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Heme-Scavenging Role of α_1 -Microglobulin in Chronic Ulcers

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Chronic venous ulcers are characterized by chronic inflammation. Heme and iron, originating from blood cell hemolysis as well as extravascular necrosis, have been implicated as important pathogenic factors due to their promotion of oxidative stress. It was recently reported that the plasma and tissue protein α_1 -microglobulin is involved in heme metabolism. The protein binds heme, and a carboxy-terminally processed form, truncated α_1 -microglobulin, also degrades heme. Here, we show the presence of micromolar levels of heme and free iron in chronic leg ulcer fluids. Micromolar amounts of α_1 -microglobulin was also present in the ulcer fluids and bound to added radiolabeled heme. Truncated α_1 -microglobulin was found in the ulcer fluids and exogenously added α_1 -microglobulin was processed

into the truncated α_1 -microglobulin form. Histochemical analysis of chronic wound tissue showed the presence of iron deposits, heme/porphyrins in infiltrating cells basement membranes and fibrin cuffs around vessels, and α_1 -microglobulin ubiquitously distributed but especially abundant in basement membranes around vessels and at fibrin cuffs. Our results suggest that α_1 -microglobulin constitutes a previously unknown defense mechanism against high heme and iron levels during skin wound healing. Excessive heme and iron, which are not buffered by α_1 -microglobulin, may underlie the chronic inflammation in chronic ulcers. *Key words: inflammation/iron/lipocalin/oxidative stress/plasma protein. J Invest Dermatol 121:640–646, 2003*

Wound healing is a complex process encompassing phases of inflammation, granulation tissue formation, angiogenesis, and epithelialization. Aberrant and defective wound healing occurs in patients with chronic ulcers. This condition represents a major health problem and an increasing burden to health care providers. The major group of ulcers, venous leg ulcers, are characterized by persistent and unregulated chronic inflammation, involving extravasation of inflammatory cells, release of proteases and degradation of matrix molecules, growth factors, and other constituents (Ågren *et al*, 2000; Falanga, 2001).

Several causative mechanisms underlying the chronic inflammation have been proposed. The sustained venous pressure of these patients leads to a reduced pressure differential between the arterial and venous systems, thus trapping leukocytes in the connective tissue. Extravasation of leukocytes also occurs in nonulcer patients with venous disease and may lead to ulcer formation (Wilkinson *et al*, 1993). Extravasation of fibrin lead to the formation of fibrin depositions (fibrin cuffs), possibly trapping and inactivating growth factors involved in wound healing in the perivascular areas (Falanga, 2001). Furthermore, all chronic ulcers are constantly colonized or infected by various bacteria such as *Pseudomonas aeruginosa*, *Escherichia faecalis*, and *P. mirabilis* and recent evidence supports the view that high bacterial loads contribute to nonhealing (Davies *et al*, 2001).

Iron, in free form or complex-bound to protoporphyrin IX in heme, has been implicated as an important pathogenic factor in inflammation in general and in leg ulcers in particular (Ackerman *et al*, 1988; Wagener *et al*, 2001; Wenk *et al*, 2001). The oxidized ferric (Fe^{3+}) forms of heme and free iron are strong oxidative agents and the ferrous iron (Fe^{2+}) can generate oxygen-derived free radicals by the Fenton reaction (Halliwell and Gutteridge, 1989). These compounds present considerable oxidative stress that leads to tissue damage and cell destruction, and have been proposed to participate in the pathogenesis of chronic ulcers (for a review, see Ågren *et al*, 2000) by initiating local lipid peroxidation of polyunsaturated fatty acids in cell membranes as well as enhancing the synthesis and activation of matrix-degrading metalloproteinases.

α_1 -Microglobulin ($\alpha_1\text{m}$) is a 26 kDa glycoprotein that is evolutionarily well-conserved and has so far been found in mammals, birds, fish, and amphibians (Åkerström and Lögdberg, 1990; Åkerström *et al*, 2000b). It belongs to the lipocalin superfamily, a group of proteins from animals, plants, and bacteria with very diverse functions but a well-conserved three-dimensional structure (Åkerström *et al*, 2000a). The lipocalins consist of a 160 to 190 amino acid polypeptide folded into an eight-stranded β -barrel surrounding a pocket that can bind hydrophobic ligands. About half of the $\alpha_1\text{m}$ -molecules are complex-bound to IgA (Grubb *et al*, 1986). Both free and IgA-bound $\alpha_1\text{m}$ are found in most tissues intravascularly and extravascularly. It was recently shown that $\alpha_1\text{m}$ has heme-binding properties and may be involved in extracellular heme catabolism (Allhorn *et al*, 2002). Formation of a processed form of $\alpha_1\text{m}$, lacking the C-terminal tetrapeptide LIPR (leucine-isoleucine-proline-arginine), is induced by hemoglobin. This processed $\alpha_1\text{m}$ form, called t- $\alpha_1\text{m}$ (t = truncated), has both heme-binding and heme-degrading properties. The results indicate a protective role of $\alpha_1\text{m}$ against heme-induced oxidative damage. Considering the proposed

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Abbreviations: $\alpha_1\text{m}$, α_1 -microglobulin; LIPR, leucine-isoleucine-proline-arginine; t- $\alpha_1\text{m}$, truncated α_1 -microglobulin (= lacking the C-terminal tetrapeptide LIPR).

pathogenic role of heme, we have investigated concentration, distribution, and biochemical properties of α_1 m in wound fluids from chronic venous leg ulcers. It is shown that α_1 m is ubiquitously distributed in wound sections, that it is a heme-binding protein in ulcer fluids, and that t- α_1 m is formed. These results are consistent with a protective role of α_1 m in the ulcer fluids as a heme antagonist.

