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An Antithrombin III product containing biologically active hepatocyte growth factor may be beneficial in deep ulcer infections

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ABSTRACT

Background: Widely studied for the past 20 years, hepatocyte growth factor (HGF) has been identified as a regenerative marker and an important factor in the development and healing of injuries. Antithrombin III (AT III) is a protein in the blood stream with anti-thrombotic and anti-inflammatory properties and has been used as an adjuvant treatment along with antibiotics in severe sepsis.
Objective: To study the content and properties of HGF in plasma-derived AT III products, and the regenerative effect in severe deep ulcer infections.
Methods: Commercial AT III products were analyzed for the presence and biological activity of HGF. One AT III product containing biologically active HGF was used to treat 18 cases of critical, deep ulcer infections scheduled for major invasive intervention. The patients were followed up for 6–60 months.
Results: The AT III products contained HGF with different biological activity. No adverse reactions were observed after local administration of AT III during the study or follow-up period. In 16 of 18 cases no surgical intervention was needed within the first 6 month of inclusion.
Conclusion: AT III products containing biologically active HGF may contribute to regeneration and healing in severe deep ulcer infections which do not respond adequately to different combinations of antibiotics alone.

1. Introduction

At what point does an acute injury become classified as chronic, and why does it become chronic? These are questions for intensive study. Up to date extensive resources have been invested to remedy symptoms and causes of chronic injuries. Infection is an important and widely studied cause of injury [1]. Efficient treatment of infection results in healing of damaged tissue, and such treatable damage is considered the result of acute inflammation. However when, despite conventional methods of therapy, injury resists healing, countless body changes occur causing chronic organ failure and cancer [2]. Predisposing factors, as the normal atherosclerotic process or the accentuated atherosclerosis in diabetes mellitus with angiopathy or chronic uremia with atherosclerosis, are such predisposing factors especially in patients treated with hemodialysis [3]. Infectious agents difficult to discover by available methods may cause such chronic damage. Several recent studies strengthen this notion, such as the discovery of *Helicobacter pylori* as the cause of chronic gastritis and cancer; hepatitis as the cause of chronic liver injury and cancer [4]; and the fact that chronicity and damage are inhibited by eliminating infection [5]. Thus the infectious agent, the body's response to injury, and several other factors such as genetic variation and environment may interact, resulting in the development of chronic injury when damage in the acute phase remains unresolved [6]. Furthermore, it is known that various cytokines and growth factors mediate inflammatory responses [7].

Hepatocyte growth factor (HGF) is a cytokine produced during injury and mediates development, regeneration and healing [8]. Knockout mice lacking the HGF gene cannot survive [9] and low concentration of circulating HGF during acute infection may indicate an unfavorable prognosis [10]. In an attempt to study the causes of healing defects during chronic inflammation we chose since 1996 to focus our studies on the presence, properties and function of HGF in various organs. Skin ulcers may be proper models to assess organ injury. The damage is visible, the ulcer contaminated with bacteria, and the acute and chronic injury well defined. Therefore we investigated HGF in chronic and acute ulcers [11–13] and observed that:

a. The HGF receptor was significantly up-regulated in chronic ulcers.

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b. The concentration of HGF was significantly increased in chronic ulcers.
c. Unlike acute ulcers, secretion from chronic ulcers had no biological activity in an *in vitro* model of cell injury.
d. Western blot analysis of ulcer secretion differed in the studied chronic ulcers compared to controls in the intensity of α and β-chains of HGF.
e. HGF from chronic ulcer secretion lacked binding affinity to heparan sulfate proteoglycan (HSPG) in a Surface plasmon resonance (SPR) system.
f. Local application of HGF to chronic ulcers, with properties of HGF found in acute ulcers, increased microcirculation.
g. HGF with no biological activity *in vitro* or no binding affinity to HSPG in SPR had no significant effect on microcirculation or healing of chronic ulcers despite relevant anti-microbial treatment.
h. Based on variations in commercially available recombinant HGF products, endogenous HGF produced in healthy subjects seemed to be appropriate for studies of the HGF effect in deeper injuries.

Antithrombin III (AT III) is a plasma protein and one of the most important inhibitor of clotting. It also has anti-inflammatory properties and is administered to patients with congenital or acquired AT III deficiency. The latter indication has been studied widely in critically ill patients with multiple organ failure [14]. In a review article by Afshari et al. [15], 20 placebo-controlled trials evaluating the therapeutic/side effects of AT III in critically ill patients were surveyed. Thirteen trials consisted of critically ill participants, mainly with sepsis. The risk of bias was evaluated, and the different studies were categorized as low/high risk of bias. The results, combining all trials, showed no statistically significant effect of AT III on mortality. However, the bleeding events significantly increased in AT III-treated patients. Thus, authors of earlier studies do not recommend AT III substitution to critically ill patients [16]. Although Afshari et al. assessed very detailed and valuable components of the studies in their review article, no information about the AT III product used was provided. Since 2004 we have studied the products developed during the process of purification and found that AT III products contain HGF. However, the properties of HGF differed among commercial products.

In the current work different commercial AT III products are investigated regarding their properties of HGF. The AT III product with biologically active HGF was used locally in cases of deep skin and soft tissue injury in which amputation or major surgery was planned because of therapy failure and life risks. Patients were followed up 6–60 months after inclusion. The result was compared to that of earlier studies in the same field (Table 1).

2. Materials and methods

2.1. Non-clinical study

2.1.1. AT III products

For the non-clinical studies of AT III the products available commercially; Atenativ® (Pharmacia), Atenativ® (Octapharma), Kybernin-P® (Aventis-Behring), Thrombhibin® (Immuno AG), and AT III Baxter® (Baxter) were used. Products developed in the production process of AT III were received from Octapharma in 2004. The different AT III products were analyzed according to the contents, binding affinity to ligands and biological activity of HGF.

