Detection of Serine Proteases Secreted by *Lucilia sericata* In vitro and During Treatment of a Chronic Leg Ulcer

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Accepted March 21, 2003.

Sir,

For centuries, larval therapy has been recognized as an aid in wound healing. During the 1930s and 1940s, before the antibiotic era, larval therapy was commonly used by surgeons in the USA and Europe when treating various soft-tissue and bone infections. The most commonly used larval species is *Lucilia sericata* (LS). From a clinical point of view, the two major effects of larval therapy have been ascribed to their antibacterial and debriding mechanisms (1–4). In regard to the latter function it has been speculated that the larvae, when introduced into the wound, secrete proteolytic enzymes that enable them to degrade and ingest necrotic tissue. Here, we address this question and demonstrate that these larvae secrete a group of serine proteases when cultured *in vitro*. Furthermore, these serine proteases were detected in the wound fluid of a patient with a chronic leg ulcer treated with larvae. The data suggest that serine proteases of LS are released during treatment.

PATIENTS AND METHODS

Materials

Glycerol and Coomassie blue were products from Sigma; Tris hydrochloride was from ICN Biomedicals; and acrylamide/bisacrylamide solution (30/0.8) was purchased from Scotlab. Other chemicals and reagents, as well as some of the equipment, have been described previously (5).

Wound fluid

Wound fluid from a 92-year-old woman with a chronic, fibrin-covered venous ulcer (in combination with peripheral arterial insufficiency) on the medial malleolus of the left leg was collected using a polyurethane (PU) foam. Sampling was performed before and after larval treatment. The wound was subjected to larval therapy for 2 days (4). A PU-foam oblate (2 mm thick) was applied to the ulcer. The ulcer area was then sealed with Tegaderm film (3 M). Wound fluid was recovered after 2 h by squeezing the oblate in a syringe (6). As additional non-treated controls, we used wound fluid extracts from patients with chronic venous leg ulcers (7).

Growth of larvae and collection of larval secretions

In the fly laboratory, LS laid eggs on meat. The eggs were removed with a pair of tweezers and separated from each other with a pipette in running tap water. The separated eggs were then thoroughly rinsed in chloramine 0.25%. BHI meat bouillon was used to keep them alive and moist. The larvae were checked for sterility, placed on a horse agar plate and incubated for 24 h in a climate chamber maintaining a temperature of 37°C. Sterility controls were performed twice. The eggs were put in a flask containing agar and BHI (50/50%) and stored in a refrigerator for a maximum of 3–5 days before hatching. Hatched instar 2 larvae, approximately 2 mm in size, were used in the ulcer. For the *in vitro* experiments, the larvae were grown on blood-agar plates (24 h), washed in phosphate-buffered saline (PBS) and partially immersed in various growth media (see below) for detection of secreted proteases.

Zymography

Between 5 and 10 μl of conditioned medium was mixed with sample buffer (0.4 M Tris HCl, 20% glycerol, 5% sodium dodecyl sulphate (SDS), 0.03% bromophenol blue, pH 6.8) and electrophoresed on 10% polyacrylamide gels (1 mg bovine gelatine per ml gel). To remove SDS, gels were incubated with 2.5% Triton X-100. Incubation was then performed for 18 h at 37°C in buffer containing 50 mM TrisHCl, 200 mM NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, pH 7.5. Gels were stained with Coomassie blue G-250 in 30% methanol, 10% acetic acid for 1 h and destained in the same solution without the dye. Gelatinase-containing bands were visualized as clear bands against a dark background.

Assessment of proteases production

LS larvae were grown on blood-agar plates for 24 h, washed in PBS and transferred to Petri dishes. The larvae were immersed to ~50% for 16 h in the following media: Todd-Hewitt growth medium (TH), minimal essential medium (MEM), or tryptic soy broth (TSB, 3% in PBS). The various media were poured of, centrifuged and the supernatants analysed by zymography.

RESULTS AND DISCUSSION

Production of 20–50 kDa proteases by LS was seen in the presence of TH, MEM and TSB media, and the strongest enzyme activity was found in TH and TSB (the results using TH are presented; Fig. 1A, lane C). Incubation in PBS did not support growth, and the larvae were dead after the incubation period. No 20–50 kDa enzymes were detected, but instead high molecular weight proteases (~100 kDa), were observed (possibly reflecting tissue necrosis and autolysis). For determination of the mechanistic class of the secreted 20–50 kDa enzymes, we incubated the LS media with the serine proteases inhibitors phenylmethanesulfonyl fluoride (PMSF) and diisopropyl fluorophosphate (DFP). The results indicated that the LS-derived enzymes were inhibited by these inhibitors (Fig. 1A). Some residual activity was noted. However, a second addition of the inhibitors (see, e.g., Fig. 1B, DFP 1 and 2) completely inhibited the enzymes. Boiling for 15–30 min inactivated the enzymes (Fig. 1A). To study whether these proteases were also secreted in patients treated with LS, we collected wound fluid after LS treatment and analysed this material for the presence of these low molecular weight enzymes. Before treatment, only high molecular weight