MATERIALS AND METHODS

Patients, wound fluid, and plasma Wound fluid and plasma were collected from patients with venous ulcers. Wound fluid was collected either by sampling on filters as described previously (Schmidtchen, 1999, 2000) or under a Tegaderm dressing (Schmidtchen, 2000). In the first procedure (used in experiments described in Fig 1; sampling from a group of 12 patients), sterilized filters (Whatman GF/D; diameter 2.5 cm) were applied to wounds for 4 h and wound fluid was extracted from the filter. Proteinase activity was blocked by the addition of the proteinase inhibitors diisopropyl phosphorofluoridate, *N*-ethylmaleimide, and ethylenediamine tetraacetic acid (Schmidtchen, 2000). The filters did not induce degradation of α_1 -m in human plasma (not shown). Wound fluid from 12 patients with venous ulcers (>3 mo duration) was used for the experiments. In the second procedure (used for all other experiments), Tegaderm dressings were applied on the wound and wound fluid was collected by gentle aspiration underneath the film after 2 h, centrifuged at 10,000 r.p.m. (3500 g) in an Eppendorf centrifuge, aliquoted and stored at -20°C until further use. The venous insufficiency was routinely determined either by a handheld Doppler (5 MHz probe; examination of reflux in the popliteal vein, great saphenous vein and small saphenous vein) or by color duplex examination. The patients had a systolic index of more than 0.8. Patients with diabetes or signs of general infection (malaise, fever) or local infection (cellulitis, erysipelas) were excluded. Plasma from healthy volunteers was collected by intravenous puncture. The research project was approved by the local Ethics Committee. Informed consent was obtained from the patients.

Proteins and reagents Human plasma α_1 m was prepared from plasma as described previously (Åkerström *et al.*, 1995; Berggård *et al.*, 1997, 1999). Protein LG was a generous gift from Dr Lars Björck, Department of Cell

and Molecular Biology, Lund University, Sweden, and was coupled to CNBr-activated Sepharose-4B (Pharmacia-Biotech, Uppsala, Sweden), 7 mg per mL gel, as described by Pharmacia-Biotech. Orosomucoid, human albumin and other chemicals were from Sigma-Aldrich Stockholm, Sweden AB if not indicated otherwise. Mouse monoclonal anti-human α_1 m antibody was raised as described (Nilson *et al.*, 1987). Rabbit anti-serum against human α_1 m was prepared in this laboratory by following the earlier description (Berggård and Bearn, 1968). Rabbit anti-LIPR was prepared by AgriSera AB (Vännäs, Sweden) by immunization with the synthetic peptide CKKLIPR conjugated to keyhole limpet hemocyanin (KLH). The preparation of goat anti-rabbit immunoglobulins has been described previously (Björck *et al.*, 1977). Proteins were labeled with [^{125}I]iodide (Bio-Nuclear AB, Stockholm, Sweden) using the chloramine T method (Greenwood *et al.*, 1963). Labeled proteins were separated from free iodide by gel filtration on Sephadex G-25 column (Pharmacia). The specific activity was approximately 0.5 MBq per μg protein.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting of α_1 m in wound fluids and plasma The molecular forms of α_1 m in wound fluids and plasma were investigated by SDS-PAGE and immunoblotting. SDS-PAGE was performed under denaturing conditions using 12% gels in the buffer system described by Laemmli (1970). Samples mixed with sample buffer 1:1 (v/v) containing 2% (v/v) β -mercaptoethanol and SDS were boiled 3 min before loading to the gel. High molecular mass standards (Rainbow markers, Amersham, International plc, Amersham, Buckinghamshire, England) were used. The gels were stained with Coomassie Brilliant Blue R-250 (BDH Chemicals, Ltd, Poole, UK) and dried. In some cases 3% to 12% polyacrylamide gradient gels with a 3% stacking gel were used. The separated proteins were transferred to polyvinylidene fluoride membranes (Immobilon, Millipore, Bedford, Massachusetts) as described (Matsudaira, 1987). Immunoblotting was performed as previously described (Schmidtchen, 1999) using polyclonal anti-sera against α_1 m diluted 1000 times. The membranes were developed using the ECL system (Boehringer, Mannheim GmbH, Mannheim, Germany).

Affinity chromatography of wound fluids To distinguish between full-length α_1 m and t- α_1 m, i.e., the form of the protein that lacks the C-terminal tetrapeptide LIPR, α_1 m was purified from wound fluids by anti- α_1 m affinity chromatography and subsequently immunoblotted both with antibodies against full-length α_1 m and antibodies against LIPR. Affinity chromatography was performed according to an earlier description (Allhorn *et al.*, 2002), using mouse monoclonal anti- α_1 m, BN11.10, which had been immobilized to Affigel Hz (Bio-Rad Laboratories, Hercules, California) at 20 mg per mL following instructions from the merchant. The eluted proteins were separated by SDS-PAGE and identified by immunoblotting (see above) using anti- α_1 m diluted 1000 times or anti-LIPR diluted 300 times. The blots were developed by incubation with ^{125}I -goat anti-rabbit immunoglobulin as described (Wester *et al.*, 1997) and analysis with a Fuji Bas 2000 Bio-imaging analyzer (Fuji Films Co., Japan).

