invest Dermatol* 115:12–18, 2000

Liyanage JA, Taylor DM, Williams DR: Chemical speciation in wound fluid leading to enhanced bioavailability for healing. *Chem Speciation Bioavailability* 8:45–52, 1996

Mast BA, Schultz GS: Interactions of cytokines, growth factors, and proteases in acute and chronic wounds. *Wound Rep Reg* 4:411–420, 1996

Meyer-Ingold W, Eichner W, Ettner N, Schink M: Wound coverings for removal of interfering factors from wound fluid. *Patent Application EP 0 945 144 A2*, 1998

Monboisse JC, Borel JP: Oxidative damage to collagen. In Emerit I, Chance B: eds. *Free Radicals and Aging. Experimentia (Suppl 62)*. Basel: Birkhäuser Verlag, 1992, pp 323–327

Motulsky AG: Hemochromatosis (iron storage disease). In Wyngaarden JB, Smith LH, eds. *Cecil's Textbook of Medicine*, 17th edn. Philadelphia: W.B. Saunders, 1985, pp 1160–1163

Okazaki I, Brinckerhoff CE, Sinclair JF: Iron increases collagenase production by rabbit synovial fibroblasts. *J Lab Clin Med* 97:396–402, 1981

Rao CN, Ladin DA, Liu YY, Chilukuri K, Hou ZZ, Woodley DT: α1-Antitrypsin is degraded and non-functional in chronic wounds but intact and functional in acute wounds: the inhibitor protects fibronectin from degradation by chronic wounds fluid enzymes. *J Invest Dermatol* 105:572–578, 1995

Rosner K, Ross C, Karlsmark T, Petersen AA, Gottrup F, Vejlsgaard GL: Immunohistochemical characterization of the cutaneous cellular infiltrate in different areas of chronic leg ulcers. *APMIS* 103:293–299, 1995

Saarialho-Kere UK: Patterns of matrix metalloproteinase and TIMP expression in chronic ulcers. *Arch Dermatol Res* 290:S47–S54, 1998

Salim AS: The role of oxygen-derived free radicals in the management of venous (varicose) ulceration: a new approach. *World J Surg* 15:264–269, 1991

Schaich KM: Metals and lipid oxidation. Contemporary issues. *Lipids* 27:209–218, 1992

Soybir GR, Koyuneu H, Koksoy F: Protective effect of desferrioxamine against TPA caused inflammation in CD-1 mouse skin. *Surg Oncol* 5:253–258, 1996

Thomas CE, Morehouse LA, Aust SD: Ferritin and superoxide-dependent lipid peroxidation. *J Mol Biol* 260:3275–3280, 1985

Thomas S, Fear M, Humphreys J, Disley L, Waring MJ: The effect of dressings on the production of exudate from venous leg ulcers. *Wounds* 8:145–150, 1996

Weckroth M, Vaheri A, Lauharanta J, Sorsa T, Konttinen YT: Matrix metalloproteinases, gelatinase and collagenase, in chronic leg ulcers. *J Invest Dermatol* 106:1119–1124, 1996

Weinstein GS, Maves MD, McCormack ML: Deferoxamine decreases necrosis in dorsally based pig skin flaps. *Otolaryngol Head Neck Surg* 101:559–561, 1989

Weiss SJ: Tissue destruction by neutrophils. *N Engl J Med* 320:365–376, 1989

Wenner A, Leu HJ, Spycher M, Brunner U: Ultrastructural changes of capillaries in chronic venous insufficiency. *Expl Cell Biol* 48:1–14, 1980

Wlaschek M, Peus D, Achterberg V, Meyer-Ingold W, Scharffetter-Kochanek K: Protease inhibitors protect growth factor activity in chronic wounds. *Br J Dermatol* 137:646–663, 1997

Wysocki AB, Staiano-Coico L, Grinnell F: Wound fluid from chronic leg ulcers contains elevated levels of metalloproteinases MMP-2 and MMP-9. *J Invest Dermatol* 101:64–68, 1993

Yager DR, Chen SM, Ward SI, Olutoye OO, Diegelmann RF, Cohen IK: Ability of chronic wound fluids to degrade peptide growth factors is associated with increased levels of elastase activity and diminished levels of proteinase inhibitors. *Wound Rep Reg* 5:23–32, 1997

Yu LW, Latriano L, Duncan S, Hartwick RA, Witz G: High-performance liquid chromatography analysis of the thiobarbituric acid adducts of malonaldehyde and trans,trans-muconaldehyde. *Anal Biochem* 156:326–333, 1986

Zeimer R, Belkin M, Leitersdorff E: A non-invasive method for the evaluation of tissue iron deposition in beta thalassemia major. *J Lab Clin Med* 91:24–31, 1978

Zhang JR, Scherch HM, Hall ED: Direct measurement of lipid hydroperoxides in iron-dependent spinal neuronal injury. *J Neurochem* 66:355–361, 1996