2.1.2. Evaluation of the biological activity of HGF in a model of cell injury

The biological activity of HGF in AT III samples was tested in an *in vitro* cell injury assay using transformed mouse skin epithelial cells (CCL-53.1 cell line). The method has been described in a previous publication [17]. Shortly, CCl-53.1 cells were grown in Kaighn's modification of Ham's F-12K medium (ATCC) supplemented with 15% horse serum and 2.5% fetal bovine serum (Sigma–Aldrich, St. Louis, MO, USA) in an atmosphere of 5% CO_2 and 95% air at 37 °C. After the cells reached confluence, they were separated with non-enzymatic cell dissociation solution (1×) (Sigma–Aldrich), suspended in an F-12K medium with 15% horse serum and 2.5% fetal bovine serum, and inoculated in a 24-well culture plate (Nunc Brand Products, Roskilde, Denmark). Cells were cultured under the exact conditions for 24–48 h until they reached confluence. Then, a line across the confluent monolayer was scraped with a sterile steel device, detached cells were washed away with PBS and fresh medium was added to the wells. The area (mm^2) of the square not covered by cells was measured by microscopy (Olympus) and documented in each well. AT III products or PBS was added (100 μl a′ 50 IU/ml AT III or 100 μl PBS as control), and the cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After 24 and 48 h, the area not covered by a monolayer was measured again and documented. A decreased area was categorized as a "positive" effect and no effect as "negative".

2.1.3. SPR measurements and ligand immobilization procedures

To analyze the binding affinity to the different ligands, SPR measurements were conducted at 760 nm in a fully automatic Biacore 2000 instrument (GE-Healthcare GmbH, Uppsala, Sweden) equipped with four flow cells. Biologically relevant ligands of HGF (monoclonal anti-HGF antibody (unknown epitope), polyclonal antibodies against different parts of HGF, and HGF receptors) were obtained commercially (Table 2) and immobilized on surface plasmon resonance (SPR) CM5 chips as previously described [18]. Briefly, the flow cell temperature was 25 °C in all experiments. The sample surfaces were carboxy-methylated dextran CM5 chips (GE-Healthcare GmbH, Uppsala, Sweden). Coupling of ligands to the carboxylic acid groups of the dextran hydrogel was carried out by conventional carbodiimide chemistry using 200 mM EDC (N-ethyl-N′-(3-diethylaminopropyl) carbodiimide) and 50 mM NHS (N-hydroxysuccinimide). Activation time was 7 min, followed by a 7 min ligand injection. Deactivation of the remaining active esters was performed by a 7 min injection of ethanolamine/hydrochloride at pH 8.5. A flow rate of 5 μl/min was used during immobilization. The ligands (Table 2) were diluted in 10 mM acetate buffer at a pH below the protein's isoelectric point, thus enhancing the electrostatic interactions between the dextran matrix and the ligands. The contact time was 7 min, which resulted in levels of

Table 1
The effect of the AT III products used as treatment of critically ill patients compared to controls in other studies. The outcome measured is overall mortality.

Study	Journal	Risk ratio	AT III product
Baudo (1992)	Thrombosis Research	0.25	Thrombhibin® (Immuno AG)
Warren (2001)	JAMA	0.96	Kybernin-P® (Aventis-Behring)
Haire (1998)	Biology of Blood and Marrow Transplantation	0.67	AT III Baxter® (Baxter)
Schorr (2000)	European Journal of Clinical Investigation	1.08	Atenativ, Pharmacia® (Octapharma)
Waydhas (1998)	Trauma, Injury, Infection and Critical Care	2.00	Atenativ, Pharmacia® (Octapharma)

Table 2
The ligands used for binding affinity analysis in SPR.

Immobilized ligands in SPR	Source/product number	Code	Goal of investigation
Monoclonal anti-HGF ab	R&D Systems/MAB294	MN	Determine amount of HGF
Recombinant HGF receptor (c-met)/fc chimera	R&D Systems/358 MT	c-Met	Analyze HGF binding to c-met receptor
Heparan sulfate proteoglycan	Sigma–Aldrich/H4777	HSPG	Analyze HGF binding to HSPG $\geqslant$ 100 μg/ml
Polyclonal anti-HGF ab, affinity isolated	Sigma–Aldrich/HH0652	PK	Determine amount of HGF
H-170 rabbit polyclonal ab	Santa Cruz/sc-13,087	H-170	Bind amino acids 1–170 of human HGF
N-19 affinity-purified goat polyclonal ab	Santa Cruz/sc-1356	N-19	Peptide mapping at the N-terminus of human HGF
N-17 affinity-purified goat polyclonal ab	Santa Cruz/sc-1357	N-17	Peptide mapping at the N-terminus of human HGFα
C-20 affinity-purified goat polyclonal ab	Santa Cruz/sc-1358	C-20	Peptide mapping at the C-terminus of human HGFα
H-145 rabbit polyclonal ab	Santa Cruz/sc-7949	H-145	Bind amino acids 32–176 of human HGFα
D-19 goat polyclonal IgG	Santa Cruz/Sc-34,461	D-19	Epitope mapping in an internal region of human HGF
S-16 goat polyclonal IgG	Santa Cruz/Sc-34,462	S-16	Epitope mapping in an internal region of human HGF

immobilization between 8000 and 30,000 response units (RU). After deactivation, the surfaces were washed with five subsequent 1 min injections of 5 mM glycine buffer, pH 2.0, with 1 M NaCl (regeneration buffer). One of the flow cells was used as a control to monitor the response due to the interaction of HGF with the carboxymethylated dextran matrix. This flow cell was treated in the same way as the others during the immobilization procedure, but the ligand immobilization step was omitted.