Cleavage of α_1 m by wound fluids The cleaving activity of wound fluids was tested by adding small amounts of radiolabeled α_1 m. One Bq of ^{125}I - α_1 m or ^{125}I -IgA- α_1 m was added to 1 μL wound fluid (10 μg total proteins as measured by the Bradford method) diluted in phosphate-buffered saline (PBS: 10 mM phosphate buffer, pH 7.4, 120 mM NaCl, 3 mM KCl). The reaction proceeded for 1 h at room temperature. The samples were then applied to SDS-PAGE and the dried gel analyzed with a Fuji Bas 2000 Bio-imaging analyzer.

Binding of [^{14}C]heme to α_1 m in wound fluids [^{14}C]heme was produced using the *Escherichia coli* strain AN344 containing the plasmid pTYR13 as described by Schiött *et al.*, 1997). AN344 is blocked in delta-amino levulinic acid (ALA) synthesis and requires ALA for growth. Thus, the addition of 31 μM (50.8 Ci per mol) [^{14}C]ALA (New England Nuclear Inc., Boston, MA) to strain AN344/pTYR13 in Luria broth (LB) medium resulted in production of [^{14}C]heme with the yield 20 nmol per 60 mL culture and with a specific activity of 2.5 Ci per mmol. [^{14}C]heme (15 pmol) was incubated with 1.5 μL wound fluid (15 μg total proteins as measured by the Bradford method) with or without added α_1 m (45 pmol) or albumin (45 pmol), or incubated with these amounts of α_1 m or albumin alone, diluted in PBS + 0.05% Tween-20 to 10 μL . After 30 min at 20°C , SDS-PAGE sample buffer was added and the samples analyzed by SDS-PAGE as described above, the gels dried and the radioactivity analyzed with a Fuji Bas 2000 Bio-imaging analyzer.

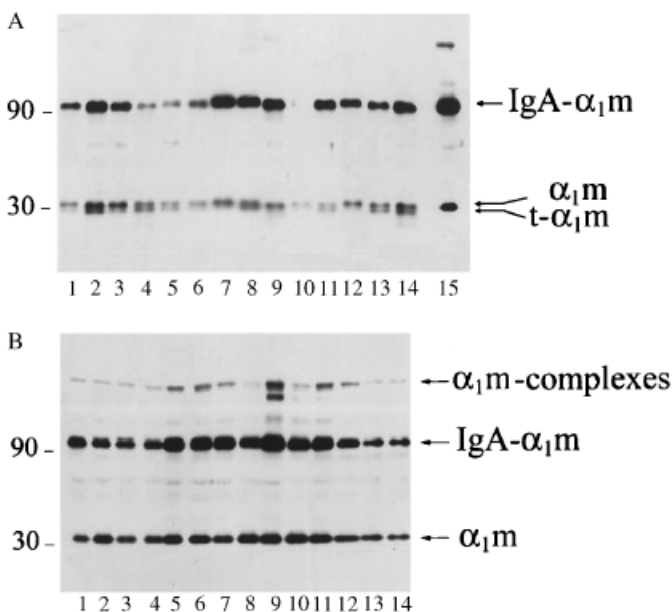


Figure 1. α_1 m in wound fluids and plasma. Twelve samples of wound fluid (A) and plasma, diluted 200 \times (B) indicated with numbers 3 to 14, each 10 μg total protein, were applied on SDS-PAGE, 3% to 12%, and immunoblotted using polyclonal anti-sera against α_1 m. Samples from one patient were collected during three visits (corresponding to lane numbers 1–3). Number 15 on (A) displays a normal plasma sample. Molecular weight standards (in kDa) are indicated to the left.

Binding of [14 C]heme in HepG2 cell cultures To measure the binding of heme to newly synthesized α_{1m} , [14 C]heme was added to cultures of the liver cell line HepG2, which synthesize and secrete α_{1m} . HepG2 cells were grown at 37°C in RPMI 1640 medium (Gibco, Life Technologies, Gaithersburg, MD) in an atmosphere of 5%. The medium contained 10% fetal bovine serum (FBS), L-glutamine, 300 μ g per mL, and 100 μ g per mL antibiotics (penicillin and streptomycin). In one experiment, 0.03 μ M Na₂SeO₃ was used instead of FBS, using conditions described previously (Åkerström *et al*, 1995). The cells were split 1:5 or 1:10 by trypsin digestion twice a week. Fresh culture medium was added to flasks with confluent cells, and 24 h later, [14 C]heme was added to a final concentration of 0.07 to 0.4 μ M and the incubation proceeded for 3 h. The cells were centrifuged and the medium removed and saved for immunoprecipitation, purification of α_{1m} by affinity chromatography (see above), and radioimmunoassay. The cells were lysed by resuspension in 15 mL 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% NaN₃, 1% Triton X-100, 0.25% nonidet P-40, 0.25% bovine serum albumin. The suspension was then centrifuged, 1000 r.p.m., (700g) 20 min 1 mL medium or 1 mL cell lysate was immunoprecipitated with 10 μ L of polyclonal antibody (100 μ g IgG) against α_{1m} . As a control, 10 μ L polyclonal anti-rosomucoid (Dakopatts Glostrup, Denmark) was used. After 20 h incubation at 4°C, 30 μ L protein LG-Sepharose (50% 1:1 suspension) was added and the incubation continued for 2 h at room temperature. After centrifugation and removal of supernatant, the immunoprecipitates were washed several times with PBS. The pellets were finally solubilized in SDS-PAGE sample buffer and analyzed by SDS-PAGE. The gels were dried and analyzed with a Fuji Bas 2000 Bio-imaging analyzer.