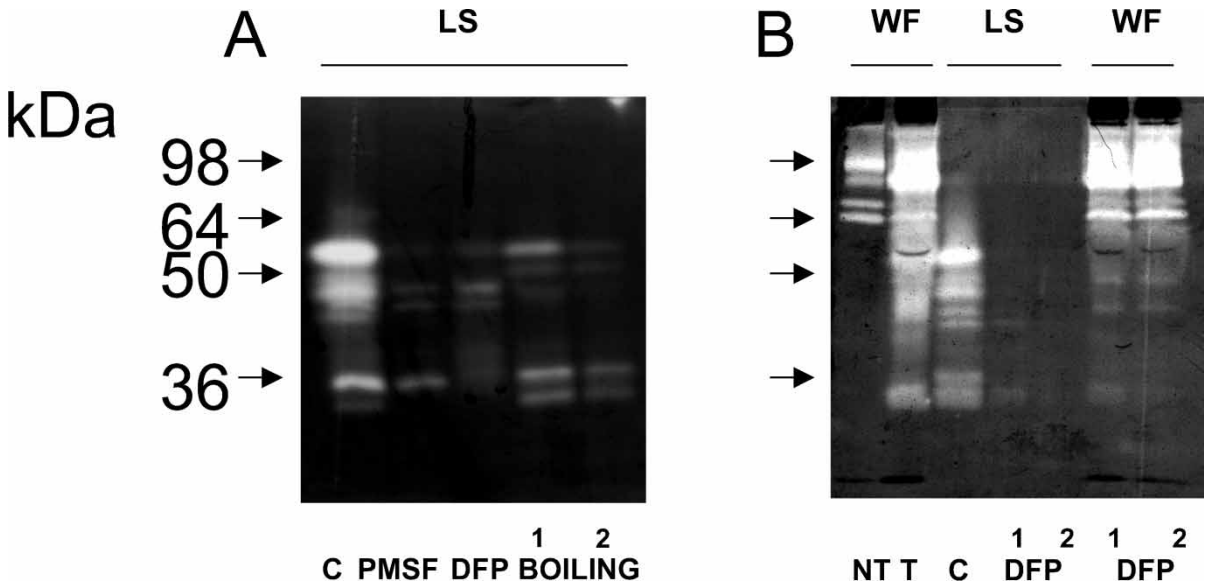

Fig. 1. Zymographic detection of larval proteases *in vitro* and *in vivo*. Panel A. Secretions from *Lucilia sericata* (LS) were analysed directly (C), after treatment with the serine proteases inhibitors phenylmethanesulfonyl fluoride (PMSF) (2 mM) and diisopropyl fluorophosphate (DFP) (2.5 mM), or after boiling for 15 min (1) or 30 min (2). Panel B. Wound fluid (WF) was analysed before treatment (not treated, NT) or after treatment (T). For comparison, larval secretions from LS were run in parallel (C). Both the wound fluid (treated material, T) and the LS material were supplemented with DFP (two additions of 2.5 mM each, 1 and 2).

proteases, probably identical to human 92 and 72 kDa matrix metalloproteinases (MMPs), were detected (Fig. 1B, lane NT). After LS treatment, wound fluid contained additional proteases of 20–50 kDa (Fig. 1B, lane T) which co-migrated with the enzymes secreted from LS. The fact that these 20–50 kDa proteases were inactivated completely by DFP (panel B, LS and WF; DFP 1 and 2) proved their identity as serine proteases. The high molecular weight MMPs were unaffected by DFP. Interestingly, the activity of a 70–80 kDa protease was significantly higher after LS treatment, suggesting that LS treatment may activate endogenous MMPs. However, this hypothesis has to be verified in more patients. Unavailability of antibodies to these serine proteases made a direct identification impossible in wound fluid, and therefore our data do not exclude the possibility that the 20–50 kDa proteases detected in wound fluid of this treated patient are unrelated to LS enzymes. However, we did not detect these enzymes in wound fluids from a group of chronic ulcer patients (12 patients, not shown). This observation, along with the fact that the enzymes were serine proteases and were detected only in relation to the LS treatment, strongly suggests that these enzymes are LS-derived. It is likely that the proteases described here are similar to a group of tryptic and chymotryptic proteases previously described in the blowfly *Lucilia cuprina* (8, 9). These proteases were inhibited by serine proteases inhibitors and of molecular weight 20 kDa and higher belonging to a multigene family of trypsins (8, 9). In conclusion, we have demonstrated that LS larvae secrete serine proteases *in vitro*, and also that similar enzymes were detected during larval therapy *in vivo*. Interestingly, after submission of this Letter a paper appeared demonstrating production of similar enzymes and their activities on extracellular matrix components *in vitro* (10).

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