AT III products were reconstituted and analyzed at different concentrations. Physiological NaCl (9 mg/ml), distilled water (B. Braun Medical AB, Bromma, Sweden), PBS (pH 7.4, Apoteket AB, Umeå, Sweden), and HBS-EP (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20; GE-Healthcare GmbH, Uppsala, Sweden) were used as dilution and running buffers. A 1:1 mixture of 1 M NaCl + 10 mM glycine, pH 2, was used as regeneration buffer. The presented SPR data was extracted from the Biacore sensorgrams after the injections were completed, i.e. during the analyte dissociation phase. One Biacore RU corresponds to a surface concentration of 1 pg protein per mm^2 [19]. The binding of HGF to ligands was tested in AT III samples after reconstitution in different dilutions. A positive and a negative control were included at the beginning and at the end of each run to confirm the reliability of surfaces.

The following experiments were analyzed in SPR:

- Epitope mapping: AT III Baxter 50 IU/ml (diluted in a ratio of 1:1 with PBS) was injected for 3 min three times until further injections did not add to the signal intensity (all binding sites were engaged) and this level was set as baseline. Epitope-specific antibodies against different parts of HGF (Table 2) were then injected for 3 min, and the signal intensity in each channel immobilized with c-Met, monoclonal anti-HGF antibody (MN), and HSPG was recorded [20].
- Stability of fractions: The effect of time on the response was tested by continuous analysis of binding over time on the same chip and during the same run.
- Affinity purified AT III products: The affinity chromatography columns (Hi-trap GE Healthcare) were immobilized with HSPG according to the manufacturer's instructions. The AT III samples (50 IU/ml) were reconstituted and diluted in PBS (pH 7.4) in a ratio of 1:1 and injected into the column. 5 mM glycine buffer pH 2.0 with 1 M NaCl was used as elution buffer. Thereafter the affinity of samples (start sample as well as eluent from the column) to immobilized ligands in the SPR system was tested.
- Incubation of AT III with glycosaminoglycan dextran sulfate (DS) or fragmin: AT III (50 IU/ml) containing biologically active HGF was incubated with DS (10 mg/ml MQ; Sigma Aldrich), or 12 IU/ml AT III with fragmin (0.02-100 IU á25 000 IU/ml; Pfizer AB, Sollentuna, Sweden), at RT for 30 min. The binding response to HSPG and MN was measured in SPR.
- Incubation of AT III with bacteria: AT III that contained biologically active HGF was diluted in a ratio of 1:5 with PBS and kept at room temperature for 48 h with or without addition of aerobic bacteria (*Enterococcus faecalis*, *Enterobacter cloacae*, *Staphylococcus aureus*, *Escherichia coli*, *Staphylococcus epidermis*) or anaerobic bacteria (*Porphyromonas gingivalis*). The binding affinity to MN and HSPG was assessed in SPR.

2.1.4. ELISA

The HGF concentration in the AT III samples (Atenativ and AT III Baxter) was determined using a commercial ELISA kit (Quantikine Human HGF immunoassay, minimum detectable limit: 0.04 ng/mL; R&D Systems) according to the manufacturer's instructions. The measurements were performed in duplicate at 450 nm using an ELISA reader (Expert 96; Asys Hitech GmbH, Eugendorf, Austria), and calibrated using the recombinant human HGF reference samples that were provided in the ELISA kit.

2.1.5. Western blotting

The AT III products were diluted in Krebs–Ringer Glucose Buffer; in a ratio of 1:11 (KRG; consisted of 120 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4$, 1.7 mM KH_2PO_4, 8.3 mM Na_2HPO_4, and 10 mM glucose, pH 7.3), and thereafter heated in a Laemmli sample buffer and 2-Mercaptoethanol (98 °C for 5 min) at a ratio of 1:1 to denature and reduce the proteins. The proteins were separated using standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE containing 12% Tris or any Kd gel) and electrotransferred to a polyvinylidene difluoride membrane. Unspecific binding was blocked by incubating the membranes in 5% milk-Tris-buffer saline (TBS; consisted of 25 mM Tris base, 150 mM NaCl, 2 mM KCl pH 7.4, and 0.1% Tween-20) for 1 h at RT prior to incubation with a goat polyclonal anti-HGF antibody (1:1000; AF-294-NA) for 1 h at RT. The membranes were thereafter incubated with a polyclonal HRP-conjugated donkey anti-goat antibody (1:1000; HAF109). Recombinant HGF (294-HA; R&D systems, Minneapolis, MN, USA) was used as a positive control.

2.2. Clinical part of the study

2.2.1. AT III product used in deep ulcer infections

AT III found to contain HGF with binding affinity to both MN and HSPG in the SPR system and showed biological activity on CCL-53.1 cells, was used in the clinical part of the study.

2.2.2. Study subjects and treatment

The study subjects were enrolled from patients admitted to hospital because of deep ulcer infection in need of systemic antibiotic treatment and invasive interventions. However, therapy had failed to resolve the infection and major surgery was planned. None of the included cases were suffering from severe sepsis and/or multiple organ dysfunctions at the time of inclusion. The

patients were asked to join the study and the study group undertook therapeutic responsibility for patients, receiving 100 μl of the AT III product (50 IU/ml) containing biologically active HGF locally once daily together with antibiotics for 5–7 days. In cases where the process of deterioration continued after inclusion abrupt surgery was planned. In other cases patients were treated and followed up by the study group for at least 6 months. Occlusive or semi-occlusive bandages containing plastic material were strictly prohibited during the study period of 6 months if the ulcer was not already closed. Choice of antibiotics was based on bacterial culture results of the wound as well as clinical judgment based on appearance, discharge, smell, and circulation. Eighteen patients were included and followed the study protocol from 2005 in an open prospective study. Four patients were included twice upon patients' request to inhibit severe progress of already healed ulcers. The local ethical committee in Linköping, Sweden, approved the study and a written consent was obtained from all study subjects.