Biopsies Four millimeter biopsies were taken from the wound edge of a chronic venous leg ulcer. Control biopsies were taken from the edges of acute wounds or from healthy skin of the thigh. The tissue samples were fixed overnight in 4% paraformaldehyde, dehydrated sequentially in ethanol and subsequently embedded in paraffin. Tissue sections (5 μ m) were cut in a microtome, mounted on slides, and air dried overnight in a 37°C incubator.

Immunohistochemistry Sections were deparaffinized by routine procedures and endogenous peroxidase activity was blocked with 3% H₂O₂ in methanol for 10 min. After rinsing in distilled water, followed by PBS, the slides were subsequently permeabilized with Tween 20 (0.05%) in PBS. Thereafter, sections were rinsed in PBS. After preincubation with normal goat serum (1%) for 30 min at room temperature, incubation was performed with primary antibody diluted 1:500 in PBS (containing 0.05% Tween 20, 0.2% bovine serum albumin) overnight at 4°C. Control sections were incubated in the same PBS solution without antibody. Antibody detection was performed with a standard avidin-biotin complex detection system after which they were developed with 3,3'-diaminobenzidine tetrahydrochloride as the chromogenic substrate (Vectastain avidin-biotin complex, Vector Laboratories, Burlingame, California). Sections were counterstained with Mayer's Hemalum (Diagnostica Merc, Darmstadt, Germany), mounted with Pertex (Histolab Products AB, Gothenburg, Sweden) and examined and photographed (Olympus BHS photomicrographic system).

Iron and porphyrin staining For iron staining, paraffin-embedded skin sections were dewaxed and rehydrated. Iron deposition was examined using Perls' Prussian blue staining. The sections were incubated in a solution containing 1% potassium ferrocyanide in 1% HCl for 1 h. After several washes in H₂O the sections were incubated with a 1% neutral red solution for 5 min and washed again in H₂O. Dehydrated and differentiated sections were mounted in DPX-type mountant. Porphyrins were stained using Fouchet technique, the method of choice for demonstration of bile pigments. After 5 min incubation in a reagent containing 12.5% trichloroacetic acid and 5% aqueous ferric chloride, the sections were washed in distilled water and counterstained with van Gieson's solution: saturated picric acid, 0.1% acid fuchsin, and 0.1% glacial acetic acid, for 3 min. The sections were washed in alcohol before dehydration.

Determination of α_{1m} , porphyrin, and iron concentrations in wound fluids and plasma Specific concentrations of α_{1m} were measured by a soluble competitive radioimmunoassay (Åkerström, 1985). Protoporphyrin IX was detected using the pyridin-hemochromogen method as described by Falk (1964). Iron concentrations were determined by a spectrophotometric procedure with hydroxylamine and thioglycolate as the reducing agents and ferrozine as the ferrous ion complexing agent, at the Department of Clinical Chemistry, Malmö General Hospital, Malmö,

Sweden. The assays were performed with SYNCHRON LX Systems, Beckman Coulter, Inc., Fullerton California.

RESULTS

α_{1m} in wound fluids The wound fluids from 12 patients with chronic venous ulcers contained three variants of α_{1m} : free monomeric α_{1m} , t- α_{1m} , and α_{1m} complex-bound to IgA (IgA- α_{1m}) when analyzing by SDS-PAGE (Fig 1). The IgA- α_{1m} complex appears on SDS-PAGE as a nonreducible 90 kDa protein band containing α_{1m} covalently linked to the α -chain. A large variation in the relative concentrations of the three forms was noted. In contrast, plasma from the same patients contained no t- α_{1m} and displayed less variation between individuals. Furthermore, the various high-molecular-weight α_{1m} complexes described previously (Berggård *et al*, 1997) could be seen in plasma samples but were absent in wound fluids. T- α_{1m} in wound fluids was identified by purification of α_{1m} on anti-human α_{1m} affinity chromatography, followed by immunoblotting with anti-LIPR (Fig 2). The results obtained confirmed that the protein band corresponding to t- α_{1m} on SDS-PAGE lacked the C-terminal tetrapeptide.

Cleavage of α_{1m} The appearance of t- α_{1m} on SDS-PAGE prompted a further investigation of the cleaving activity of wound fluids. Thus, ¹²⁵I- α_{1m} or ¹²⁵I-IgA- α_{1m} was added to wound fluid samples and the mixtures were then analyzed on SDS-PAGE. The results obtained revealed the presence of a radioactive protein band corresponding to t- α_{1m} , approximately 3 to 4 kDa smaller compared with native α_{1m} (Fig 3). ¹²⁵I-IgA- α_{1m} was also processed, which caused disappearance of the 90 kDa band and appearance of free α_{1m} .

Binding of [14 C]heme to α_{1m} α_{1m} was added to wound fluid and the binding to exogenously added [14 C]heme was tested (Fig 4). A clear binding of radiolabeled heme to α_{1m} in the wound fluids could be seen (lane 1) and to α_{1m} alone without wound fluid present (lane 2). Albumin, a known physiologic heme-binding protein, also bound the added heme when added to wound fluid (lane 3). The binding of [14 C]heme to wound fluid without previous addition of α_{1m} or albumin was not detected (not shown), possibly due to low concentrations of the proteins and/or because endogenous α_{1m} is saturated with pre-existing heme in the wound fluids.

