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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)