2.3. Statistical analysis

Data were statistically analyzed by paired and un-paired Student *t* tests by using Graph Pad Prism version 5.0. $P \leqslant 0.05$ was considered statistically significant Representative experiments are presented.

3. Results

3.1. The presence and biological activity of HGF in AT III products

AT III products; AT III Baxter, Atenativ, Kybernin-P, and Thrombhibin used in previous studies analyzing the effect of AT III in critically ill patients (Table 1) were examined in the present study regarding the HGF containment, concentration and biological activity.

Beside the ELISA measurements (Table 3), the presence of HGF in the AT III products was also confirmed by western blotting (Fig. 1). AT III Baxter and Thrombhibin, both from Baxter, showed several bands, compared to Atenativ and Kybernin-P that did not contain biologically active HGF and showed bands of about 60 kDa in western blotting.

In the *in vitro* model of cell injury, the addition of 100 μl AT III Baxter or Thrombhibin (50 IU/ml) caused a decrease of the injured area and was considered to contain biologically active HGF, while Atenativ and Kybernin-P did not show any effect on the cultured cells (Table 3), neither the negative control PBS.

AT III Baxter and Thrombhibin also showed the presence of HGF with a high binding affinity to HSPG, as well as monoclonal anti-HGF antibodies in SPR (Table 3). The affinity of HGF to the ligands in the SPR system did not decrease significantly when incubated overnight in RT or during several SPR runs ($P = 9$) thus indicating the stability of HGF in the AT III products. After purification in the HSPG-bound affinity column the eluent had still affinity towards HSPG in the SPR system.

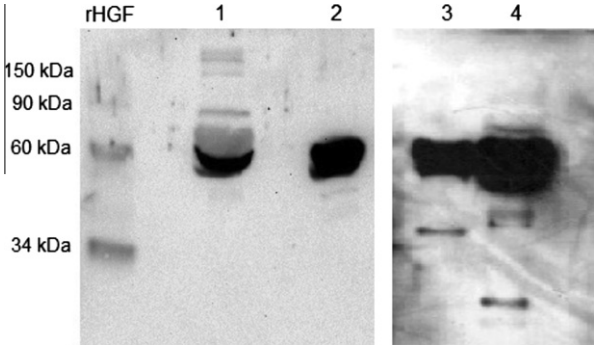

Fig. 1. Western blot analysis of the AT III products. The AT III products; Thrombhibin® (Immuno AG; 1), Kybernin-P® (Aventis-Behring; 2), Atenativ® (Octapharma; 3) and AT III Baxter® (Baxter; 4) were reduced by heating 98 °C, 5 min in Laemmli sample buffer, and the electrophoretically separated proteins were detected by western blotting using a goat polyclonal anti-HGF antibody (1:1000) and a polyclonal HRP-conjugated donkey anti-goat antibody (1:1000). Recombinant HGF (rHGF) was used as a positive control.

By epitope mapping it was found that when binding (HGF in) AT III Baxter to ligands in SPR without a following wash, the specific anti-HGF antibodies H-170 (binds to amino acids 1–170 of human HGFβ), H-145 (binds to amino acids 32–176 of human HGFα), S-16 (binds to an internal region of human HGFβ) (Table 2) that sequentially was run over the sample, did not bind to the AT III sample in all immobilized channels (C-met, MN and HSPG) ($P < 0.0001$) (Table 4). This indicates that these peptides, which the antibodies are directed against, are involved in the binding affinity of biological active HGF in the AT III product. Furthermore, the addition of glycosaminoglycan dextran sulfate or fragmin to the AT III product caused reduced binding to HSPG and MN ($P < 0.00001$) (Tables 5 and 6) in SPR.

The effect of different bacteria on AT III product that contained biologically active HGF (AT III Baxter) was analyzed by SPR after incubation with bacteria. Several bacterial species, especially the anaerobic bacterium *P. gingivalis*, decreased the binding affinity to MN and HSPG in SPR (Table 7).

3.2. The clinical study of patients with critical deep ulcer infections

After inclusion all patients continued the study after the fifth day of the treatment (Table 8), thus the situation of injury was improved and conservative therapy continued. The end point of the study was total closure of the ulcer within 6 months. Inclusion of patients from 2005 gave the advantage of meeting and following the patients for several years. No signs of adverse reactions or malignancy were observed during the study or within the follow-up period. Fresh bleeding from chronic infectious injury was seen in the first week and predicted a favorable outcome. During the study occlusive bandaging was strictly prohibited and beside from washing with tap water and mild soap and sterile cotton bandages no other local treatments or active bandages were used. In two patients (Nos. 3 and 13, Table 8) in spite of partial improvement,

Table 3
AT III products and responses in SPR, ELISA and on cultured cells. Affinity chromatography and ELISA were performed on Atenativ® and AT III Baxter®.

	MN anti-HGF SPR (RU)	HSPG SPR (RU)	Biological activity on CCL-53.1 cells	ELISA
Atenativ 50 IU/ml (1:1 PBS)	1762	53	Negative	3.0 ng per 50 IU
Atenativ affinity purified in HSPG column	−11	−9	nd	nd
Kybernin-P 50 IU/ml (1:1 PBS)	731	2	Negative	nd
Thrombhibin, (Baxter) 50 IU/ml (1:1 PBS)	3055	2386	Positive	nd
Antithrombin III Baxter 50 IU/ml (1:1 PBS)	1053	865	Positive	5.5 ng per 500 IU
Antithrombin III Baxter affinity purified in HSPG column	158	86	nd	nd

Table 4
Affinity to epitope-specific antibodies. AT III Baxter® (50 IU/ml) was diluted in a ratio of 1:1 in PBS and was injected into the SPR channel, with immobilized C-met, monoclonal-anti-HGF ab (MN) and HSPG, several times until binding sites were saturated. This value was then set as baseline. Epitope-specific antibodies (diluted in PBS 1:5) against HGF were then injected into the channel, and the affinity of antibody for the HGF + ligand complex was measured in response units (RU). An un-immobilized channel was used as control of the binding to the dextran-surface of the chip.