Binding of [14 C]heme by α_{1m} in HepG2 cells To examine the heme-binding properties of newly synthesized α_{1m} not

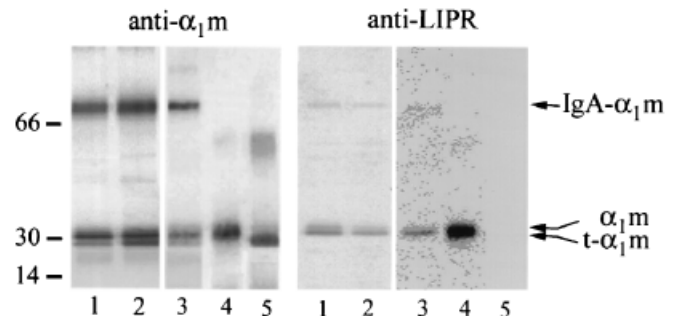


Figure 2. α_{1m} and t- α_{1m} purified from wound fluids. α_{1m} from 100 μ L wound fluid, collected from each of three patients with chronic leg ulcers, were purified by affinity chromatography on a column with immobilized monoclonal antibodies against α_{1m} . The eluates (lanes 1–3) were electrophoresed on 10% SDS-PAGE under reducing conditions and immunoblotted with anti- α_{1m} (A) and anti-LIPR (B). Lane 4 shows plasma α_{1m} and lane 5 corresponds to t- α_{1m} , obtained by incubation of plasma α_{1m} with erythrocyte membranes as described (Allhorn *et al*, 2002).

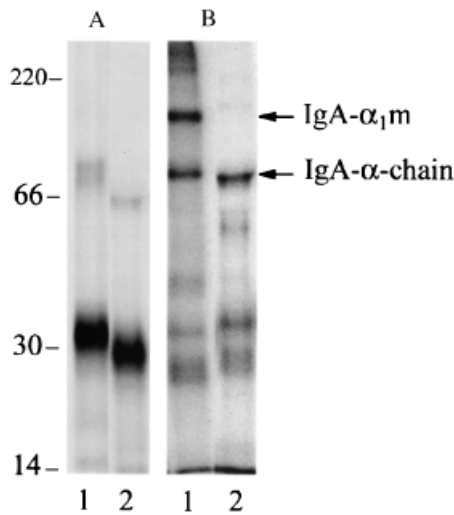


Figure 3. Cleavage of exogenously added α_1 m in wound fluid. One becquerel of 125 I-plasma α_1 m (A) or 125 I-IgA- α_1 m (B) was added to wound fluid, 5 μ g total proteins, in PBS. Lanes 1 and 2 correspond to samples before and after addition of wound fluids, respectively. After 1 h incubation at room temperature, the samples were separated by SDS-PAGE.

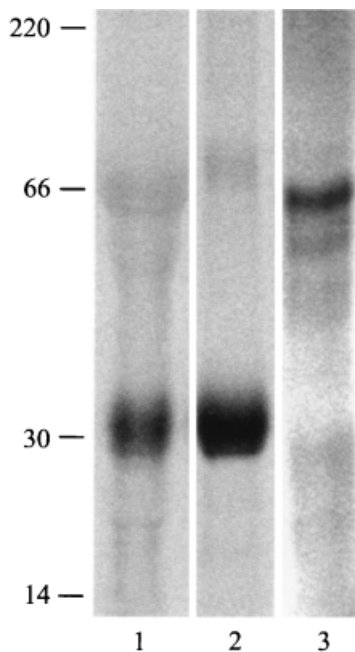


Figure 4. Binding of [14 C]heme to α_1 m in wound fluid. Wound fluid (1.5 μ L = 15 μ g protein), with added 45 pmol plasma α_1 m (1.5 μ g), was incubated with 15 pmol [14 C]heme in PBS, 0.05% Tween (lane 1). In lane 2, the same amount (45 pmol) of purified plasma α_1 m was incubated with [14 C]heme (15 pmol) without wound fluid. As a control, 45 pmol human albumin was incubated with 15 pmol [14 C]heme in 1.5 μ L wound fluid (lane 3). The samples were incubated for 30 min and then applied to SDS-PAGE and the dried gel analyzed in a Fuji Bas 2000 Bio-imaging analyzer.

previously exposed to heme, human hepatoblastoma (HepG2) cells were used. These cells synthesize α_1 m as a precursor protein, α_1 m/bikunin, which is cleaved intracellularly and the proteins are secreted separately. Thus, [14 C]heme was added to the culture medium containing newly synthesized and secreted α_1 m. This medium was then immunoprecipitated with polyclonal antisera. The results displayed in Fig 5(A) show that the [14 C]heme

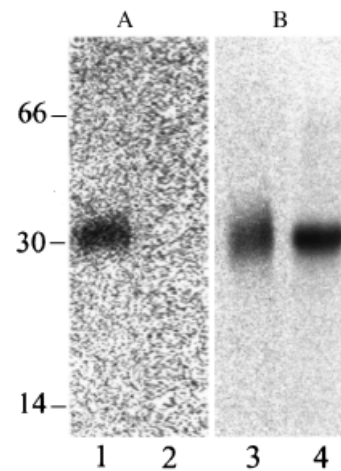


Figure 5. Binding of [14 C]heme by α_1 m in HepG2 cells. (A) Medium from HepG2 cells, grown in the presence of 1 nmol [14 C]heme, was immunoprecipitated with polyclonal anti- α_1 m (lane 1) or anti-orosomucoid (lane 2). The α_1 m samples were then loaded on SDS-PAGE. (B) Five nanomol [14 C]heme were added to HepG2 cells grown in medium supplemented with 10% FBS (lane 3) or 0.03 μ M Na_2SeO_3 (lane 4). The medium was then purified on an affinity column with immobilized monoclonal antibodies against α_1 -m and the eluates analyzed by SDS-PAGE.

added to HepG2 cells bound to α_1 m, but not to orosomucoid also synthesized by the HepG2 cells. Medium was also applied to a monoclonal affinity chromatography column and α_1 m isolated by affinity chromatography could be seen as a radioactive band on SDS-PAGE (Fig 5B). The lysed cells from the same cultivation did not show any radioactivity on SDS-PAGE, suggesting that there were too small amounts of [14 C]heme bound intracellularly to be detected (not shown). To exclude the possibility that [14 C]heme bound to proteins from the FBS in the medium, FBS was replaced by Na_2SeO_3 . In this case the binding of [14 C]heme to α_1 m was even more pronounced, as indicated in Fig 5(B), possibly as a result of the absence of competing heme-binding factors in FBS. The synthesis rate of α_1 m was similar with or without FBS (not shown).

Iron, heme, and α_1 m in wound fluids and sections The skin sections from patients with chronic ulcers and from healthy individuals were investigated histologically using polyclonal anti-sera directed against α_1 m. The results revealed a global distribution of α_1 m in the dermis layer, with more intense staining in basement membranes and fibrin cuffs surrounding the blood vessels (Fig 6B). The normal skin section prepared from a healthy individual also displayed a ubiquitous distribution of α_1 m with increased amounts around vessels (Fig 6A). Porphyrin-specific staining showed the presence of porphyrins in basement membranes and fibrin cuffs around vessels, i.e., a colocalization with α_1 m, as well as in infiltrating blood cells (Fig 6C). In contrast, much less porphyrins were found in sections from patients with acute wounds (Fig 6E) or normal skin sections. Furthermore, pronounced iron deposits were seen in chronic ulcers (Fig 6D), as opposed to acute wounds or skin sections from healthy individuals, which were negative with respect to iron (not shown). These results suggest that tissue deposition of heme and iron is accentuated in chronic ulcers as compared with acute wounds and normal skin, whereas α_1 m is ubiquitously present both in normal skin and chronic ulcers.

Heme (protoporphyrin IX), free iron, and α_1 m concentrations were determined in wound fluids and plasma from patients with chronic ulcers (Table I). The heme content was estimated to