Samples and antibodies	C-Met (RU)	MN anti-HGF ab (RU)	HSPG (RU)	Control (RU)
AT III Baxter	859	490	395	272
H-145	−38	−23	−15	−4
C-20	70	36	160	142
N-19	272	223	353	329
H-170	−32	−18	−14	−4
N-17	88	79	163	175
S-16	−23	−10	2	11
D-19	86	84	149	166

Table 5
The affinity of AT III products (50 IU/ml) to ligands decreased after addition of dextran sulfate (DS, 10 mg/ml) to the samples.

	H 170 (RU)	N19 (RU)	C-20 (RU)	HSPG (RU)
DS + PBS	22	93	100	−7
Atenativ + PBS	70	62	663	58
Atenativ + DS	39	114	50	−6
AT III Baxter + PBS	1550	1563	3394	214
AT III Baxter + DS	37	109	36	−21

Table 6
AT III products incubated with fragmin (25,000 IU/ml) and the effect on affinity of HGF in AT III to ligands analyzed with SPR (in response units, RU).

	MN anti-HGF ab (RU)	HSPG (RU)
Thrombhibin 1.2 IU/50 μl (1:1 PBS)	1616	1111
+0.02 IU fragmin	971	533
+0.1 IU fragmin	10.5	−2.7
+20 IU fragmin	4.2	−15
+40 IU fragmin	12	−3
+60 IU fragmin	10	−2
+80 IU fragmin	10	−1
+100 IU fragmin	9	−1
Kybernin-P 1.2 IU/50 μl (1:1 PBS)	2.3	−3
Kybernin-P 1.2 IU + 0.02 IU fragmin	−3	−3

Table 7
Incubation of AT III product (AT III Baxter®), or physiological (9 mg/ml) NaCl, with different bacteria and binding affinity to ligands in SPR (measured in response units, RU).

Bacteria	HSPG SPR (RU)	MN anti-HGF ab SPR (RU)
E. faecalis in NaCl	−1.6	−47
E. faecalis in AT III	59	67
Enter. cloacae in NaCl	−0	−20
Enter. cloacae in AT III	90	168
E. coli in NaCl	−1.4	0
E. coli in AT III	48	131
P. aeruginosa in NaCl	−42	21
P. aeruginosa AT III	26	66
S. aureus in NaCl	−3	0
S. aureus in AT III	30	0
S. epidermis in NaCl	−3	−40
S. epidermis in AT III	14	121
P. gingivalis in NaCl	−6	−19
P. gingivalis in AT III	−4	1
AT III 1:5 in NaCl	45	134

new ulcer revision was needed within 6 months and these were considered as therapy failure. In the remainder the primary ulcer, and in three patients the new ulcers, were healed within 6 months (Nos. 4, 8 and 18, Table 8). The ulcers relapsed in eight cases following the completed study period.

In spite of the growth of gram-negative bacteria in the cultures of some patients, they received wide range antibiotics during the first week of treatment intravenously, but we chose to focus on gram-positive bacteria with oral treatment in the majority of cases (Table 8), and still the ulcer situation improved. Below we present four case reports to describe the medical history in greater detail.

3.2.1. Patient No. 3

Patient No. 3 (Table 8) was a woman born in 1959, a heavy smoker with type 1 diabetes mellitus, and a chronic venous ulcer and ulcer with necrosis in digits (Dig) II–IIII of the right foot since 1998. She was under oral antibiotic treatment because of X-ray-verified osteitis of the right foot and underwent partial amputation in 1999. She had developed a fistula at the amputation area and ulcer revisions were performed several times. She was admitted on January 4th 2006 to the Department of Infectious Diseases because of septicemia with growth of *Pasteurella multocida* in cultures taken from blood and ulcer secretion. She received ampicillin intravenously and beta-blockers because of progressing heart failure, and acute amputation of the right leg below the knee was planned. She was included on the study January 19th 2006. Antibiotic treatment was changed to piperacillin–tazobactam and the patient received AT III 100 μl (50 IU/ml) injected in the fistula daily. After the third day of treatment the fistula started bleeding. The patient was dismissed the 19th of January with ampicillin–clavulanic acid + ciprofloxacin orally. However, she had several episodes of fever of unknown origin with negative cultures. The fistula on her right foot was closed but the patient developed skin necrosis at the top of Dig I of the right foot and along the lateral side of the foot. She underwent ulcer revision in May 2006 and amputation of Dig I and V in August 2006, and foot amputation on the left side 2008. She developed a hemolytic uremic syndrome, renal failure, endocarditis, blindness, and died in septicemia caused by *E. faecalis* in August 2008.

3.2.2. Patient No. 8

Patient No. 8 (Table 8) was an 80-year-old woman with diabetes mellitus from 2002, cerebrovascular infarction, thyroid cancer, atrial fibrillation, and critical ischemia in both lower extremities verified by arterial duplex and angiography 2009. X-ray verified osteomyelitis of Dig I. The cultures from an ulcer beneath the right leg and foot yielded *Pseudomonas aeruginosa*, *Morganella morganii* and Coliform bacteria. She had received the oral antibiotics

Table 8
Study subjects' with inclusion, location of deep injury, culture results from ulcer secretion, antibiotic treatment and outcome.