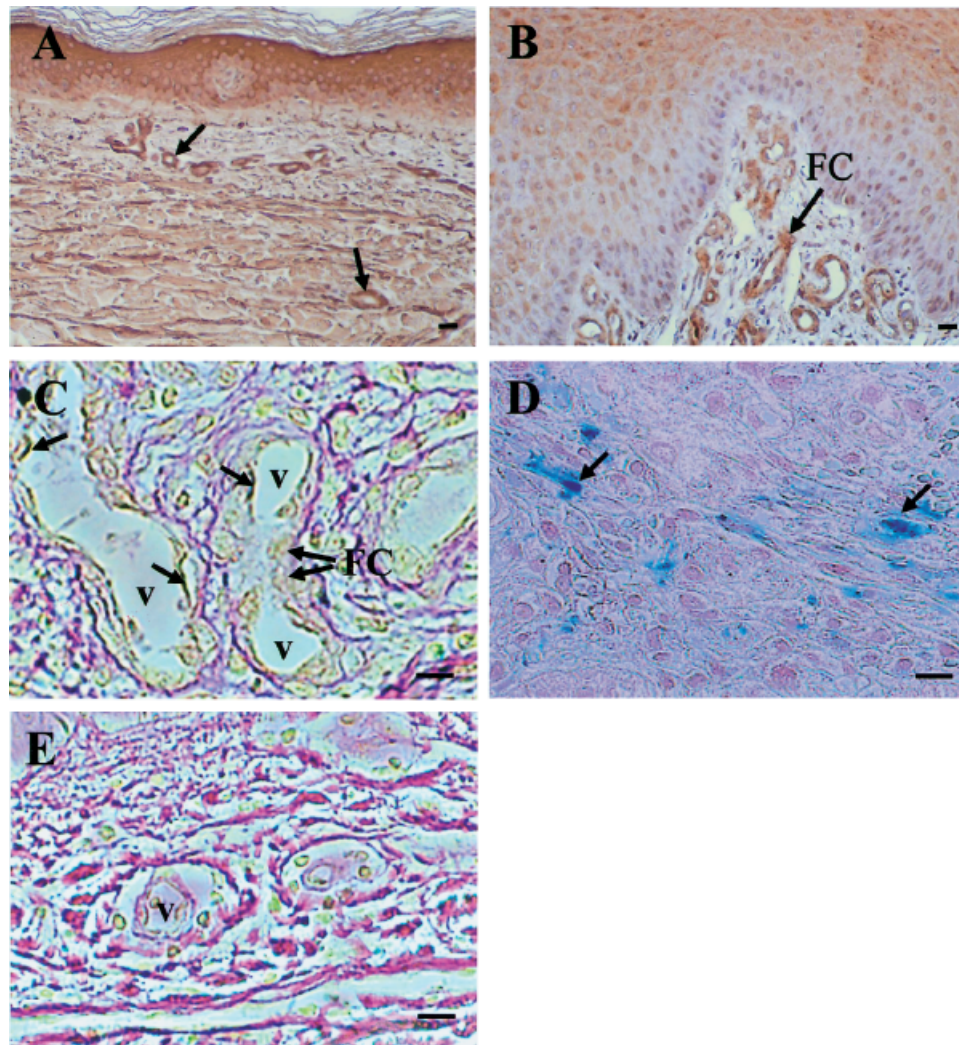


Figure 6. Histochemical distribution of $\alpha_1\text{m}$, porphyrins, and free iron in chronic venous ulcers. $\alpha_1\text{m}$, porphyrins and iron in skin sections from chronic ulcers, acute wounds and normal skin, including epidermis and dermis. (A) $\alpha_1\text{m}$ in normal skin ($\times 20$). (B) $\alpha_1\text{m}$ in skin from a patient with chronic ulcers ($\times 20$). The sections were immunostained for $\alpha_1\text{m}$ using polyclonal anti-serum against $\alpha_1\text{m}$ followed by peroxidase/diaminobenzidine tetrahydrochloride incubation as described in *Materials and Methods*. $\alpha_1\text{m}$ appears in high density in basement membranes and fibrin cuffs surrounding the blood vessels (arrows). (C, $\times 40$) and (D, $\times 40$) show porphyrins and iron depositions, respectively, in the tissue from chronic leg ulcers. Porphyrins were stained with Fouchet technique and iron with Perls' Prussian blue stain. High-density depositions are marked with arrows. (E) Porphyrin staining in the skin section from a patient with acute wound ($\times 40$). The magnification bars in the lower-right corner in each panel represent 0.02 mm. FC, fibrin cuffs, v, vessel.

19.8 μM (SEM 6.7) in wound fluids and 13.4 μM (SEM 3.6) in plasma. The iron concentration was 6.5 μM (SEM 2.7) in wound fluids and 7.3 μM (1.5) in plasma. The $\alpha_1\text{m}$ concentration was 1.7 μM (SEM 0.8) in wound fluids and 1.9 μM (SEM 1.5) in plasma. As a control, the heme, iron, and $\alpha_1\text{m}$ concentrations were determined in plasma from normal donors. The heme and $\alpha_1\text{m}$ concentrations were 12.1 μM (SEM 3.6) and 1.8 μM (SEM 0.3), respectively (Table I), i.e., similar values as in plasma from patients with chronic ulcers. The iron concentration in normal plasma was 13.9 (SEM 0.7), which is higher than in plasma and ulcer fluids from patients with chronic wounds.

DISCUSSION

Recent results indicate that the lipocalin $\alpha_1\text{m}$ is a heme scavenger, and that it participates in protection against toxic effects of extracellularly exposed heme. Heme has been proposed as an inducer of inflammation and constitutes one of several pathogenic factors in chronic leg ulcers. Chronic venous ulcers, characterized by accumulated heme, may therefore serve as a relevant model for

Table I. Concentrations of heme (protoporphyrin IX), free iron, and $\alpha_1\text{m}$ in chronic wound fluids and in plasma from patients with chronic ulcers and normal donors. Mean values of determinations on five samples ($n = 5$) and SEM values in each category are shown

	Heme (μM)	Iron (μM)	$\alpha_1\text{m}$ (μM)
Chronic ulcers			
Wound fluid	19.8 (6.7)	6.5 (2.7)	1.7 (0.8)
Plasma	13.4 (3.6)	7.3 (1.5)	1.9 (1.5)
Normal plasma	12.1 (3.6)	13.9 (0.7)	1.8 (0.3)

investigation of physiologic interactions between $\alpha_1\text{m}$ and heme. The results in this study show the simultaneous presence of heme, iron, and $\alpha_1\text{m}$ in chronic wound fluids and a colocalization of $\alpha_1\text{m}$ and heme in tissue sections to basement membranes and fibrin cuffs around vessels. Moreover, $\alpha_1\text{m}$ binds heme in the wound fluids and is processed into the truncated form $\text{t-}\alpha_1\text{m}$, which has heme-degrading properties. The results obtained here

thus support a view of $\alpha_1\text{m}$ as a heme scavenger during inflammation.

It was previously noted that the C-terminal processing of $\alpha_1\text{m}$ is induced by purified hemoglobin or by membranes from ruptured erythrocytes (Allhorn *et al*, 2002). It was speculated that an oxidized form of hemoglobin, which can be found deposited in erythrocyte membranes, may be responsible for the cleavage. The identity of the cleaving factor(s) in the leg ulcer fluids was not determined in this investigation; however, hemoglobin is usually found in this type of chronic leg ulcers (Blomgren *et al*, 2001) and this paper as well as others (Wenk *et al*, 2001) has shown the presence of heme and iron. It is therefore possible that the processing of $\alpha_1\text{m}$ in chronic leg ulcers is induced by hemoglobin, hemoglobin variants, or free heme. We have previously shown that t- $\alpha_1\text{m}$ is excreted in normal urine and that urinary t- $\alpha_1\text{m}$ may be elevated in urine from hemolytic patients (Allhorn *et al*, 2002). In analogy, an increased t- $\alpha_1\text{m}$ formation in patients with chronic ulcers may be reflected as an elevated urinary t- $\alpha_1\text{m}$ concentration in these patients, and therefore, it is interesting to speculate that the urinary $\alpha_1\text{m}$ /t- $\alpha_1\text{m}$ ratio could be a useful parameter for evaluation of the clinical status of the ulcer patients.