	Born/sex/inclusion year	Major disease/planned major surgery	Deep ulcer location	Cultures	Antibiotics	Outcome
1	1939/female/2005	Diabetes type 2 edema, blisters	Lower leg bilateral	*E. coli* + CNS	Ampicillin–clavulanic acid oral	Healed 2005 new blisters 2011
2	1948/female/2006	Diabetes, hemodialysis/amputation	Osteomyelitis foot bilateral	*S. aureus*, group *G Streptococci*	Clindamycin intravenously/Clindamycin oral	Healed 2006, dead 2008 in pulmonary edema
3	1959/female/2006	Diabetes type 1, Chronic renal failure/amputation	Osteomyelitis and fistula right foot	*Pasteurella multocida*, *enterobacter*, *pseudomonas*	Piperacillin–tazobactam iv, Ciprofloxacin oral	Fistula healed but continued ulcer, dead 2009 in *E. faecalis* septicemia
4	1939/male/2006, 2008	Diabetes type 1, hypertension/amputation	Osteomyelitis MTP 1 therapy failure	*S. aureus* + *E. faecalis*	Piperacillin–tazobactam iv/Ampicillin–clavulanic acid + metronidazole oral	Healed 2006, relapse 2008, healed, relapse 2011 amputation
5	1948/male/2007	Diabetes type 2/revision	Osteomyelitis Dig V	*S. aureus*	Ceftriaxon iv/Clindamycin oral	Healed 2007, relapse 2010
6	1939/female/2007	Heavy smoker, Trombangitis oblitirans/amputation	Osteomyelitis Dig V	*S. lugdunesis*	Ampicillin–clavulanacid + Moxifloxacin oral	Healed 2007, relapse 2011
7	1931/female/2009	Seropositive Rheumatoid arthritis (RA), several joint prosthesis, severe ischemia left leg/amputation	Necrosis, deep ulcer circumference left leg	*E. faecalis* + *E. coli*	Piperacillin–tazobactam iv/ampicillin–clavulanic acid + metronidazole oral	Healed 2009, relapse 2011, amputation under knee
8	1931/female/2009, 2010	Diabetes type 2, thyroid cancer, cerebro vascular accident/amputation	Arterial ischemia foot, osteomyelitis, necrosis	*S. aureus* + *E. faecalis*, gram negative bacteria	Cefotaxim + clindamycin iv, ampicillin–clavulanic acid oral	Healed 2009, contralateral foot ischemia 2010, healed 2010
9	1992/male/2009	Tympanic membrane perforation, myringoplastic 2008/major surgery	Chronic deep infection ear canal	MDR[a], *Burkholderia cepacia*	Trimetoprim sulfamethoxazole oral	Healed 2009
10	1942/female/2010	Smoker, spondylolisthesis, colitis/surgery	Postoperative infection, Fusion L4-S1	*S. aureus* + *E. faecalis*	Piperacillin–tazobactam iv/ampicillin–clavulanic acid oral	Healed 2010
11	1941/male/2010	Tibialis fracture 1970, osteotomy/plastic surgery	Tibialis fistula osteomyelitis	*S. aureus*, group *B. Strep*	Moxifloxacin + rifampicin oral	Healed radiologic 2010, new fistula 2011, healed
12	1943/female/2010	Seropositive RA, several prosthesis, arterial occlusion right leg 2008/amputation	Chronic osteomyelitis + fistula right lateral Malleolus	*Corynebacterium* + *peptostreptococcus*	Piperacillin–tazobactam iv/Fucidinacid + Metronidazole oral	Healed 2010,
13	1939/male/2010	Diabetes, RA, immunosupp. Aortic valvular stenosis/reamputation	Osteomyelitis Dig I, II right foot. sepsis, therapy failure	*S. aureus* + *E. faecalis*	Piperacillin–tazobactam iv/Moxifloxacin	Clinically improved 2010, relapse 2011, dead 2011 in cerebrovascular infarction
14	1954/male/2010	Diabetes type 2/amputation	Osteomyelitis Dig III right foot, necrosis	Group *G. Strep*	Ertapenem iv/Clindamycin oral	Healed 2010
15	1957/male/2010	Diabetes, hypertoni Abscess left heel 2009/amputation	Deep ulcer and osteitis left tuber calcanei, therapy failure	*S. aureus* + *E. faecalis* + *Morganella morganii*	Ampicillin–Clavulanic acid oral	Improved slowly, healed 2011
16	1950/male/2010, 2011	Necrotiserande fascitis right leg 2000 Left knee prosthesis planned	Deep ulcer lateral right foot	*S. aureus* + *E. faecalis* + *P. aeruginosa*	Ampicillin–clavulanacid + Rifampicin oral	Healed 2010, left knee prosthesis 2010 relapse ulcer 2011
17	1955/female/2011	Heavy smoker, sepsis, empyema 2010	Osteomyelitis right leg, therapy failure	MDR *P. aeruginosa*	Ceftazidim iv	Healed 2011, relapsed 2011, healed
18	1957/male/May 2011, November 2011	Diabetes, alcohol cirrhosis, myocardial infarction, renal failure/amputation	Foot osteomyelitis	*ESBL*[b], *E. faecalis*, MDR *Acinetobacter*, *S. aureus*	Piperacillin–tazobactam iv/Ampicillin–clavulanic acid oral	Healed 2011, new ulcers same foot. Healed 2011

[a] Multi-drug resistant.
[b] Extended spectrum betalactamase.

erythromycin + ciprofloxacin and later clindamycin but the situation deteriorated rapidly. New cultures showed growth of ciprofloxacin resistant *P. aeruginosa*, *S. aureus* and *Stenotrophomonas*. She received intravenous antibiotics piperacillin–tazobactam and ceftazidim for 2 weeks and the symptoms were relieved. She was admitted to the Department of Infectious Diseases with symptoms of necrosis (Dig I), intense pain, and edema of the right leg on September 29th 2009 and acute amputation was planned. She was included in this study on the same day and initially received cefotaxim + clindamycin changed to piperacillin + tazobactam on September 30th + AT III locally on the ulcers of the lower part of the leg and toes. Continuous improvement followed and the

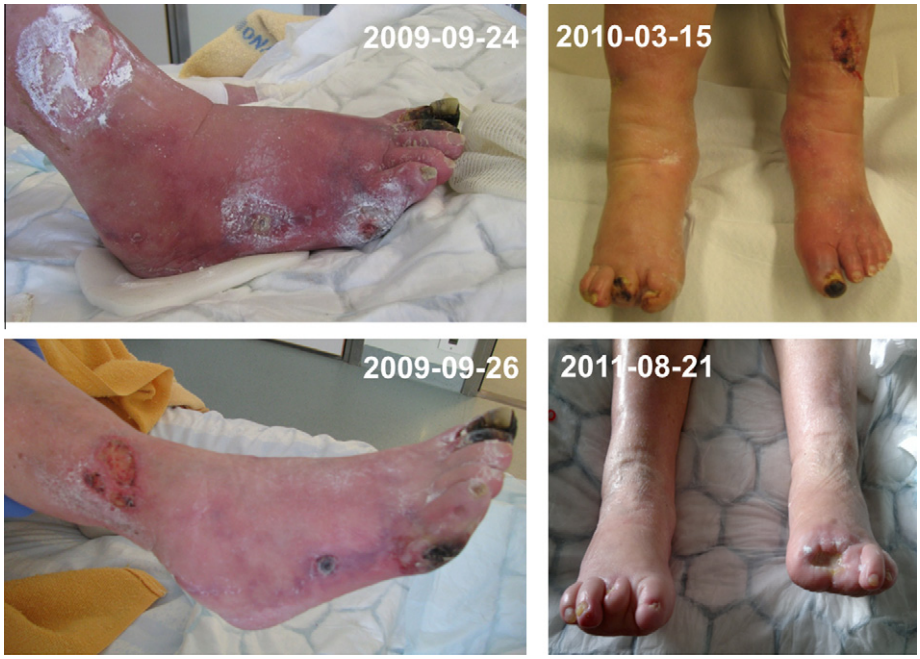

Fig. 2. An example of the process of treatment of one patient (patient No. 8, Table 8), included the first time on September 24th 2009, and the second time on March 15th 2010.

patient was discharged on October 6th 2011 with ampicillin–clavulanic acid + metronidazole orally. Ulcers healed within 2 months together with self-amputation of black toes (Dig I–II). In April 2010 the left foot showed signs of critically decreased arterial circulation. She was admitted to the Department of Infectious Diseases on October 12th 2010 and received piperacillin–tazobactam. The orthopedic consultant recommended amputation. She was included in the study again and treated with meropenem + AT III and was dismissed on October 29th 2010 with continued oral antibiotics ampicillin + trimethoprim–sulfamethoxazole. The ulcers healed and the patient had no further relapses. The blood glucose level normalized without treatment and she spontaneously returned to sinus rhythm (Fig. 2).

3.2.3. Patient No. 9

Patient No. 9 (Table 8) was a 19-year-old man with a long deep infection of several months duration in the ear canal. The cultures yielded growth of *Burkholdera cepasia* that developed resistance successively during on-going antibiotic treatment. At the time of inclusion the bacteria were resistant to piperacillin–tazobactam, ertapenem, ciprofloxacin, ceftazidim, tobramycin, meropenem, imipenem, azteronam, cefotaxim, ampicillin clavulanic acid, ticarcillin, and polymixin B. There was a profuse purulent discharge despite several weeks of intravenous antibiotic treatment. Surgery might have caused a massive tissue loss. The patient was included in the study in October 2009 and treated with a combination of trimetoprim sulfamethoxazole orally and local application of AT III in the ear canal. Antibiotic treatment continued for 2 months. Cultures were negative after 1 week, and no relapse has been documented since.

3.2.4. Patient No. 18

Patient No. 18 (Table 8) was a 56-year-old man with type 2 diabetes mellitus, alcoholic cirrhosis and myocardial infarction that was admitted to hospital in Thailand because of a deep left foot ulcer. He underwent several revisional surgeries. He was admitted to the Department of Infectious Diseases, University Hospital of Linköping, Sweden April 27th 2011 and Cultures taken on arrival yielded growth of *E. coli* and the patient was treated with cefotaxim intravenously for 1 week. Rapid deterioration was observed and the patient underwent revisional surgery and deep cultures were taken on May 2nd 2011. Necrotic tissue and osteomyelitis were observed. In spite of treatment by vacuum pump and intravenous antibiotics there was no improvement. A second operation was performed on May 20th with amputation of the second metatarsal head of the left foot. Amputation at a higher level was planned in case of failure. Deep cultures yielded growth of extended spectrum betalactamase (ESBL) *E. coli*, multiple drug resistant *Acinetobacter*, *E. faecalis* and *S. aureus*. The patient received polymyxin E (Colistin) intravenously + oxacillin and fusidic acid orally. He developed an acute renal failure and the treatment was interrupted on May 25th. He was included in the study on the same day receiving piperacillin–tazobactam + AT III for 5 days and he was discharged on June 5th 2011 with ampicillin–clavulanic acid. The ulcer cultures again yielded growth of *E. coli* ESBL and *Acinetobacter* but the healing process continued. The ulcer healed but he acquired new deep ulcers on the first metatarsal head of the same foot after antibiotic treatment was interrupted in October 2011. New treatment with AT III Baxter, and ampicillin–clavulanic acid resulted in the rapid regression of new ulcers.

4. Discussion

The current work is not just a clinical study but also case reports presenting the concerns of physicians facing the advanced stages of chronic disease in patients where conventional therapies had failed and surgery was planned, not to cure but to postpone the acute phase of deterioration. This was the case with long, unplanned follow-up periods of several years in the study protocol, which occurred because contacts between the researcher and the study subjects led to relationships formed between patient and physician. This might be the most important bias of the study, but the study group had in fact met so many patients with a critical status that could not be included in the study and undergone numerous revisions including salami-amputations, until their deaths. So this study report cases of therapy failure in spite of several treatment

attempts, however, after treatment with AT III containing biologically active HGF most patients survived, but the majority of patients suffered relapses.