About 50% of $\alpha_1\text{m}$ in blood is complex bound to monomeric IgA (Grubb *et al*, 1986; Berggård *et al*, 1997). The IgA- $\alpha_1\text{m}$ complex is also found in extravascular compartments (Berggård *et al*, 1999; Bouic *et al*, 1985; Vincent and Revillard, 1987). It was shown that IgA- $\alpha_1\text{m}$ is processed by erythrocyte membranes and hemoglobin, forming intact IgA and t- $\alpha_1\text{m}$ (Allhorn *et al*, 2002). IgA- $\alpha_1\text{m}$ may therefore be regarded as a depot from which the activated t- $\alpha_1\text{m}$ is released. Leg ulcer fluids cleaved exogenously added IgA- $\alpha_1\text{m}$, suggesting that a mobilization of activated, heme-binding and heme-degrading $\alpha_1\text{m}$ from the IgA- $\alpha_1\text{m}$ depot takes place in leg ulcers *in vivo*. IgA is a normal component of skin, and granular deposits containing various forms of IgA were found in skin from patients with dermatitis herpetiformis (Egelrud and Bäck, 1985), suggesting a role of this molecule in homeostasis and pathology of the skin. It should therefore be of interest to investigate the normal distribution of IgA- $\alpha_1\text{m}$ as well as the presence of the complex in the pathologic granular deposits. Other high-molecular-weight plasma forms with masses around 200 kDa, representing complexes with albumin, prothrombin, and other as yet unknown molecules, have also been found to be associated with $\alpha_1\text{m}$ (Berggård *et al*, 1997). Interestingly, these high-molecular-weight complexes are still present in plasma from patients with chronic leg ulcers but absent in wound fluids, as judged by SDS-PAGE. The reason for this is unclear but demonstrates that the distribution and metabolism of the $\alpha_1\text{m}$ complexes are different.

The liver is the main site of synthesis of $\alpha_1\text{m}$ and only traces of $\alpha_1\text{m}$ -encoding mRNA are found in other organs (Kaumeyer *et al*, 1986; Itoh *et al*, 1996; Daveau *et al*, 1998). No production of $\alpha_1\text{m}$ in keratinocytes or fibroblasts could be detected (not shown). The $\alpha_1\text{m}$ and $\alpha_1\text{m}$ complexes found in ulcer fluids therefore most likely originate from the liver and are transported there via the blood, rather than being synthesized locally. Plasma $\alpha_1\text{m}$ and hepatocyte-derived $\alpha_1\text{m}$ could indeed bind to radiolabeled heme in wound fluids, supporting this view (Figs 4 and 5). Such a transport of $\alpha_1\text{m}$ from blood to tissues was demonstrated experimentally after injection of ^{125}I -labeled $\alpha_1\text{m}$ and $\alpha_1\text{m}$ complexes intravenously into rats (Larsson *et al*, 2000). The protein composition in chronic wound fluids closely resembles that of plasma (Schmidtchen, 2000), suggesting a leakage of plasma proteins into wounds and further supporting the notion that $\alpha_1\text{m}$ in wound fluids is indeed plasma derived.

Heme was found both in ulcer fluids and in plasma of ulcer patients (Table I). Heme was also found widely distributed in tissue sections from the chronic ulcers as opposed to acute wound sections or normal skin sections (Fig 6). Owing to venous hypertension in chronic ulcers (Ågren *et al*, 2000; Falanga, 2001), a flow of heme from plasma to the extravascular compartments across the endothelium and basement membranes is expected. Heme

may therefore be trapped and accumulated in the membranous compartments of the endothelium and basement membranes and accumulated in pericapillary fibrin cuffs, which probably develop as a result of venous hypertension and extravasation of fibrinogen (Van de Scheur and Falanga, 1997). Such a distribution of heme to basement membranes and fibrin cuffs was indeed found in this investigation. Interestingly, especially high amounts of $\alpha_1\text{m}$ were also found in these compartments (Fig 6), supporting the view of $\alpha_1\text{m}$ as a heme scavenger. This localization of $\alpha_1\text{m}$ also agrees with previous immunohistochemical studies of skin and placenta (Bouic *et al*, 1985; Berggård *et al*, 1999).

Free heme, released from hemoglobin and other heme proteins as a result of oxidation, generates reactive oxygen species leading to subsequent cell and tissue damage. Excess accumulation of heme causes an increased vasopermeability, increased expression of endothelial adhesion molecules and infiltration of leukocytes, signs of inflammation (Wagener *et al*, 2001). These events appear to be linked to the chronic inflammation in venous leg ulcers. The pro-inflammatory effects of heme induce expression of heme oxygenase, an intracellular, heme-degrading enzyme (Panchenko *et al*, 2000; Wagener *et al*, 2001). The products of the heme degradation by heme oxygenase, CO, and bilirubin, have cyto-protecting properties against oxidative stress (Otterbein and Choi, 2000). It may be speculated that t- $\alpha_1\text{m}$, which seems to have its heme-degrading property in common with heme oxygenase (Allhorn *et al*, 2002), affects heme in a similar way giving rise to these anti-oxidative mediators.

Another product of heme degradation is, of course, free iron. The results in this paper, in agreement with previous reports, demonstrate the presence of iron in the ulcer fluids, as well as iron deposits in chronic wound sections. Interestingly, the levels of iron (free + protein bound) were lower in wound fluids and plasma from chronic ulcer patients than in normal plasma, whereas iron deposits were seen in chronic wound tissue but not in acute wound or normal tissue. This suggests that the iron-chelating mechanisms are insufficient in the inflammatory tissue. Iron represents an oxidative threat to the tissue components, but heme oxygenase has been suggested to promote sequestering of the iron into the storage protein ferritin, which serves a dual role by hiding the iron and itself possesses a cyto-protective and anti-oxidant activity (Balla *et al*, 1992; Otterbein and Choi, 2000; Ryter and Tyrell, 2000). It may be speculated that $\alpha_1\text{m}$ has a similar effect as heme oxygenase also in this case, promoting iron storage; however, additional observations are necessary before any conclusions can be drawn about the role of $\alpha_1\text{m}$ in iron metabolism.

Unlike heme oxygenase, $\alpha_1\text{m}$ operates extracellularly, possibly providing the main source of protection against unsequestered heme in the extracellular space. In conclusion, our results support the concept of $\alpha_1\text{m}$ as part of a novel endogenous defense mechanism against heme-induced oxidative stress.

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