Our group have previously studied recombinant HGF, kindly provided from Professor Nakamura, in local applications to chronic ulcers and positive results were obtained with the objective sign of increased microcirculation in the ulcerous area [13]. This recombinant HGF showed the same properties as endogenous HGF found in ulcerous secretion from acute ulcers with a high affinity for HSPG in an SPR system and biological activity in a model of cell injury in mouse skin epithelial cells (CCL-53.1), accelerated hair growth in mice, and two separate bands resembling α and β chains of HGF in western blot [11]. However, later studies by our group using commercial recombinant HGF showed no increased regeneration or healing, and further studies of these products showed that one or several of the named properties were missing [12]. Thus, we decided to use purified HGF from healthy blood. The products, obtained during Cohn fractioning of blood for the production of AT III, was hypothesized to contain HGF. This was the case after the examination of fractions during the process of purifying blood for the production of AT III.

The presence of HGF in the AT III products was confirmed with ELISA, Western blot and SPR. However, the biological activity and stability of HGF could be assessed by its affinity to HSPG [18]. Two of the AT III products showed high binding affinity to HSPG and also cell growth in the model of cell injury, and were therefore considered to contain biologically active HGF. The sites on the HGF molecule that interacts with the receptors have been studied previously [21]. Epitope mapping of the AT III products containing biologically active HGF in the present study showed that they, after interaction of HGF in AT III products to receptors (c-Met and HSPG) in the SPR system, did not bind to antibodies, directed against specific epitopes in α and β chains of HGF, that subsequently were run over the sample, indicating that both chains of HGF are involved in the interaction of HGF with receptors. Thus attenuation of bands (α and β chains) of HGF by strains, such as those found during the process of virus inactivation during the production of some AT III products, may result in the inactivation of HGF. In the western blot analysis the AT III products with biologically active HGF presented several bands, compared to the products without biologically active HGF (Fig. 1), these may be both α and β chains, and pro-HGF that are bigger in size. When incubating AT III products with dextran sulfate, a glycosaminoglycan analog, the binding response to ligands decreased, especially in AT III containing biologically active HGF (Table 6). This shows that the binding site for HSPG, which dextran sulfate occupies during the incubation, is necessary for the binding of AT III to HSPG and the biological activity (Table 5). Likewise, incubation with fragmin, a low molecular weight heparin which also belongs to the glycosaminoglycan family, decreased the binding response proportionally with increased concentration. In a clinical study by Warren et al. [16] it was shown that concomitant administration of heparin, even when given in relatively low doses as unfractionated or low molecular weight heparin, interacted with AT III treatment and the patients with severe sepsis did not respond to the AT III treatment and it was associated with increased risk of hemorrhage.

Elimination of bacteria in chronic injuries is an important step in therapy response [22]. However, not one but several bacteria contaminated ulcers. The question is which bacteria we should focus treatment on? The pathogenic bacteria in ulcers are apparently not those that grow first. Culture results and the ultimately beneficial antibiotic regime chosen were shown by following diseases and their outcome in included patients (Table 8). The results indicate the importance of recognizing the symptom-producing bacteria in the ulcer. Improvements may partly depend on the fact that we avoided occlusive/semi-occlusive bandages and washed away the contaminating bacteria. Interestingly, *P. gingivalis* that is an etiological agent strongly associated with periodontal disease [23] and correlates with numerous inflammatory disorders, such as cardiovascular and rheumatic disease. The bacterium was found to eliminate the biologic activity of HGF. *P. gingivalis* express a broad range of virulence factors, such as cysteine proteinases (gingipains) that could cleave proteins of the host [24] and perhaps this could be one mechanism in the reduced biological activity of HGF.

Some study patients suffered from serious arterial insufficiency in the lower extremity (patient Nos. 6, 7, 8 and 12 in Table 8) in addition to decreased microcirculation. Still, they showed favorable results from the study intervention. HGF is a known angiogenetic factor [25] and we have shown increased microcirculation in ulcers after the administration of recombinant HGF [13]. However, AT III is an anti-coagulatory factor and the combination of AT III and biologically active HGF may have a positive effect on the stability of HGF and circulation of injured tissue in which overgrowth of bacteria might have caused intravascular obstruction and rapidly deteriorated circulation. Besides, HGF has also been shown to inhibit platelet aggregation *in vitro* [26]. Appropriate antibiotic treatment against underlying bacteria together with AT III and HGF may have caused improved circulation.

It is known that blood products, including AT III, contain plasmatic trace impurities. The beneficial effect of AT III in critically ill patients suffering from multiple organ deficiency has been an area of investigation. However, there is a discrepancy between study results. We have shown that AT III products contain a very potent form of HGF. Effects on organ failure during sepsis and regenerative properties of HGF have been reported [27]. Thus, this should be given consideration in the studies of AT III reporting beneficial effects on organ failure during sepsis (Table 1). It is noteworthy that the AT III products which contained biologically active HGF and that had beneficial effects in the treatment of the patients in this study were the ones that also showed the lowest risk ratio of mortality as outcome after treatment in critically ill patients in the review article by Afshari et al. Further studies, both *in vitro* and comparing AT III with and without biologically active HGF in a double-blind clinical study might be indicated.

5. Conclusion

The reason for an acute inflammation that otherwise should heal rapidly becoming chronic is not yet known. In the process of chronic inflammation, inactivation of growth factors, such as HGF, is seen and the application of HGF, with similar properties as found in healthy subjects, supports the healings process. In the present work commercial AT III products have been studied regarding the presence and quality of HGF, and patients with critical, deep, chronic ulcer infections are treated locally with an AT III product containing biologically active HGF. The outcome and follow-up of patients indicate favorable results of the intervention.

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